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Mapping the molecular determinants of positive allosteric modulators of the mGlu2 receptor

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Table of contents

Abbreviations

$[^{35}S]GTP\gamma S$ - Guanosine 5'-(γ-thio)triphosphate $[^{35}S]$-

1GZM - rhodopsin receptor

2RH1 - β_2-adrenergic receptor

3D – 3-dimensional

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

CRD – cystein rich domain

DAG - *sn*-1,2-diacylglycerol

DMSO - dimethyl sulphoxide

E. coli - Escherichia coli

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

FBS - fetal bovine serum

GABA - γ-amino butyric acid

GDP – guanosine 5' diphosphate

GPCR – G-protein coupled receptor

GRK – G-protein coupled receptor kinases

GTP – guanosine 5' triphosphate

hmGluR - human metabotropic glutamate receptor

IL – intracellular loop

IP_3 - inositol (1,4,5)-trisphosphate

JAK – Janus kinase

KA - kainate

K_D – apparent equilibrium dissociation constant

LB - Luria-Bertani

LTD - long-term depression

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

mGluR – metabotropic glutamate receptor

MOE - Molecular Operating Environment

MTOR - mammalian target of rapamycin

NAM - negative allosteric modulator

NFDM - Non-Fat Dry Milk

NMDA - N-methyl-D-aspartate

PAM – positive allosteric modulator

PBS - phosphate-buffered saline

PIP_2 - phosphatidylinositol (4,5)-bisphosphate

PKA –protein kinase A

PKC – protein kinase C

PLC_β - phospholipase C_β

RGS – regulators of G-protein signaling

rmGluR - rat metabotropic glutamate receptor

RT - room temperature

TBS-T – Tris-Buffered Saline and Tween 20

TM – transmembrane

VFT – venus flytrap domain

Resumo

O sucesso dos receptores acoplados à proteína G (GPCRs) enquanto alvos terapêuticos para o tratamento de doenças do sistema nervoso central torna estes receptores alvos pertinentes de investigação. Especificamente, a activação de receptores mGlu2 tem mostrado reduzir a transmissão glutamatérgica em áreas do cerebro onde o excesso de sinalização glutamatérgica parece estar implicado na patofisiologia de doenças como a ansiedade e a esquizofrenia. Deste modo, a activação dos receptores mGlu2 está actualmente a ser encarada como uma potencial estratégia para o tratamento destas doenças. Diversos moduladores alostéricos positivos (PAMs), que se ligam a um local do receptor diferente do local de ligação do glutamato, têm revelado modular os receptores mGlu2 de uma forma selectiva. Com o objectivo de identificar aminoácidos potencialmente importantes para a interacção entre o PAM e o receptor mGlu2, foi efectuada a modelação molecular e o docking de PAMs de receptores mGlu2 em paralelo com mutagénese dirigida. Receptores mGlu2 mutantes foram produzidos e o impacto dessas mutações na actividade de diversos PAMs foi avaliado, de forma a confirmar o papel destes aminoácidos na actividade destes compostos. Este estudo identifica aminoácidos importantes para a actividade dos PAMs e sugere um potencial local de ligação destes compostos.

Palavras-chave: GPCR, receptor mGlu2, alosterismo, modulador alostérico positivo, mutagénese.

Abstract

The proven success of G protein-coupled receptors (GPCRs) as drug targets for the treatment of CNS disorders renders them attractive targets of research. Specifically, activation of the G protein-coupled or metabotropic glutamate (mGlu2) receptor has been shown to reduce glutamatergic transmission in brain regions where excess glutamate signaling may be implicated in the pathophysiology of disorders such as anxiety and schizophrenia. Hence, activation of the mGlu2 receptor is being pursued as a novel therapeutic approach for the treatment of these diseases. Multiple positive allosteric modulators (PAMs), which bind to a site other than that of the endogenous mGlu2 receptor agonist glutamate, have been shown to modulate mGlu2 receptors in a selective way. In order to identify amino acids potentially important for the interaction between PAMs and the mGlu2 receptor, homology modelling and docking of mGlu2 receptor PAMs was performed in parallel with experimental site directed mutagenesis. Mutant mGlu2 receptors were produced and the impact of the selected receptor mutations on the activity of several PAM compounds towards the receptor was evaluated, in order to confirm the role of these amino acids in the activity of the compounds. This study identifies crucial amino acids for the activity of mGlu2 receptor PAMs and suggests a potential binding pocket of PAMs.

Keywords: GPCR, mGlu2 receptor, allosterism, positive allosteric modulator, mutagenesis.

Chapter 1

Introduction

1.1. G Protein-Coupled Receptors

Membrane-bound receptors have a leading role in the recognition of intercellular messenger molecules and sensory messages, which makes them fundamental for the communication of the cells with each other and with the environment (Bockaert and Pin, 1999). They have been classified into several families, the most common of which is the G Protein-Coupled Receptors (GPCR) family (Bockaert and Pin, 1999). GPCRs currently constitute one of the principal drug targets in pharmacology (Vauquelin and von Mentzer, 2007; Urwyler, 2011). More than 50% of the marketed therapeutics are based on GPCRs, and a large share of total drug sales and prescriptions (~25%) are directed at these receptors (Lündstrom and Chiu, 2006), rendering them important and pertinent targets of research.

GPCRs constitute one of the largest and most diverse protein superfamilies (Urwyler, 2011). Human genome sequencing has revealed that more than 1000 genes encode GPCRs, which represents a substantial part ($\pm$ 3%) of the human genome (Vauquelin and von Mentzer, 2007). Vassilatis *et al.*, (2003) proposed that 367 human GPCRs have endogenous ligands ('endoGPCRs'), 143 of which are orphan receptors (i.e. receptors with no known ligand). The remaining receptors are chemosensory (taste, odorant or pheromone receptors), and they respond only to sensory signals of external origin (Vassilatis *et al.*, 2003; Urwyler, 2011).

GPCRs are receptors for a variety of extracellular stimuli, such as hormones, neurotransmitters, chemokines, calcium ions, light, odorants (Lündstrom and Chiu, 2006), pheromones, gustative molecules, lipids, peptides, proteins (Urwyler, 2011), small molecules including amino-acid residues and nucleotides (Figure 1). Upon binding of these ligands, they mediate several cellular signal transduction events that are crucial for the cells, tissues, organs and whole organisms to react properly to environmental requirements (Bockaert and Pin, 1999).

GPCRs regulate different physiological processes that affect neurological and neurodegenerative functions, cardiovascular mechanisms and metabolic control (Lündstrom and Chiu, 2006).
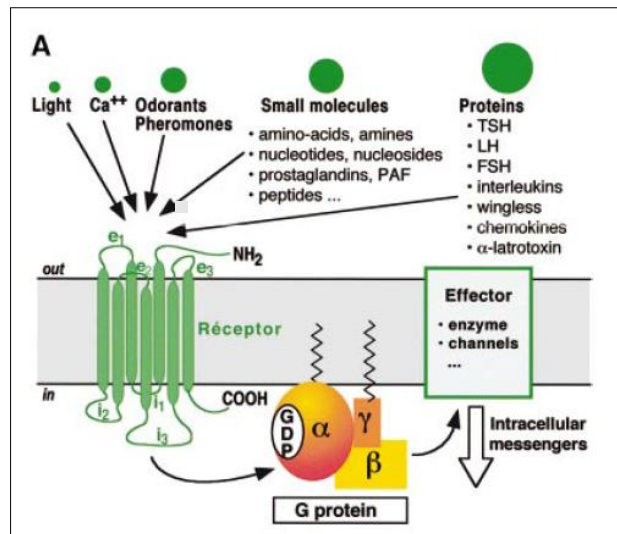
Figure 1: Diversity of messages which activate G-protein Coupled Receptors (GPCRs). GPCRs have a central common core made of seven transmembrane helices (TM1-7) connected by three intracellular (i1, i2, i3) and three extracellular (e1, e2, e3) loops. Bockaert and Pin, 1999

1.1.1. GPCR structure

GPCRs consist of a single peptide, whose length can vary from 400 to 1200 amino-acids (Vauquelin and von Mentzer, 2007). They have in common a central core domain composed of seven transmembrane α-helices (TM1 – TM7) connected by three intracellular (IL1 – IL3) and three extracellular (EL1 – EL3) loops (Baldwin, 1993). Each GPCR possesses also an extracellular N-terminal domain and an intracellular C-terminal domain (Lündstrom and Chiu, 2006) (Figure 1). The length and function of the N- and C- terminal domains and of the intracellular loops varies between the different GPCRs and each of these domains confers specific properties to these receptor proteins (Bockaert and Pin, 1999). Two cystein residues, one in EL1 and the other in EL2, are conserved in most GPCRs. These two residues form a disulfide bond which is thought to be important for the packing and stabilization of a restricted number of conformations of the seven TMs (Bockaert and Pin, 1999).

1.1.2. GPCR mode of action – signaling pathways

GPCRs become functionally active when coupled to guanine nucleotide-binding proteins (G proteins). Typically, the receptor is in its resting or low affinity state in the absence of agonists. This inactive conformation is caused by a number of intramolecular constraining interactions (Vauquelin and von Mentzer, 2007). Upon agonist binding to the receptor, a modification in these constraints occurs, leading to a shift in the receptor conformation. The receptor is now in an active state that leads to the formation of a complex with intracellular G proteins.

G proteins constitute a family of closely related membrane-associated polypeptides (Vauquelin and von Mentzer, 2007). They are heterotrimeric proteins that consist of a guanine nucleotide binding Gα subunit (38-52 kDa), a Gβ subunit (35 kDa) and a Gγ (8-10 kDa) subunit. G proteins are anchored to the cytoplasmic side of the plasma membrane (Vauquelin and von Mentzer, 2007). The β and γ subunit are always closely associated, thus creating a βγ complex that is presumed to be interchangeable from one G protein to another (Vauquelin and von Mentzer, 2007). Gα subunit is predominantly hydrophilic but it is anchored to the plasma membrane through its coupling to the βγ complex; this subunit constitutes the receptor-recognizing part of G proteins (Vauquelin and von Mentzer, 2007).

As previously mentioned, in the resting state the receptor and the G protein do not interact with each other. In this state, Gα subunit contains tightly bound guanosine-5'-diphosphate (GDP) (Vauquelin and von Mentzer, 2007). Once an agonist binds to the receptor, the conformation of the receptor changes which enables the interaction of the receptor with the Gα subunit of the G protein. GDP is then released and replaced by guanosine-5'-triphosphate (GTP), and the Gα subunit is dissociated from the Gβγ dimer. Gα subunit and Gβγ dimer can then both activate cellular effector molecules (Lündstrom and Chiu, 2006). The G-protein linked effector components are either enzymes or ion channels (Table 1).

Table 1: Principal G protein subunits and their primary effectors (Adapted from Hermans, 2003)

Subunit	Family	Main Subtypes	Primary effector
α	α_S	$G\alpha_S$, $G\alpha_{olf}$	+ Adenylyl cyclase
	$\alpha_{i/o}$	$G\alpha_{i-1}$, $G\alpha_{i-2}$, $G\alpha_{i-3}$	- Adenylyl cyclase
		$G\alpha_{oA}$, $G\alpha_{oB}$	+ K^+ channels
		$G\alpha_{t1}$, $G\alpha_{t2}$	- Ca^{2+} channels
		$G\alpha_z$	Cyclic GMP
			+ Phosphodiesterase
	$\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$	- Phospholipase C
		$G\alpha_{15}$, $G\alpha_{16}$	
	α_{12}	$G\alpha_{12}$, $G\alpha_{13}$	?
β	$\beta_{1-5\,(6)}$	Different assemblies of β/γ subunits	+/- Adenylyl cyclase
			+ Phospholipases
			Phosphatidylinositol
			+ 3-kinase
γ	γ_{1-11} (12)		+ Protein kinase C
			+ Protein kinase D
			+ GPCR kinases
			Ca^{2+}, K^+ (and Na^+) channels

The Gα subunit possesses an endogenous GTPase activity, which will cause GTP hydrolysis into GDP and lead to the termination of receptor activity. Rapid termination of GPCR function can occur also through other mechanisms. One of them is receptor phosphorylation mediated by Protein Kinase A (PKA) or Protein Kinase C (PKC), which will lead to the uncoupling of the receptor and the G protein. Another mechanism involves G protein-coupled receptor kinases (GRKs) which can only phosphorylate the receptor when it is activated or agonist-occupied, stabilizing a conformation state necessary for the interaction of the GPCR with arrestins. Binding of arrestin prevents further GPCR and G protein interactions or leads to receptor endocytosis. Receptor degradation in lysosomes can also occur, as well as regulation of gene transcription or translation. At a post-expression level, regulators of G protein signaling (RGS) serve as GTPase-activating proteins for G proteins, enhancing the hydrolysis rate of GTP and thus leading to termination of receptor activity (Lündstrom and Chiu, 2006).

GPCRs are known to induce signaling pathways not only through direct interaction with G proteins, but also through interaction with other proteins. These alternative signaling pathways may confer a higher specificity to the GPCR signaling and may also allow distinct physiological functions of the different GPCRs (Lündstrom and Chiu, 2006). Arrestins act as adapters or scaffolds for signaling proteins involved in the ERK/mitogen-activated protein

kinase signaling pathway (Vauquelin and von Mentzer, 2007). GPCRs have also been suggested to interact with the family of the PDZ domain-containing proteins (Kornau *et al.*, 1997). Several GPCRs interact with members of the janus kinase (JAK) family of nonreceptor protein tyrosine kinases, which will recruit and phosphorylate members of the STAT (signal transducers and activators of transcription) family of transcription factors (Ritter and Hall, 2009).

1.1.3. Families of GPCRs

GPCRs are presumed to have evolved from a common ancestor (Vauquelin and von Mentzer, 2007). They have been classified into several families/classes based on sequence and structural similarity (Lündstrom and Chiu, 2006, Vauquelin and von Mentzer, 2007 and Yarnitzky *et al.*, 2010).

1.1.3.1. Family A or Class 1 GPCRs

Family A is the largest group of GPCRs and the subfamily of rhodopsin/β_2-adrenegic receptor-like receptors is by far the most studied (Vauquelin and von Mentzer, 2007). Family A consists of light receptors (rhodopsin), adrenaline receptors and olfactory receptors. The homology among the members of this family is overall low (Vauquelin and von Mentzer, 2007) but they all possess an arginine in the highly conserved Asp/Glu-Arg-Tyr (D/ERY) motif positioned at the cytoplasmic side of TM3 (Figure 2) (Lündstrom and Chiu, 2006, Vauquelin and von Mentzer, 2007). The ligands of family A GPCRs bind within the 7TM domain.

18
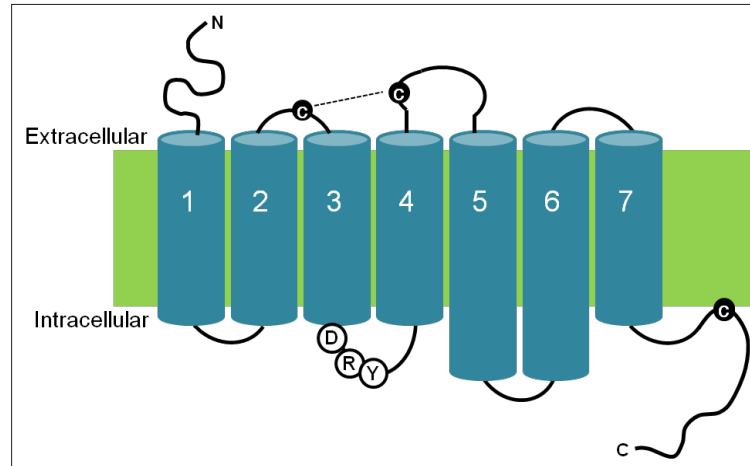
Figure 2: Two-dimensional structure of family A GPCRs and rhodopsin. Adapted from Vauquelin and von Mentzer, 2007.

1.1.3.2. Family B or Class 2 GPCRs

Based on the homology shared by family B receptors (Vauquelin and von Mentzer, 2007), they were divided in three subfamilies: subfamily B1) those recognizing peptide hormones (secretin, glucagon, VIP (Vauquelin and von Mentzer, 2007), calcitonin and corticotrophin-releasing (Lündstrom and Chiu, 2006) hormone receptor family), B2) those containing a GPCR proteolytic site domain (so far, orphan receptors) and B3) those with cystein-rich domains (frizzled and smoothened receptors) (Vauquelin and von Mentzer, 2007). Family B receptors have a propensity to associate with partner/accessory proteins (Vauquelin and von Mentzer, 2007). For the subfamily of secretin/glucagon/VIP receptors, it is shown that the N-terminal domain is required for ligand binding (Unson *et al*., 1995).

1.1.3.3. Family C or Class 3 GPCRs

Family C receptors include γ-amino butyric acid (GABA) receptors, calcium-sensing receptors, three receptors involved in taste perception and metabotropic glutamate (mGlu) receptors, which represent the focus of this thesis (Vauquelin and von Mentzer, 2007; Lündstrom and Chiu, 2006).

19
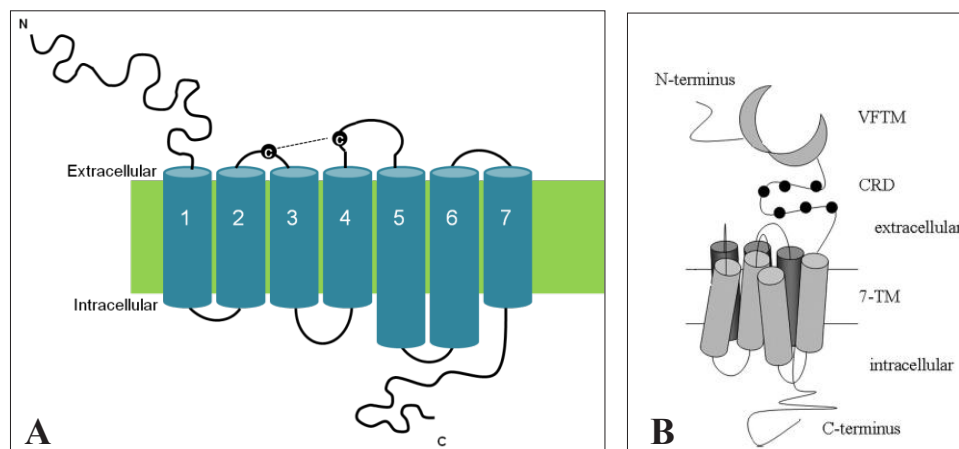
Figure 3: Structure of family C GPCRs. A) Two-dimensional structure of family C GPCRs and rhodopsin (Adapted from Vauquelin and von Mentzer, 2007); B) Family C GPCRs all have a common core composed of seven-transmembrane helices (the 7TM domain comprised of TM1-7) with a large, bilobal Venus flytrap extracellular N-terminal domain and an intracellular C-terminal domain. (Adapted from Urwyler, 2011).

The typical feature of this GPCR family is the exceptionally large extracellular N-terminal region (600 amino acids; Vauquelin and von Mentzer, 2007) that is essential for ligand binding and receptor activation (Lündstrom and Chiu, 2006) (Figure 3A). The large extracellular N-terminal domain is also termed the Venus flytrap domain (VFD) (Pin *et al.*, 2003), which is connected to the 7TM domain through a cystein-rich domain (CRD), composed of nine conserved cysteins and present in all family C receptors, except for GABA$_B$ receptors (Vauquelin and von Mentzer, 2007; Niswender and Conn, 2010). Each VFD is composed of two lobes, and the cleft between them constitutes the binding site for the natural messenger (Vauquelin and von Mentzer, 2007, Niswender and Conn, 2010) (Figure 3B). Interestingly, the VFD shares sequence similarity with bacterial periplasmic-binding proteins, which are involved in the transport of small molecules (Vauquelin and von Mentzer, 2007). The TM loops of family C GPCRs show an overall low sequence similarity with the homologous loops of family A. However, the two families share several conserved amino acid residues, as well as a conserved disulfide bond between the top of TM3 and the second extracellular loop, suggesting a common ancestor gene for both and, for family C GPCRs, a fusion of that gene with the gene for a periplasmic-binding protein (Vauquelin and von Mentzer, 2007).

Class C GPCRs have been found to form homo- and heterodimers (Vauquelin and von Mentzer, 2007). For mGlu receptors, there is evidence suggesting that they form homodimers stabilized by a disulphide bridge (through VFD or CRD). Accordingly, studies focusing on the structure of mGlu receptors revealed that the binding of the agonist to one or both domains induces large conformational changes (Jingami *et al.*, 2003). There are three main

configurations of the VFD dimer: open-open (inactive), which is stabilized by antagonists; open-closed and closed-closed, which are induced by the binding of the agonist to one or two protomers, respectively (Figure 4). The configuration of the flytrap domains is important for receptor activation; it has for example been shown that mutations in the residues that prevent closure of the VFD are capable of switching the pharmacology of antagonists to agonists (Bessis *et al.*, 2002). In the case of mGlu receptors, VFDs not only bind glutamate, but also bind magnesium and calcium, which can potentiate or activate the receptor (Francesconi and Duvoisin 2004; Kubo *et al.*, 1998; Kunishima *et al.*, 2000).
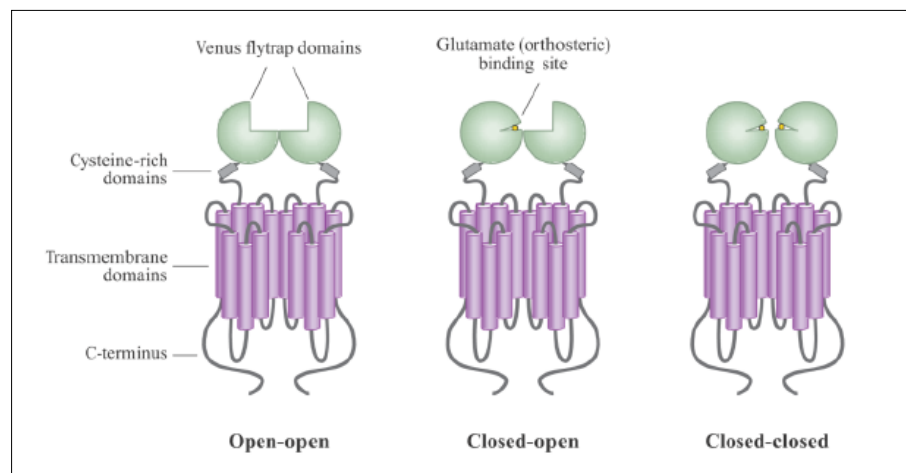
Figure 4: Scheme of the mGlu receptor dimer in different activity states. mGlu receptor dimers possess two large extracellular domains called the Venus flytrap domains (VFDs), which bind glutamate and other so-called orthosteric ligands. The cysteine-rich domain links the VFDs to seven transmembrane-spanning domains; the C-terminus is intracellular and is often subject to alternative splicing to generate different C-terminal protein tails. The open-open state is the inactive state and can be stabilized by antagonists. One or both VFDs can bind glutamate, which will lead to active receptor conformations. Niswender and Conn, 2010

Once an agonist binds to the VFD, it induces conformational changes that are propagated to the transmembrane domain (TM) and C-terminal tail, through the CRD (Huang *et al.*, 2011). A binding pocket located in the TM is the binding site for the majority of characterized allosteric modulators (discussed in Section 1.3) of mGlu receptors, thus allowing the modulation of receptor activity by those ligands (Brauner-Osborne *et al.*, 2007). The C-terminal domain is target of alternative splicing, regulation by phosphorylation, and modulatory protein-protein interactions in several GPCRs, which make it an important region for the modulation of G protein coupling (Niswender and Conn, 2010; Bockaert and Pin, 1999).

21

1.1.3.4. Families D, E and F or Class 4, 5 and 6 GPCRs

The yeast pheromone receptors – families D (STE2 receptors) and E (STE3 receptors) and four cAMP receptors from *Dictyostelium discoideum* (family F) form three smaller families of GPCRs (Vauquelin and von Mentzer, 2007; Lündstrom and Chiu, 2006).

1.1.4. GPCRs in the Central Nervous System - Overview

GPCRs are widely expressed in the central nervous system (CNS) (Lündstrom and Chiu, 2006; Lim, 2007). They are expressed in different types of neurons and in astrocytes and glial cells, and mediate a slow modulation of neuronal activity, in contrast to ion channels, which mediate fast synaptic excitatory neurotransmission (Lündstrom and Chiu, 2006, Dingledine *et al*., 1999). Postsynaptically, GPCRs can induce changes in intracellular cAMP levels, increase phosphoinositol turnover and/or indirectly modulate ion channel activity through the activation of kinases. Presynaptically, they act as autoreceptors to decrease the release of the respective neurotransmitter or as heteroreceptors that modulate the release of neurotransmitters other than their equivalent ligands (Lündstrom and Chiu, 2006). GPCRs play a critical role in developmental processes and synaptic transmission, and they also mediate other important physiological processes such as cognition, thought and emotional state, motor and hormonal control and pain sensation (Lündstrom and Chiu, 2006; Lim, 2007).

Given the wide distribution of GPCRs in the CNS, their involvement in CNS disorders is expectable. Abnormalities in the regulation of GPCRs have been related to psychiatric disorders such as schizophrenia and mood disorders (Catapano and Manji, 2007). GPCRs have also been shown to play important roles in key neurotransmitter systems that are disrupted in Alzheimer's disease and hence have been related to Alzheimer's disease pathogenesis (Thathiah and De Strooper, 2011).

GPCRs have been proven successful as drug targets in the CNS: they constitute the target for some of the most efficacious analgesics, and many drugs for depression, anxiety and schizophrenia act on GPCRs. Additional GPCRs are emerging as potential targets for Parkinson and Alzheimer's diseases (Lündstrom and Chiu, 2006).

In the past decade, mGlu receptors have received much attention due to their strong potential as a drug target (Lavreysen and Dautzenberg, 2008). mGlu receptors are of interest for the treatment of several neurological and psychiatric disorders, such as depression, anxiety, schizophrenia, chronic pain, epilepsy, Alzheimer's disease and Parkinson's disease (Conn *et al.*, 2009b); Marino and Conn, 2006). mGlu receptors will be discussed in more detail in Section 1.2.2.2.

1.2. The glutamatergic system in the central nervous system

Glutamate is the major excitatory neurotransmitter in the mammalian brain. Approximately 90% of neurons in the brain utilize glutamate as their primary neurotransmitter, and 80-90% of the synapses in the brain are glutamatergic (Breitenberg and Schüz, 1998). Glutamate is the major mediator of sensory information, motor coordination, emotions and cognition (Siegel *et al.*, 2006). The concentration of this neurotransmitter in brain gray matter ranges between 10 and 15 μmol per gram of tissue, whereas in the white matter it varies between 4 and 6 μmol/g (Siegel *et al.*, 2006). Once synthesized in the pre-synaptic cytoplasm, glutamate accumulates in synaptic vesicles, by specific transporters, termed vesicular glutamate transporters.

Glutamate participates in many reactions in the brain: its formation is a step in the metabolism of glucose and amino-acids; it is a precursor for GABA in GABAergic neurons and for glutamine in glial cells; it is a constituent of proteins and peptides. As a result, glutamate is found in all cells of the brain (neuronal and glial cell bodies and their processes), both in the cytosol and the mitochondria (Siegel *et al.*, 2006).

Abnormal glutamate neurotransmission has been related to many neurological disorders, including Alzheimer's disease, Parkinson's disease, addiction, depression, epilepsy, pain, anxiety, and schizophrenia (Rowe *et al.*, 2008).

1.2.1. Glutamate receptors

Glutamate binds to and activates two main categories of receptors: ionotropic and metabotropic (Figure 5).

1.2.1.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors – or ligand-gated ion channels – mediate the vast majority of fast synaptic excitatory neurotransmission in the brain (Dingledine *et al.*, 1999) and they comprise two functional domains: an extracellular domain which is the neurotransmitter binding site and a membrane-crossing domain that forms an ion channel (Purves *et al.*, 2004).

Neurotransmitter binding induces a conformational change in the receptor, increasing the probability of channel opening and thus a cation influx. Three classes of ionotropic glutamate receptors have been identified and named after the agonist that activates them: *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and kainate (KA) receptors. They all have a different affinity for glutamate (Javitt, 2004). Ligand-gated ion channel receptors appear to be a multimeric aggregation of different protein subunits. For NMDA receptors, NMDAR1 is the obligatory subunit that combines with NMDAR2A-2D subunits to form a gated channel. AMPA receptors consist of four different subunits, GluR1-4, all of which can exist in two different forms. Kainate receptors are formed by five kainate subunits, GluR5-7 and KA1-2, which can undergo alternative splicing and editing (Nakanishi, 1992).

Due to the fact that ionotropic glutamate receptors are expressed by nearly all types of neurons and mediate fast excitatory neurotransmission throughout the brain, direct pharmacological manipulation of these receptors is undesired as their inhibition could produce widespread disruption of brain function, which could lead to serious side effects (Rowe *et al.*, 2008; Swanson *et al.*, 2005).
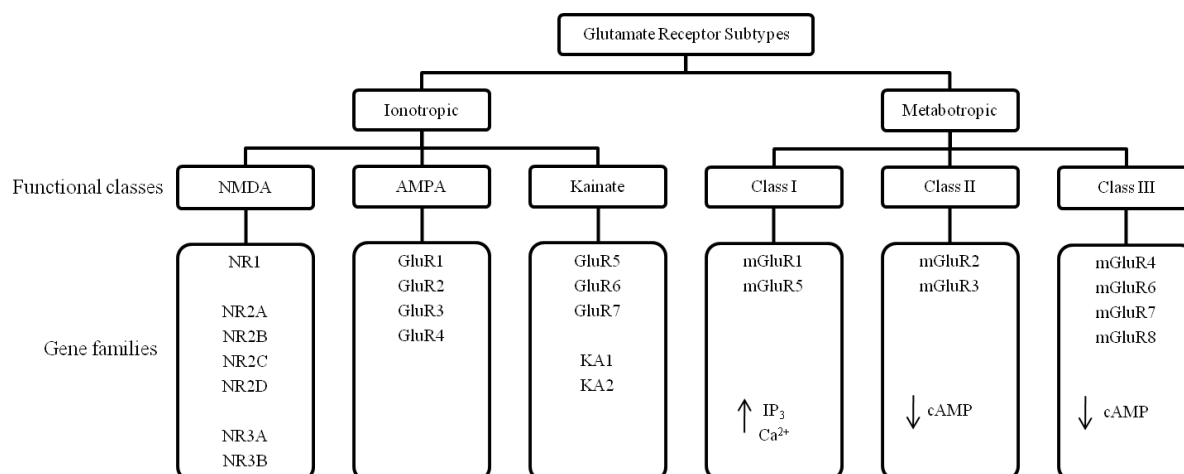
Figure 5: Molecular subtypes of glutamate receptors. Each subtype includes three functionally defined groups (classes) of receptor, which are made up of numerous individual subunits that are encoded by different genes. Adapted from Siegel *et al.*, 2006

1.2.1.2. Metabotropic glutamate receptors

mGlu receptors are, as discussed above (see section 1.1.3.3.), members of the family C GPCRs and they are involved in the slower action of glutamate (Lim, 2007). These neuromodulatory receptors enable glutamate to modulate cell excitability and synaptic transmission through second messenger signalling pathways (Niswender and Conn, 2010).

1.2.1.3. The mGlu receptor Family

To date, eight mGlu receptors have been identified and they can be divided in three groups (I, II and III) according to their amino acid sequence homology, pharmacology and the preferred signal transduction mechanisms they couple to when expressed *in vitro* (See Table 2 for a summary) (Swanson *et al.*, 2005). In addition to these eight mGlu receptors, several isoforms of these subtypes are produced by the alternative splicing of their RNAs, which generates splice variants in the cytoplasmic C-terminal domain (Litschig *et al.*, 1999; Urwyler, 2011).

Signal Transduction Mechanisms

Group I receptors (mGlu1/5 receptor) preferentially activate phospholipase C_β (PLC_β) via G_q/G_{11} proteins. This results in the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) and the consequent generation of inositol (1,4,5)-trisphosphate (IP3) and *sn*-1,2-diacylglycerol (DAG), which initiate a second messenger cascade: IP3 will mobilize Ca^{2+} from intracellular calcium stores, and DAG will increase the activity of membrane-bound PKC (Figure 6A). These receptors have been shown to modulate additional signaling pathways, including other cascades downstream of $G\alpha_q$, as well as pathways originating from $G\alpha_{i/o}$, $G\alpha_S$ and other molecules independent of G proteins (Hermans *et al.*, 2001). Accordingly, depending on the cell type or neuronal population, group I mGlu receptors are capable of inducing the activation of several downstream effectors: phospholipase D, protein kinase pathways such as casein kinase 1, cyclin-dependent protein kinase 5, Jun kinase, components of the mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) pathway, and the mammalian target of rapamycin (MTOR)/p70 S6 kinase

pathway (Niswender and Conn, 2010). The last two pathways are presumably important for the regulation of synaptic plasticity by group I mGlu receptors (Niswender and Conn, 2010). Additionally, when mGlu1a receptors are expressed in several cellular systems they can enhance the formation of cAMP and the release of arachidonic acid (Aramori and Nakanishi, 1992).

Groups II (mGlu2/3) and III (mGlu4/6/7/8) inhibit adenylyl cyclase activity – which will lead to decreases in the cyclic adenosine monophosphate (cAMP) levels – and directly regulate ion channels and other downstream signaling partners via $G\alpha_{i/o}$ proteins (Figure 6B). As for group I, group II and III mGlu receptors have been associated with other signaling pathways, including activation of MAPK and phosphatidyl inositol 3-kinase PI3 kinase pathways (Iacovelli *et al.*, 2002), which shows that the regulation of synaptic transmission by these receptors involves different mechanisms (Spooren *et al.*, 2003; Niswender and Conn, 2010; Wood *et al.*, 2011).
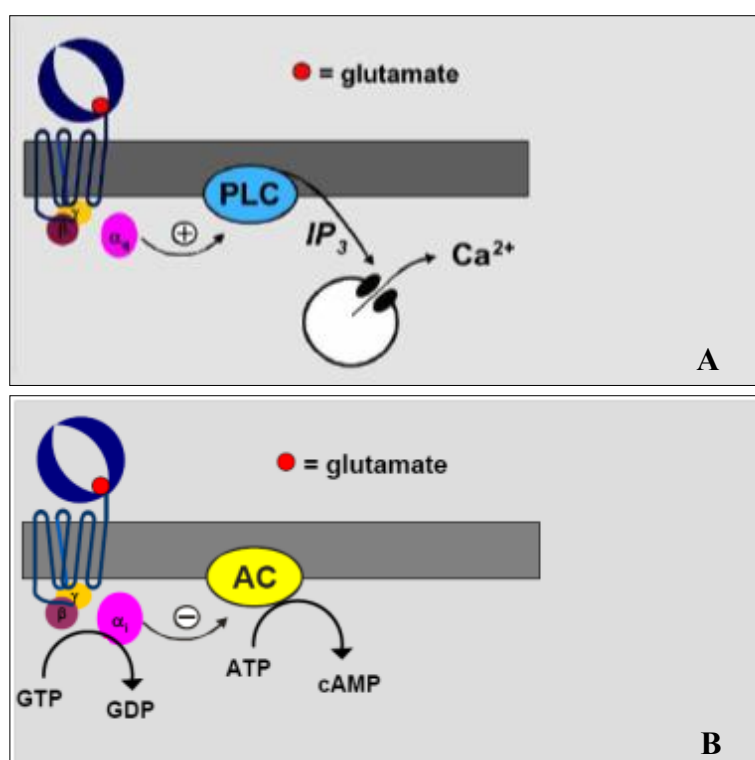
Figure 6: General signal transduction mechanisms of mGlu receptors. **A)** Group I mGlu receptors signal transduction pathway; **B)** Group II and III mGlu receptors signal transduction pathway (Adapted from Gasparini and Spooren, 2007).

Pharmacology

The pharmacology of mGlu receptors has been ascertained by studies in both native preparations (as brain slice and cultured neurons) and non-neuronal cell lines expressing recombinant rat or human mGlu receptors.

L-glutamate, (1*S*, 3*R*)-ACPD, ABH x D-I (agonists) and LY341495 (antagonist) are common ligands for the three different mGlu receptor groups (Niswender and Conn, 2010; Schoepp *et al*., 1999), but specific agonists and antagonists for each of the three groups of mGlu receptors have been identified. Table 2 summarizes the pharmacological profiles of group I, II and III mGlu receptors (full name of the compounds can be found in Appendix 1). Besides orthosteric ligands, allosteric ligands – negative (NAM) and positive (PAM) – are also presented. The two concepts (orthosteric and allosteric) will be discussed in section 1.3.

The pharmacology of group III mGlu receptors is not as extensively described as for groups I and II, due to the lack of subtype-selective pharmacological tools to study these targets (Schoepp *et al.*, 1999). Some of the compounds, of which MPEP and SIB-1893 are examples, have shown activity at multiple mGlu receptor subtypes, which require caution when interpreting their effect, as it can be arising from different mGlu receptor subtypes.

Table 2: Pharmacological profile of mGlu receptors. PAM: positive allosteric modulator; NAM: negative allosteric modulator

	Pharmacodynamic profile	Selectivity	Compound	References
Group I	Orthosteric agonist	Group I	(*S*)-3,5-DHPG Quisqualate	Desai *et al*., 1995 Parmentier *et al.*, 1998
		mGluR5	*Z*-CBQA *E*-CBQA CHPG	Littman *et al*, 1999 Doherty *et al*., 1997
	Orthosteric antagonist	Group I	MCPG and derivatives LY367385	Niswender and Conn, 2010 Clark *et al*., 1998
	NAM	mGluR1	CPCCOEt R214127 CTZ Bay 36-7620 JNJ 16259685 FTIDC YM 298198 EM-TBPC	Litschig *et al.*, 2000 Lavreysen *et al.*, 2003 Surin *et al*., 2007 Carroll *et al*., 2001 Lavreysen *et al*., 2004 Suzuki *et al*., 2007 Kohara *et al*., 2005 Malherbe *et al*., 2003 a)
		mGluR5	SIB-1757 SIB-1893	Varney *et al*., 1999
			MPEP MTEP	Gasparini *et al*., 1999 Cosford et al., 2003
	PAM	mGluR1	Ro 67-7476 Ro 67-4853 Ro 01-6128	Knoflach *et al*., 2001
			VU60 VU54 VU48 VU34 VU71	Hemstapat *et al*., 2006
		mGluR5	DFB CPPHA	Zhang *et al.*, 2005
			CDPPB VU29 ADX47273	de Paulis *et al*., 2006 Chen, 2007 Liu *et al*., 2008
Group II	Orthosteric agonist	Group II	DCG-IV (2*R*,4*R*)-APDC 1*S*,3*S*-ACPD LY354740 LY379268 *S*-4MeGlu 2S, 4*S*-4MG NAAG L-CBG-I L-F2CCG-I LY389795 (?) LCCG-I MGS0028	Hayashi et al., 1993 Schoepp et al., 1996 Pin *et al*., 1994 Schoepp *et al*., 1997 Monn *et al*., 1999 Bräuner-Osborne *et al*., 1997a) Bräuner-Osborne *et al*., 1997a) Wroblewska *et al*., 1997 Tsujishima *et al*., 1998 Saitoh *et al., 1998* Monn *et al*., 1999 Hayashi *et al*., 1992 Nakazato *et al*, 2000
	Orthosteric antagonist		LY341495 HYDIA	Kingston *et al.*, 1995 Lundström *et al.*, 2009
	PAM	mGlu2	LY487379 BINA Acetophenone series	Schaffhauser *et al*., 2003 Galici *et al*., 2006 Trabanco *et al*., 2011
	NAM	Group II	MNI-135 MNI-136 MNI-137	Hemstapat *et al.*, 2007
			RO4988546 RO5488608	Lundström *et al*., 2011
Group III	Orthosteric agonist	Group III mGluR8 mGluR6 mGluR4	AP4 *S*-SOP (*RS*)-PPG (+)-ACPT-III ACPT-I *S*-Homo-AMPA *S*-AP4	Cartmell *et al*., 1998 Laurie *et al*., 1997 Flor *et al.*, 1998 Acher *et al*., 1997 Acher *et al*., 1997 Ahmadian *et al*, 1997 Laurie *et al*., 1997
	Orthosteric antagonist	Group III	CPPG	Kowal *et al*., 1998
	PAM	mGluR4	SIB-1893 MPEP	Mathiesen *et al.,* 2003

1.2.1.4. Distribution and Functional Roles of mGlu receptors

Distribution

      mGlu receptors are widely distributed in the CNS, suggesting that these receptors have the ability to play a role in numerous functions throughout the brain (Niswender and Conn, 2010). They are specifically concentrated at discrete synaptic and extrasynaptic sites in neurons and glia in nearly every major brain region and their activation results in diverse actions on neuronal excitability and synaptic transmission through the modulation of ion channels and other regulatory and signaling proteins (Niswender and Conn, 2010) (Figure 7).

      In general, group I mGlu receptors are localized in the postsynaptic terminal, and, when activated, often lead to cell depolarization and increases in neuronal excitability. mGlu1 receptor immunoreactivity was shown in the caudate putamen, nucleus accumbens and globus pallidus (Martin *et al*., 1992; Baude *et al.*, 1993; Petralia *et al.*, 1997; Testa *et al.*, 1998; Lavreysen *et al.*, 2004). Moderate expression was found in the substantia nigra pars reticulata. Additionally, mGlu1 receptor binding in rat brain was found in the cerebellum, thalamus, dentate gyrus and medial central gray. Moderate binding was also found in the CA3 of the hippocampus and in the hypothalamus, and, in lower levels, in the basal ganglia and cortex (Lavreysen *et al*., 2004). mGlu5 receptors are widely expressed in the hippocampus, caudate/putamen, lateral septum, cortex and olfactory bulb (Shigemoto *et al.*, 1993; Romano *et al.*, 1995). Astrocytes from the cortex, thalamus, tegmentum, hippocampus and striatum have also been found to express mGlu5 (Bradley and Challiss, 2012).

      In contrast to group I, group II and III mGlu receptors are often localized presynaptically or in preterminal axons where they inhibit neurotransmitter release (Figure 7). This can take place on excitatory, inhibitory and neuromodulatory synapses (Niswender and Conn, 2010).
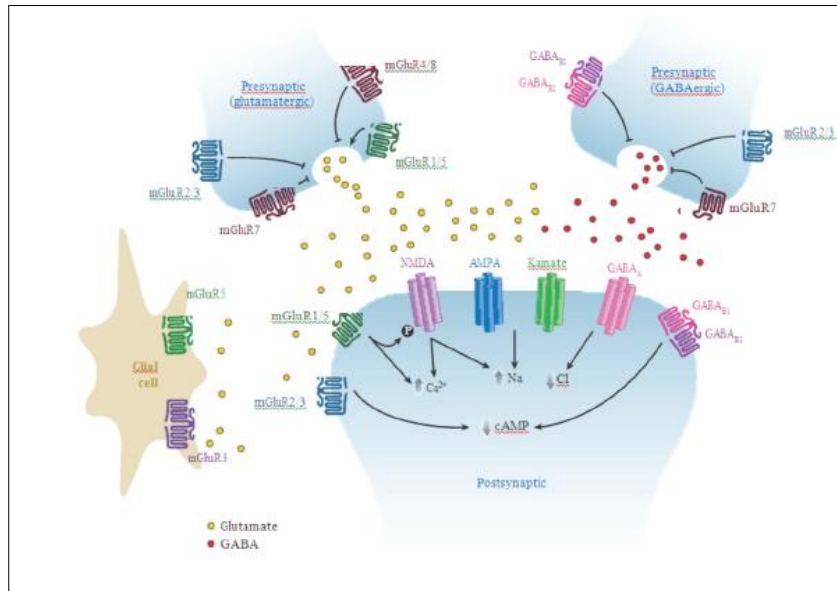
Figure 7: Scheme of general mGlu receptor localization at the synapse. Niswender and Conn, 2010.

Accordingly, group II mGlu receptors (mGlu2 and mGlu3) localize primarily presynaptically in the hippocampus, cortex, striatum, thalamus and amygdala (Ohishi *et al.*, 1993a,b). Studies of *in situ* hybridization (Ohishi *et al.*, 1993a,b), immunohistochemistry (Carlton *et al.*, 2001) and autoradiography (Schaffhauser *et al.*, 1998) demonstrated the expression of mGlu2/3 in the hippocampus, olfactory bulb, neocortical regions, cerebellar Golgi neurons and, with a lower level of expression, in thalamic nuclei and striatum (Schaffhauser *et al.*, 2003). Astrocytes have also been found to express mGlu3 (Bradley and Challiss, 2012). Group III mGlu receptors show an heterogeneous distribution: mGlu4 is highly expressed in cerebellar granule cells (Lavreysen and Dautzenberg, 2008) and is present at lower expression levels in regions like the hippocampus, amygdala, striatum and olfactory bulb (Lavreysen and Dautzenberg, 2008); mGlu6 is found primarily in retinal ON bipolar cells (Nakajima *et al.*, 1993), but there are indications of low level expression in the hippocampus, limbic, cerebellar and cerebral areas (Lavreysen and Dautzenberg, 2008); mGlu7 is broadly distributed in the entire brain, and localizes in the active zones of the synapses (Shigemoto *et al.*, 1997; Kinoshita *et al.*, 1998); finally, mGlu8 is also widely distributed throughout the brain but it is expressed in lower levels than mGlu4 and mGlu7 (Niswender and Conn, 2010); it localizes predominantly presynaptically, but also in some postsynaptic locations and in the periphery (Lavreysen and Dautzenberg, 2008).

30

Functional Roles

The physiological roles of different mGlu receptor subtypes are greatly specific to the neuronal population and even subcellular localization (Niswender and Conn, 2010). mGlu receptor genetic deletion in mice has helped revealing the potential roles for each receptor.

High expression of mGlu1 in the hippocampus indicates a potential role in learning and memory (Niswender and Conn, 2010). In the same lines, mGlu1 deficiency has been shown to lead to a deficit in long-term depression in the cerebellum (Aiba *et al.*, 1994). Lack of mGlu1 seems to lead to abnormal levels of regression of climbing fibers from cerebellar Purkinje cells, suggesting that mGlu1 plays a critical role in maintaining adequate levels of innervations of cerebellar neurons (Levenes *et al.*, 1997). Additionally, it was recently shown that several spontaneous mutations which cause spontaneous ataxia in mice are related to the ligand-binding domain of mGlu1 (hence, mGlu1 function is suggested to be critical for this ataxic phenotype) (Sachs *et al.*, 2007; Niswender and Conn, 2010). mGlu5 is understood to have a role in learning and memory, addiction, motor regulation and obesity and it has been recently related to the treatment of fragile X syndrome (Niswender and Conn, 2010). Deficits in prepulse inhibition (measure of sensorimotor gating that is impaired in schizophrenia patients) were seen in mGlu1 and mGlu5 knock-out animals (Brody *et al.*, 2003 and 2004).

Group II and III mGlu receptors play important roles in the induction of long-term depression (LTD), thus reducing the efficacy of transmission (Bellone *et al.*, 2008; Pinheiro and Mulle, 2008). Activation of mGlu2/3 decreases synaptic transmission and glutamate release in the hippocampus (Macek *et al.*, 1996). mGlu3 seems to have an important role in astrocytes: studies with wild-type, mGlu2 and mGlu3 knock-out mice showed that an agonist of group II mGlu receptors has neuroprotective effects when NMDA is administered to the cells, but that the neuroprotective effect is lost when mGlu3 is absent from astrocytes in the cell culture. In the same studies, mGlu2 activation seemed harmful in terms of excitotoxicity (Corti *et al.*, 2007). mGlu4 knock-out mice show impairments in cerebellar synaptic plasticity and in learning complicated motor tasks (Pekhletski *et al.*, 1996). They also exhibit impaired abilities in spatial memory performance (Gerlai *et al.*, 1998). mGlu4 has furthermore been shown to modulate GABA$_A$ receptor-mediated seizure activity (Snead *et al.*, 2000). mGlu6, which is found primarily in the retinal ON bipolar cells, seems to have a fundamental role in ON responses to light (Masu *et al.*, 1995; Sugihara *et al.*, 1997). mGlu7 has low affinity for glutamate and the deletion of this receptor caused an epileptic phenotype, suggesting that this receptor only becomes active when the levels of glutamate are very high, working as a break

for overstimulation by glutamate (Niswender and Conn, 2010). Furthermore, knockout animals revealed abnormalities in learning tasks. As such, mGlu7 has been implicated in amygdala-dependent learning (Masugi *et al.*, 1999).

The wide presence and range of functions of mGlu receptors throughout the CNS renders them important players in CNS disorders. Based on the distribution and physiological roles of each mGlu receptor subtype, it is possible to relate them to brain circuits or regions implicated in the pathology of CNS-related disorders. Indeed, members of the three groups of mGlu receptors have been related to neurological and psychiatric disorders, such as pain, schizophrenia, anxiety, depression, epilepsy, fragile X syndrome, cognitive disorders, Alzheimer's and Parkinson's disease (Gregory *et al.*, 2011).

Conversely to ionotropic glutamate receptors, whose pharmacological manipulation would need to be highly sensitive to avoid disruption of brain function, mGlu receptors represent an opportunity for developing drugs that regulate glutamate neurotransmission in an indirect and selective manner (Rowe *et al.*, 2008). Due to the wide and heterogeneous distribution of these receptors in the CNS, they may act as selective targets for the development of new treatment strategies for psychiatric and neurological disorders (Niswender and Conn, 2010).

1.2.3. mGlu2 receptor receptor in central nervous system diseases – mGlu2 receptor as a drug target

The distribution and major functions of group II mGlu receptors in neuronal excitability and synaptic transmission, renders the modulation of these receptors a promising strategy for the treatment of neurological and neuropsychiatric disorders (Rowe *et al.*, 2008). Preclinical and clinical studies strongly suggest that several agonists of this group of receptors have potential as a new strategy for the treatment of anxiety disorders and schizophrenia (Niswender and Conn, 2010). mGlu2 receptor, particularly, was shown to selectively mediate the beneficial effects of group II agonists in rodent models of psychosis (Fell *et al.*, 2008; Woolley *et al.*, 2008). The mGlu2 receptor role and its potential for the treatment of schizophrenia and anxiety disorders will be discussed in more detail here.

DCG-IV and (2R, 4R)-APDC were the first selective group II mGlu receptor agonists (Schoepp *et al.*, 1999). More recently, systemically active and highly selective agonists of group II mGlu receptors have been developed, enabling the investigation of *in vitro* and *in*

vivo functions of these receptors. The mGlu2/3 receptor agonists LY379268 and LY354740 cause anxiolytic-like effects and antipsychotic-like activity in animal models used to predict efficacy in the treatment of anxiety disorders and schizophrenia (Conn *et al*., 2009a; Carter *et al*., 2004; Linden *et al*., 2005) and there are even clinical data supporting the use of mGlu2/3 agonists for the treatment of anxiety in humans (Grillon *et al*., 2003). Additionally, LY-2140023, the prodrug of the mGlu2/3 agonist LY-404039, has been shown to improve positive and negative symptoms in schizophrenic patients (Patil *et al.*, 2007; Mezler *et al*., 2010). Unlike currently marketed antipsychotic drugs, there were no major adverse effects reported for the mGlu2/3 agonist in the clinical studies to date. However, chronic administration of group II mGlu receptor agonists induces tolerance in at least one rodent model that has been used to predict antipsychotic efficacy (Conn *et al*., 2009b).

While these compounds are highly selective for group II mGlu receptors, they do not distinguish between mGlu2 and mGlu3, possibly representing a disadvantage since preclinical studies with mGlu2 and mGlu3 knockout mice suggest that mGlu2 is likely to be the responsible for preclinical efficacy (Spooren *et al*., 2000). However, novel compounds that specifically act on mGlu2 have been identified; they act as so-called positive allosteric modulators (PAMs) of mGlu2. Early examples of mGlu2 receptor PAMs include LY487379 and BINA (Conn *et al*., 2009a). These compounds have been shown to be highly selective for mGlu2 (Conn *et al*., 2009a) and they have efficacy in animal models that predict antipsychotic activity that are very similar to those observed with mGlu2/3 agonists (Galici *et al*., 2005). In recent years there was great progress in the discovery, optimization and clinical development of several GPCR subtypes allosteric modulators (Conn *et al.*, 2009 b). Hence, the concept of allosteric modulation will now be further discussed.

1.3. Allosteric modulation of GPCRs

Allosteric – from the Greek "αλλο" (other) and "στερεο" (object, shape) – modulation refers to the regulation of a protein by the binding of a ligand to a site that is topographically distinct from its orthosteric – "ορτηο" (correct) – site (the binding site for the natural or exogenous/competitive agonists on a receptor) (Urwyler, 2011; Conn *et al.*, 2009b). Allosteric modulators induce a change in the three-dimensional receptor conformation, thus affecting the affinities and/or efficacies of orthosteric ligands (Urwyler, 2011; Conn *et al.*, 2009b). Allosteric ligands are, in general, structurally diverse and different from orthosteric ligands, particularly from the endogenous natural agonists (Urwyler, 2011).

Allosteric modulators can be classified according to their effect on the affinity/ efficacy of the natural agonist to the receptor: ligands that enhance agonist-induced receptor activity are referred to as positive allosteric modulators (PAMs) (or allosteric activators), whereas these that decrease agonist activity are termed negative allosteric modulators (NAMs) (or allosteric inhibitors); neutral allosteric ligands bind to the allosteric site, but have no effects on the responses to the orthosteric agonist. Furthermore, these compounds have one or more of the following properties (Figure 8): affinity modulation (the receptor conformational change affects the receptor binding pocket, inducing modifications in the association and/or dissociation rate of the orthosteric ligand), efficacy modulation (the allosteric modulator binding induces a change in the intracellular responses, thus modifying the orthosteric ligand signalling capacity) and agonism/inverse agonism (the allosteric modulator affects receptor signalling in either a positive (agonism) or negative (inverse agonism) way, irrespective of the presence or absence of an orthosteric ligand) (Conn et al., 2009 b).
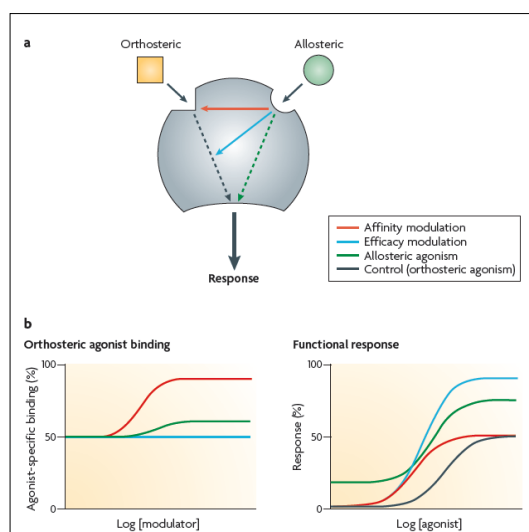
Figure 8: Modes of action of allosteric modulators. a) Allosteric ligands can affect orthosteric ligand affinity (red) and/or efficacy (blue), and even directly perturb signalling in their own right (green). b) Effect of three different allosteric potentiators on the binding (left) or function (right) of an orthosteric agonist: the first (red) enhances orthosteric agonist affinity only; the second (blue) enhances orthosteric agonist efficacy only; the third (green) modestly enhances both affinity and efficacy, and also shows allosteric agonism. Conn *et al.*, 2009b

34

As mentioned before, GPCRs, including mGlu receptors, have been implicated in several human disorders, so they are the target of many therapeutic agents that are currently in use. However, synthetic ligands exist for only a fraction of the known GPCRs and the production of highly selective compounds suitable as drug leads has proven to be difficult (Wood *et al.*, 2011). Several important issues contribute to the difficulty of discovering small-molecule selective agonists or antagonists that act on the orthosteric site of some GPCRs: orthosteric binding sites are highly conserved for a particular endogenous ligand in members of a single GPCR subfamily, which will limit the achievement of subtype selectivity; moreover, ligands at orthosteric sites for some GPCRs have other physicochemical and pharmacokinetic properties that are incompatible with scaffolds that are useful for small-molecule drug discovery (Conn *et al*., 2009b); also, in case of mGlu receptors, ligands that bind the orthosteric binding site are frequently glutamate analogues, and for that reason they typically lack the bioavailability and/or CNS penetration desired in a probe or drug candidate (Wood *et al*., 2011). Finally, a main issue with classical GPCR agonists is that after repeated dosing they often cause receptor desensitization (Rowe *et al.*, 2008). Allosteric modulators can, however, overcome some of these obstacles.

Whereas orthosteric agonists stimulate a receptor independently of its physiological state, allosteric ligands that have no agonist activity only exhibit their effects in receptors activated by endogenous agonists, being quiescent in the absence of endogenous orthosteric ligand. Therefore, these modulators act much more in concert with the temporal and spatial organization of endogenous physiological signalling (Conn *et al.*, 2009b), having a lower side effect potential and a lower propensity to induce receptor desensitization (Urwyler, 2011). As opposed to orthosteric binding sites, allosteric binding sites show a higher sequence divergence and hence allosteric modulators can display greater selectivity than orthosteric ligands (Urwyler, 2011; Christopoulos, 2002). Due to the size and number of attachment sites of the recognition domain of the endogenous ligands, it is difficult to design low-molecular-weight synthetic orthosteric ligands that exhibit good bioavailability and/or CNS penetration (Urwyler, 2011).

There are, however, some negative aspects of allosteric modulators that are worth to be mentioned: sequence divergence of allosteric binding sites can result in significant differences between species, which will constitute a problem whenever rodent receptors/models are used for drug screening or for *in vivo* trials of an allosteric compound characterized *in vitro* at human receptors; the activity-dependence of PAMs might be seen as a disadvantage in neurodegenerative diseases in which the loss of neurons results in

decreased availability of the endogenous agonist; the "flat", non-tractable structure-activity relationships that often characterize GPCRs allosteric modulators would be a barrier to the improvement of lead molecules; finally, the complexity of the biology of a disease, which is often not completely understood, makes it still impossible to find an optimal profile of an allosteric drug (Urwyler, 2011).

The ubiquity of GPCRs in the CNS renders it important to create a mechanism of smoother and selective activation of these receptors and allosteric modulators can provide that mechanism. This is considered an emerging area of drug discovery. Whilst GPCR ligands likely represent ~50% of all marketed drugs, only two of these, Cinacalcet and Maraviroc, are marketed allosteric modulators. This is due to the different approaches needed to identify allosteric modulators compared to orthosteric ligands and the relative infancy of the field.

Although allosteric modulators generally display much better features in specificity when compared to orthosteric ligands, there are, as mentioned before, a few examples of allosteric modulators interacting with multiple receptor subtypes: MPEP and SIB-1893 are both mGlu5 NAMs and mGlu4 PAMs; DFB and CPPHA, mGlu5 PAMs, also display weak NAM activity on mGlu4; and PHCCC has combined mGlu4 PAM and mGlu1 NAM activity. This duality does suggest similarities in allosteric binding sites across different receptors (Gregory *et al*., 2011). For that reason, the characterization of the binding sites of these compounds is a fundamental step towards the identification of drugs with increased potency and selectivity. Moreover, as more allosteric modulators will likely reach the clinic, it will be crucial to understand more about their binding site and mechanism of action. Several mutagenesis studies, which have been performed to that aim, have been reported in the literature, and will now be further discussed.

1.4. Mutational Studies on mGlu receptors

13 years ago, Litschig *et al.* (1999) reported the first compound acting at and inhibiting an mGlu receptor without affecting the binding of the endogenous agonist. Based on the sequence alignment of hmGlu1b and hmGlu5a, Litschig *et al.* identified which amino acids could be playing a role in the selectivity of CPCCOEt for hmGlu1b over hmGluR5a. Accordingly, they systematically exchanged segments and non-conserved single amino acids

between these two subtypes to precisely identify the amino acids mediating the effect of this compound (Listchig *et al*., 1999). Since then, a number of mutagenesis studies on mGlu receptors have been published (Table 3) with the purpose of investigating the molecular determinants of these receptors. These studies have been performed through the use of chimeric receptors, which share segments from different receptor groups/subtypes, point mutations on these receptors, and molecular modelling through the alignment of the receptor to the TM membranes of the bovine rhodopsin receptor and *in silico* docking of compounds.

Mutational studies generate important information on the study of mGlu receptors and can be important to reveal how extracellular signals are transmitted into the cells (Listchig *et al*., 1999). Not only have these studies allowed the mapping of the regions of mGlu receptors that are critical for modulation of agonist activity (Schaffhauser *et al*., 2003) they also bring insight to the pharmacological profile, site of action and the potential binding mode of novel mGlu receptors ligands, allosteric modulators included. Furthermore, the need to identify novel selective ligands that are specific for each mGlu receptor subtype to characterize the physiological role of individual receptors (Litschig *et al*., 1999) and to improve selectivity and potency – potentially useful for pharmacological modulation of mGlu receptors – makes these studies extremely relevant (Pagano *et al*., 2000; Malherbe *et al*., 2003).

In order to predict which amino acids can be playing a role in the interaction ligand/receptor, it is essential not only to know the amino acid sequence of the receptor, but also its structure, which is inferred using a template from a known GPCR structure. This structure can then be used for the docking of allosteric modulators, allowing the establishment of a putative model of ligand/receptor interaction. For this purpose, sequence alignment and homology modelling have to be performed.

Tabe 3: Summary of the amino acids identified as important important molecular determinants of mGlu receptors through mutagenesis studies performed on mGlu receptors. b.s-binding site; hmGluR (human metabotropic glutamate receptor; rmGluR (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	Ro 01-6128 Ro 67-4853		Ser668		Val757			Knoflach et al., 2001	
rmGluR5	Ro 67-7476 (PAMs)		Cys671		Pro654 Ser657				
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.

Sequence alignment and homology modelling

Homology modelling is used to predict the structure of a protein from its sequence (Krieger *et al*., 2003). It is based on two major observations: the structure of a protein is uniquely determined by its amino acid sequence (Epstain *et al*., 1963), which means that, theoretically, the structure of a protein can be obtained once its sequence is known (Krieger *et al*.., 2003); in terms of evolution, the structure is more stable than the associated sequence, so that similar sequences still adopt the same structure (Chothia and Lesk, 1986; Sander and Schneider, 1991). Homology modelling can be described as a seven step procedure: 1) template recognition and initial alignment, 2) alignment correction, 3) backbone generation, 4) loop modelling, 5) side-chain modelling, 6) model optimization and 7) model validation.

High-resolution 3-dimensional (3D) structures of proteins provide important information on the form and function of those proteins at a molecular level. Not only does this information elucidate the aspects of protein structure which underlie physiological processes, it is also helpful to examine the interactions of proteins to their ligands and to small molecule drugs (Congreve and Marchall, 2010). Palczewski *et al.* determined for the first time the 3D crystal structure of a mammalian GPCR, the visual pigment rhodopsin (Figure 9) (Palczewski *et al*., 2000). The 3D crystal structure of bovine rhodopsin built by Palczweski *et al.* provided a structural template for other GPCRs, enabling the modeling of other members of this receptor family. The structure of bovine rhodopsin contains many features common to most GPCRs (Palczweski *et al.*, 2000). Furthermore, the molecular size of rhodopsin is intermediate among the members of the GPCR family, and the length of its 7TM helices and extracellular loops are comparable to most of the GPCRs (Palczweski *et al.*, 2000). Homology models based on rhodopsin structure have been used for the study of allosteric interactions at members of class C GPCRs. Even though the sequence similarity between class C GPCRs and rhodopsin-like class A GPCRs is low, the allosteric binding site of class C receptors share structural features with the orthosteric binding site of the class A family (Lundström *et al.,* 2011).
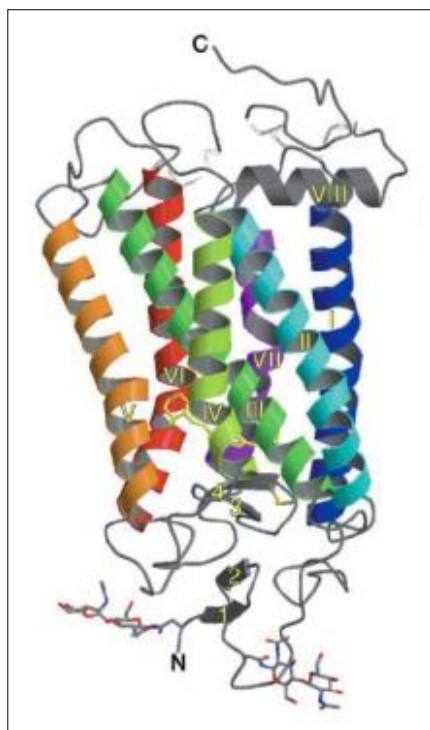
Figure 9: Three-dimensional representation
of bovine rhodopsin (Palczewski *et al.*, 2000)

Until recently, bovine rhodopsin was the best structure of GPCR receptors and the investigation on drug-receptor interaction was limited to models based on homology with this structure or from site-directed mutagenesis experiments (Congreve and Marshall, 2010). Recently, however, the structures of other GPCRs have been identified (namely β1 (Warne *et al*., 2008) and β2 (Cherezov *et al*., 2007) adrenergic receptors and adenosine A2a receptor (Jaakola *et al*., 2008)) enabling a wider analysis of structural differences between the different receptors.

1.5. Goal of the project

Janssen Pharmaceutica has an mGlu2 receptor PAM in clinical development for the treatment of schizophrenia. In addition, as part of an internal mGlu2 receptor PAM program, other potentially important compounds from different chemical series were identified. In order to understand the mechanisms through which these compounds might produce their effects, it is important to clarify where they bind to the receptor molecule, which receptor region is involved and which amino acids are critical for the binding and/or activity.

In order to identify amino acids potentially important for the interaction between PAMs and the mGlu2 receptor, homology modelling and docking of mGlu2 receptor PAMs was performed in parallel with experimental site directed mutagenesis. Accordingly, mutant mGlu2 receptors were produced. The goal of this study is to evaluate the impact of the receptor mutations on the activity of several PAM compounds in order to confirm the role of the selected amino acids in the binding and/or activity of PAMs. This work is an important contribution to the characterization of the allosteric binding site of mGlu2 receptors.

Chapter 2
Materials and Methods

2.1. Materials

The fourteen PAMs tested in this study (Appendix 2 for name and chemical series) 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) 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 purchased from Abcam (Cambridge, UK).

2.2. Positive allosteric modulators tested

11 compounds used in this study are part of an internal mGlu2 receptor PAM program, and were identified by lead optimization of hits originating from high-throughput screening through a calcium mobilization assay performed on the mGlu2 receptor. The compounds belong to the following chemical classes: 1,4-pyridones, 1,5-pyridones, triazolopyridines, isoquinolones and imidazopyridines. 3 reference PAMs were also tested: BINA (Galici *et al.*, 2006), the acetophenone LY2605740 (Fell *et al.*, 2011) and LY487379 (Schaffhauser *et al.*, 2003). Appendix 2 summarizes the chemical series and name of each compound used.

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

The identification and selection of mGlu2 receptor amino acids potentially important for PAMs binding, and therefore important targets of mutagenesis studies, was based on different approaches. The work described in this section was performed by a co-worker, Gary Tresadern (Molecular Informatics).

Due to the significant difference in the conformation of the EL2, and given the fact that this domain has an important role in ligand binding, the mGlu2 receptor structure was aligned to both rhodopsin (1GZM) and β2-adrenergic receptor (2RH1) structures, and certain conserved motifs between the different receptors were used as constraints to guide the

44

alignment. The alignment was performed using Molecular Operating Environment (MOE) software with the Protein Align tool (Chemical Computing Group, Canada).

In order to establish a possible binding mode of mGlu2 receptor PAMs, an mGlu2 receptor reference PAM was manually docked into the mGlu2 receptor models built from the bovine rhodopsin and β2-adrenergic receptor templates, using MOE. Figure 10 shows the best ranked docking pose, which was obtained from the mGlu2 receptor model built from the β2-adrenergic receptor template. The potentially important amino acids were then selected based on 1) their proximity to the ligand (Figure 10) and 2) literature referring amino acids at the same position in other mGlu receptors (Figure 11A). Hence, Arg636, Leu639, Phe643, Asp725, Leu732, Trp773, Phe776 and Phe780 were selected as candidates for site-directed mutagenesis studies.

Given the fact that most of the PAMs are mGlu2 receptor-selective, the sequences of the mGlu2 and mGlu3 receptor were compared in order to identify which amino acids differ between the two sequences and, therefore, are potentially important for the selective binding of mGlu2 receptor PAM compounds (Figure 11B). The main differences were seen in the extracellular portion of TM3, TM4, EL2 and TM5.

Overall, based on these different approaches (molecular modeling and docking, sequence alignment and also literature), 40 amino acids were identified as potentially important for the PAM-mGlu2 receptor interaction (Table 4). Mutant mGlu2 receptor cDNA constructs were prepared by GeneArt® (Life Technologies) and for this project 21 mGlu2 receptor mutants (highlighted in blue in Table 4) were further tested.

Figure 10: Ribbon diagram of the binding mode of a reference PAM compound in the mGlu2 model structure built from β2-adrenergic template

Figure 11: 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

45

Table 4: mGluR2 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). The first 21 mutations, highlighted in light blue, were tested on this study.

WT AA	Position	Mutation	WT codon	Mutant codon	Name	Receptor region	
Ser	688	Leu	TCG	TTG	$S_{688}L$	TM4	*Literature (PAM [1])*
Gly	689	Val	GGC	GTC	$G_{689}V$		
Asn	735	Asp	AAT	GAT	$N_{735}D$	TM5	
Ser	688	Leu	TCG	TTG	$S_{688}L$	TM4	
Gly	689	Val	GGC	GTC	$G_{689}V$		
Ser	688	Leu	TCG	TTG	$S_{688}L$	TM4/5	
Gly	689	Val	GGC	GTC	$G_{689}V$		
Asn	735	Asp	AAT	GAT	$N_{735}D$		
Arg	636	Ala	CGT	GCT	$R_{636}A$	TM3	*Docking*
Leu	639	Ala	TTG	GCG	$L_{639}A$		
Phe	643	Ala	TTC	GCC	$F_{643}A$		
Asp	725	Ala	GAT	GCT	$D_{725}A$	TM5	
Leu	732	Ala	CTG	GCG	$L_{732}A$		
Trp	773	Ala	TGG	GCG	$W_{773}A$	TM6	
Phe	776	Ala	TTC	GCC	$F_{776}A$		
Phe	780	Ala	TTC	GCC	$F_{780}A$		
Ser	644	Ala	TCT	GCT	$S_{644}A$	TM3	*mGluR2/3 comparison*
Val	700	Leu	GTC	CTC	$V_{700}L$	TM4	
His	723	Val	CAC	GTC	$H_{723}V$	EL2	
Ser	644	Ala	TCT	GCT	$S_{644}A$	TM3/4/EL2	
Val	700	Leu	GTC	CTC	$V_{700}L$		
His	723	Val	CAC	GTC	$H_{723}V$		
Arg	635	Ala	AGA	GCG	$R_{635}A$	TM3	*Literature (NAMs [2])*
Met	728	Ala	ATG	GCG	$M_{728}A$	TM5	
Ser	731	Ala	AGC	GCC	$S_{731}A$		
Val	736	Ala	GTG	GCG	$V_{736}A$		
Val	798	Ala	GTG	GCG	$V_{798}A$	TM7	
Ala	681	Phe	GCC	TTC	$A_{681}F$	TM4	*mGluR2 vs mGluR3*
Ile	693	Met	ATT	ATG	$I_{693}M$		
Val	695	Ser	GTG	TCG	$V_{695}S$		
Ala	696	Val	GCC	GTC	$A_{696}V$		
Gly	706	Arg	GGA	CGA	$G_{706}R$	EL2	
Glu	708	Tyr	GAG	TAC	$E_{708}Y$		
Ala	710	Leu	GCC	CTC	$A_{710}L$		
Pro	711	Ala	CCC	GCC	$P_{711}A$		
Val	716	Thr	GTG	ACG	$V_{716}T$		
Thr	718	Ile	ACC	ATC	$T_{718}I$		
Ala	726	Ser	GCC	TCC	$A_{726}S$	TM5	
Gly	730	Ile	GGC	ATC	$G_{730}I$		
Ala	733	Thr	GCC	ACC	$A_{733}T$		
Ala	740	Ile	GCC	ATC	$A_{740}I$		
Cys	616	Ser	TGC	TCC	$C_{616}S$	TM2	
Ile	622	Phe	ATC	TTC	$I_{622}F$		
Thr	641	Ser	ACC	TCC	$T_{641}S$		
Ala	642	Ser	GCC	TCC	$A_{642}S$		

[1] Baez *et al*, 2002; Schaffhauser *et al*., 2003; Hemstapat *et al*., 2007; Rowe *et al*., 2008.

[2] Lündstrom *et al*., 2011

46

2.4. Chemical transformation of One Shot® Top10 *E. coli* cells with point-mutated human mGlu2 receptors and DNA purification

1 µl of cDNA was added to 50 µl of One Shot™ competent cells (Life Technology) and the mixture was incubated 30 min on ice. After that, cells were heat shocked (30 sec at 42ºC) and rapidly returned on ice for 2 min. 250 µl of S.O.C. medium (Invitrogen™; formulation per liter: 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose) was added and the cells were incubated for 1 h at 37ºC in a rotary shaking incubator (InnovaTM 4230, New Brunswick Scientific, Edison, NJ, USA), at 225 rpm. From this, 20 µl was spread in a plate and incubated for 18 h at 37ºC.

In order to prepare a starter culture, one colony was picked from the transformation plate to inoculate 5 ml Luria-Bertani (LB) medium containing 100 µg/ml ampicilin. The culture was incubated for approximately 6 h at 37 ºC and 300 rpm, in a rotary shaking incubator (InnovaTM 4230, New Brunswick Scientific, Edison, NJ, USA).

For large scale preparation, 500 ml LB medium supplemented with 100 µg/ml ampicilin were inoculated with 2.5 ml of the starter culture and incubated overnight at 37ºC and 300 rpm (Model G25 Incubator Shaker, New Brunswick Scientific, Edison, NJ, USA). The cells were then harvested by centrifugation (Multifuge 3 S-R, Heraeus, Buckinghamshire, England).

DNA purification from the recombinant *E. coli* cells was performed using the Endofree® Plasmid Maxi/Mega Purification kit (Qiagen GmbH, Hilden, Germany), according to manufacturer's instructions. After purification, the DNA was analyzed through sequencing.

2.5. 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. Solution A consists of Penicillin G (Serva, Bioconnect, Huissen, The Netherlands) 5.10e6 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 37ºC in a humidified atmosphere with 5% CO_2.

2.6. Transient transfection of human mGlu2 receptor cDNA into CHO-K1 cells

CHO-K1 cells were seeded in 145 cm^2 Petri dishes (Nunc^{TM}, Roskilde, Denmark) at a seeding density of 20000 cells/cm^2 in DMEM supplemented with 10% heat-inactivated FBS and 2% Solution A. 24h later, when a confluence of 50-70% was reached, human mGlu2 receptor (hmGluR2) cDNA, WT and mutated (Table 4, mutation 1 – 21) was transiently transfected into CHO-K1 cells using Lipofectamine$^®$ (Invitrogen^{TM}, Life Technologies^{TM}), and the recombinant cell lines were incubated overnight in the same medium, at 37ºC and in an atmosphere of 5% CO_2. One plate was used for transfection with the plasmid encoding the Enhanced Green Fluorescent Protein (EGFP-N1) to be used as a control of the transfection efficiency. 20-24 h after the transfection, the medium was replaced by fresh medium, and 20-24 h after the medium replacement butyrate (final concentration of 5 mM) was added to each plate. The transfection efficiency was qualitatively evaluated at this point through the visualization of the cells transfected with EGFP-N1, using a fluorescence microscope (Axiovert 135, Zeiss, N.V., S.A.). 20-24h after the addition of butyrate, the plates with recombinant cells were washed twice with ice-cold phosphate-buffered saline (PBS) and stored at -80 ºC, or used readily for membrane preparation.

2.7. Membrane preparation

Confluent plates with transfected CHO-K1 cells expressing the wild-type or mutant hmGlu2 receptors were harvested with a cell scraper and resuspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4. The cell suspension was, from this moment on, always kept on ice. The cell suspension was centrifuged for 10 min at 16000 rpm in a Sorvall 5C PLUS SS34 centrifuge at 4ºC. The resulting cell pellet was resuspended and homogenized in ice-cold 5 mM Tris-HCl, pH 7.4, using an Ultra Turrax homogenizer (IKA TE5) at 24000 rpm. Additional 5 mM Tris-HCl was added to the homogenate and it was centrifuged again for 20 min at 18000 rpm in a Sorvall RC 5B/RC 28S centrifuge at 4ºC. The final *pellet* was resuspended and homogenized in 50 mM Tris-HCl using the Ultra Turrax homogenizer, and

the membrane suspension was aliquoted in cryovials and frozen at -80ºC. The protein concentration was measured using the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munich, Germany) using bovine serum albumin (Sigma, St. Louis, MO, USA) as standard.

2.8. Western Blot analysis

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 lysate was incubated 30 min on ice, and centrifuged at 14000 rpm (20 min, 4ºC). The supernatant fraction was recovered to a fresh tube and the protein concentration was determined by BCA™ Protein Assay (Sigma-Aldrich).

3 µg of protein was loaded on NuPAGE® Novex® Tris-Acetate 4-12% Bis-Tris Gel (Life Technologies). The electrophoresis ran initially at 100 V, after which the voltage was increased to 170-200 V. The proteins on the gel were blotted for 8 min on a nitrocellulose membrane through a dry blotting system (iBlot, Life Technologies). Membranes were blocked for 1h at RT with Non-Fat Dry Milk (NFDM) (Santa Cruz, Technology) (5% w/v) diluted in Tris buffer (10mM Tris pH8.0, 150mM NaCl and 0.05% v/v Tween 20) (Amersham Biosciences, GE Healthcare, Little Chalfond Buckinghamshire, UK) (TBS-T), after which they were washed three times in TBS-T. The membranes were then 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 4ºC, with gentle agitation. Primary antibodies were detected through the 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).

2.9. Radioligand binding assay – [³H]-LY341495 binding

<u>Theoretical background</u>

The binding of a ligand to its receptor is the first and indispensable step in the chain of reactions that will lead to a pharmacological effect (de Jong *et al*., 2005). Therefore, the process of ligand binding can be measured to investigate receptor molecules and the binding properties of a given compound acting on the receptor (Vauquelin and von Mentzer, 2007).

Radioligand binding assays are based on the ability of an analyte (unlabelled ligand) to displace a radioactively labelled ligand (radioligand) that binds to the receptor of interest. The radioactively labelled ligand must be appropriate to be used in a radioligand binding assay, and therefore it must obey several criteria: it should exhibit high affinity and selectivity to the receptor that is being studied; it should display a high specific activity towards the receptor; and it should be radiochemically pure, stable and resistant to enzymatic degradation (de Jong *et al*., 2005; Vauquelin and von Mentzer, 2007). Tritium (^{3}H) and iodine (^{125}I) are the most commonly used isotopes for ligand radio labelling: tritium exhibits a longer half-life than iodine (12.3 years over 60 days, respectively), which enables longer periods of storage, but since it has a lower specific radioactivity than iodine (29 Ci/mmol over 2125 Ci/mmol, respectively) it is only suitable when the tissue under study contains sufficient amounts of the receptor of interest (Vauquelin and von Mentzer, 2007).

Radioligand binding studies involve three important concepts: *specific binding* (the binding to the receptor of interest), *non-specific binding* (the observed binding in the presence of an appropriate excess of unlabeled competitor ligand – corresponds to binding to filters, absorption to the tissue, dissolution in membrane lipids) and *total binding* (the combination of specific and non-specific binding). Specific binding is determined by subtracting the non-specific binding from the total binding. Receptor-bound radioligand is separated from free through filtration, centrifugation or suction, followed by the measurement of the remaining radioactivity.

Radioligand binding studies can be performed through different experimental approaches. Saturation binding experiments are performed using a constant amount of target receptor and increasing concentrations of radioligand. From these experiments the amount of the receptor of interest can be determined. Furthermore, the K_D of a radioligand, *i.e.* the concentration of radioligand needed to bind 50% of the receptors and hence a measure of the affinity of the radioligand for the receptor, can also be determined. Competition binding

experiments use constant amounts of protein and radioligand, but increasing concentrations of an unlabeled ligand to be tested. These experiments provide information on the affinity of that ligand to a receptor. Finally, kinetic experiments enable the determination of the rate constants for radioligand association and dissociation.

Procedure

In this study, both saturation and competition binding experiments were performed. hmGluR2-CHO-K1 membranes were thawed 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). Membranes were diluted in ice-cold binding buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 2 mM $CaCl_2$. The reaction mixture contained 10 µg of membrane protein and 3 nM of [^{3}H]-LY341495 in a total volume of 500 µl. In order to measure the non-specific binding, 1 mM glutamate was used. The reaction mixture was incubated for 60 min at room temperature (RT). The incubation was stopped with a filtration step using Unifilter-96 GF/B filter plates in a 96-well PerkinElmer filtermate harvester, and the plates were dried overnight at RT. The remaining radioactivity was measured in a Microplate scintillation and luminescence counter (Packard).

51

2.10. [^{35}S]GTPγS binding assay

Theoretical background

This functional assay measures the level of G protein activation that follows agonist occupation of a GPCR. The activation of a GPCR involves the exchange of GDP for GTP at the Gα-subunit of G-protein heterotrimers. This event is followed by the dissociation of the G-protein α-subunit from the βγ complex, which will then both elicit downstream signal transduction events. The Gα subunit possesses an endogenous GTPase activity, which will cause the GTP hydrolysis into GDP, an event enabling the reassembly of Gα and Gβγ subunits and the termination of the receptor activity, providing a mechanism to restrain the duration of the signal (Figure 2.1a). ^{35}S-labeled guanosine 5′-O-(γ-thio)triphosphate ([^{35}S]GTPγS) is an analogue of GTP that is also able to bind to the α-subunit, but is resistant to its GTPase activity; because this non-hydrolysable form of GTP remains bound for a sufficient period of time, it allows counting of the amount of [^{35}S] label incorporated (Lazareno, 1997; Kowal *et al*., 1998) (Figure 2.1 b).
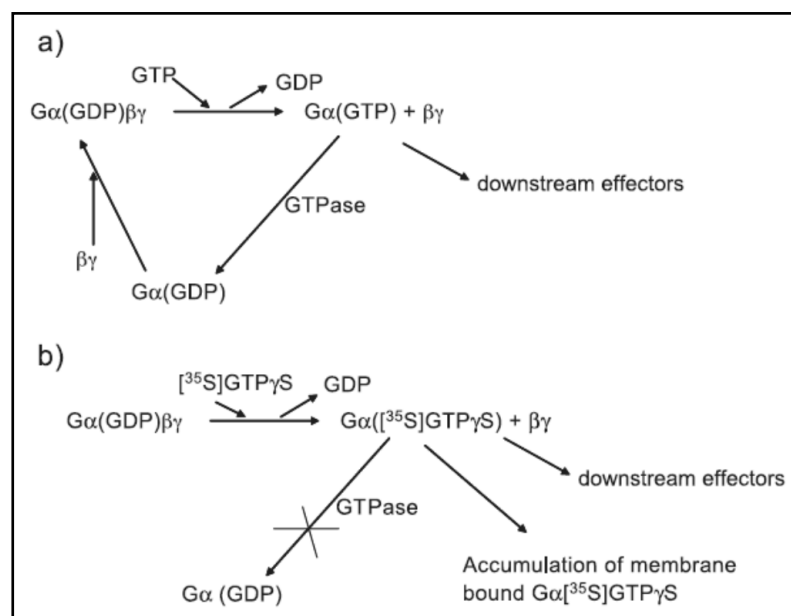
Figure 12: **a)** Once an agonist binds to a receptor, GDP is replaced by GTP on the Gα subunit of the G protein; Gα subunit and Gβγ complex both activate cellular effectors; the GTPase activity of α subunit hydrolyses GTP to GDP, the three subunits reassemble and the receptor is turned off. **b)** When [^{35}S]GTPγS is present, its exchange for GDP also occurs, but the GTPase activity of the Gα subunit is unable to hydrolyse [^{35}S]GTPγS, which accumulates. Harrison and Traynor, 2003

52

This functional assay measures an early receptor-mediated functional response – important to avoid amplification that can occur when analyzing parameters further downstream of the receptor – and it enables the determination of basic pharmacological parameters of compounds acting at GPCRs, such as potency, efficacy and antagonist affinity (Harrison and Traynor, 2003).

<u>Procedure</u>

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 kit. The membranes were diluted in assay buffer (pH 7.4 solution of 10 mM HEPES acid and 10 mM HEPES, containing 100 mM NaCl, 3 mM $MgCl_2$, 10 μM GDP and 14.3 μg/ml saponin).

The assay mixture (final total volume of 200 μl) contained 18 μl of assay buffer, 2 μl of test compound (at 10-fold final concentration), 20 μl of glutamate and 10 μg of membrane protein, after the addition of which a pre-incubation step of 30 min at 30 ºC was performed. Finally, 0.1 nM [^{35}S] GTPγS was added and the assay mixture was incubated for another 30 min at 30 ºC. The reaction was stopped through filtration using Unifilter-96 GF/B filter plates (PerkinElmer Life Sciences, Boston, MA) in a 96-well PerkinElmer filtermate harvester, in order to remove the unbound [^{35}S] GTPγS. 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^{TM}O (Perkin Elmer, MA, USA) was added to each well, covered with a plate sealer, and 30 min later the remaining radioactivity was counted in a Microplate scintillation and luminescence counter (Packard). When testing compounds for their PAM effect, the GTPγS assay was performed using 4 μM glutamate (corresponding to the EC_{20} of glutamate) as agonist.

2.11. 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.

Chapter 3

Results

3.1. Expression of WT and mutant mGlu2 receptors in CHO-K1 cells

Once purified, DNA from WT and mutant hmGlu2 receptors was analyzed through sequencing, which confirmed the correct sequence for all cases.

The efficiency of transient transfections into CHO-K1 was qualitatively evaluated through EGFP-N1 expression and consequent fluorescence each time a new transfection was performed, which always revealed an efficiency of about 30-35% (Figure 13).
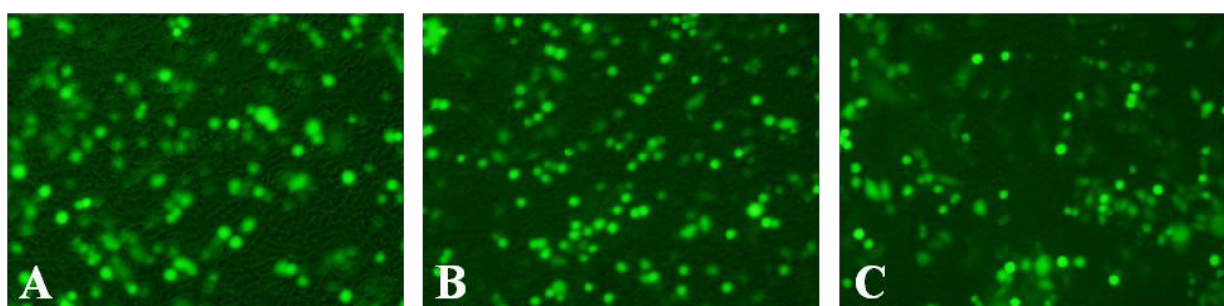
Figure 13: Visualization of transient transfection efficiency in CHO-K1 cells, through expression of EGFP-N1; this procedure was repeated each time a transfection was performed; the displayed images are representative examples of this evaluation, and correspond to the transfection of A: WT hmGlu2, B: Mutation G689V and C: Mutation R636A

In order to assess the expression of WT and mutated receptors in membranes of CHO transfected cells, Western blots were performed using an antibody that recognizes a 47 amino acid sequence of the C-terminal tail of mGlu2. Non-transfected CHO-K1 (referred to as CHO WT), CHO-K1 stably transfected with WT hmGlu2 (referred to as CHO mGlu2 Stable) and CHO-K1 transiently transfected with WT hmGlu2 (referred to as CHO mGlu2 Trans.) were used as controls. Except for the non-transfected cells, for each sample, two main bands were detected: a immunoreactive band running at ~ 100 kDa corresponding to the monomeric form of mGlu2 and a band running at ~200 kda corresponding to the homodimer. The immunoblot analysis (Figure 14) revealed the differences in expression levels for the different samples, but overall confirms the expression of each mGlu2 receptor (WT and mutant) in CHO-K1 cells. Mutant W773A (#11) displays a rather low expression level of the mGlu2 homodimer.
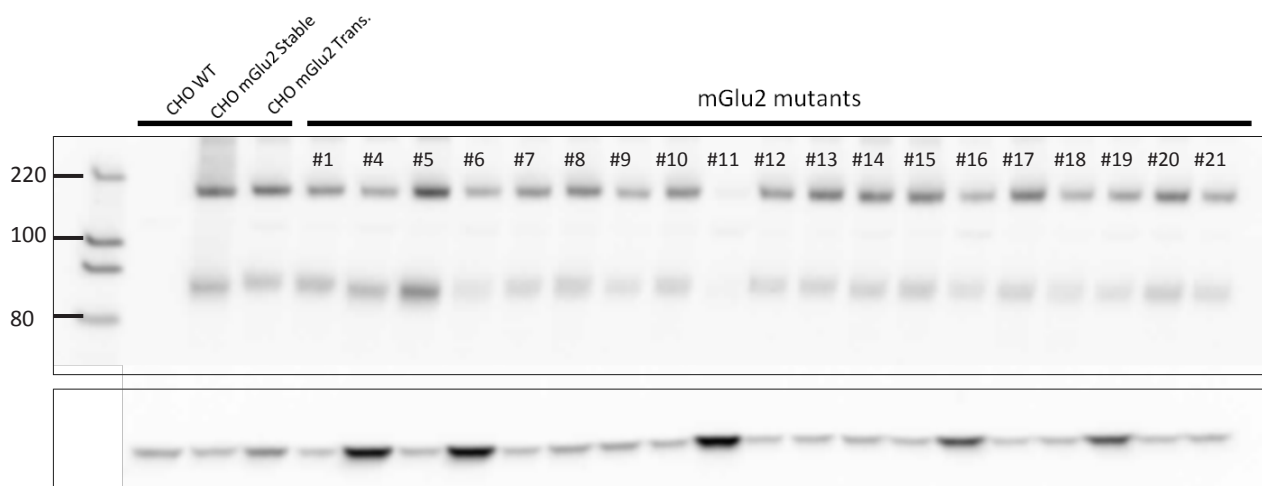
Figure 14: Western blot analysis on mGlu2 WT and mutant receptors to illustrate the expression of the receptors in CHO-K1 cells after stable (for WT hmGlu2) or transient transfection (for WT and mutant hmGlu2). Mutants are indicated with the corresponding mutation number (See Table 6, Methods section). As expected, the monomer (~100 kDa) and dimer (~200 kDa) are observed and this staining pattern is the same for WT and mutant mGlu2. Actin (antibody diluted 1:2000 in TBS-T) is presented as a loading control. Molecular weight markers are indicated in kDa. A representative immunoblot of 2 independent experiments is shown.

To further confirm the expression of mGlu2 in CHO-K1 cells, and to observe whether any of the mutations affected the orthosteric binding site or the receptor conformation needed for binding of orthosteric molecules like glutamate, radioligand binding studies were performed using the orthosteric mGlu2/3 receptor antagonist [3H]-LY341495.

All mutated receptors showed similar specific [3H]-LY341495 binding (in the range of 90%), confirming that the orthosteric site had remained intact in the mutated receptor forms. Furthermore, glutamate was able to displace [3H]-LY341495 in a concentration-dependent manner, both from the receptor stably expressed in CHO-K1 cells and from transiently transfected WT or mutant receptors (Figure 15), confirming that mutant receptors were still able to bind glutamate.
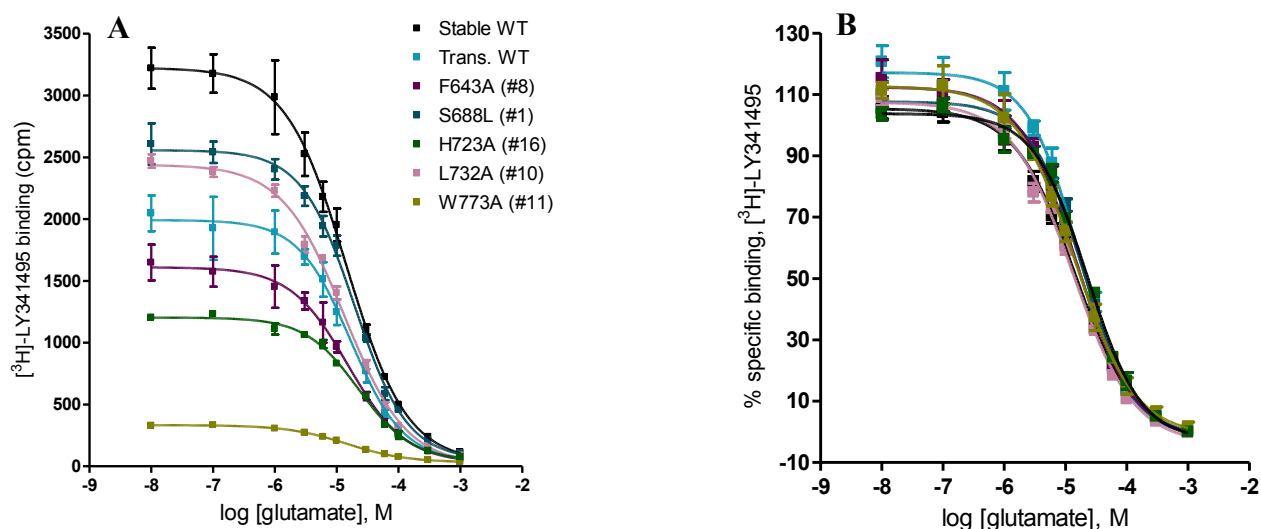
Figure 15: Displacement of [³H]-LY341495 binding from hmGlu2-WT (stably and transiently transfected) and hmGlu2 mutants by glutamate. The affinity of glutamate is not altered by any of the mutations (see Table 5 for all the results). **A)** Total binding results are presented as counts per minute (cpm); data presented as mean±S.D. of one experiment performed in triplicate. **B)** Results are presented as percentage of specific binding, i.e. total binding (binding in the absence of glutamate; corresponds to 100%) minus non-specific binding (binding in the presence of 1 mM glutamate)); data is presented as mean±S.D. of one experiment performed in triplicate.

Figure 15A does show that, for different mGlu2 constructs, the absolute values of total binding vary, which is likely due to differences in receptor expression levels. Namely mutation W773A (#11) shows a prominent decrease in total binding, consistent with the results obtained in Western blot analysis. pIC_{50} values for glutamate binding inhibition were similar for all receptors (pIC_{50} of ~5 corresponding to an IC50 of 10 μM) (Table 5) (n=1 or n=2).

Table 5: Summary of pIC50 values for displacement of [3H]-LY341495 binding from hmGlu2-WT (stably and transiently transfected) and hmGlu2 mutants by glutamate. Mutations are sorted by receptor region. For WT, data is presented as mean±SD of at least 3 concentration-response measurements performed in triplicate; for mutants S688L (#1), F643A (#8), L732A (#10), W773A (#11) and H723V (#16) data is presented as mean±SD of at least 2 concentration-response measurements performed in triplicate; for the restant mutations, the results of one concentration-response measurement performed in triplicate are presented.

Receptor Region	Mutation	pIC50 glutamate
---	Stable WT	4.9 ± 0.10
---	Transient WT	4.9 ± 0.07
TM3	R635A (#18)	4.9
TM3	R636A (#6)	4.8
TM3	L639A (#7)	4.9
TM3	F643A (#8)	4.8 ± 0.06
TM3	S644A (#14)	4.7
TM4	S688L (#1)	4.7 ± 0.06
TM4	G689V (#2)	4.7
TM4	S688L/G689V (#4)	4.7
TM4	S688L/G689V/N735D (#5)	4.7
TM4	V700L (#15)	4.9
TM4	S644A/V700L/H723V (#17)	4.8
EL2/TM 5	H723V (#16)	4.6
EL2/TM 5	D725A (#9)	5.0
EL2/TM 5	M728A (#19)	4.9
EL2/TM 5	S731A (#20)	5.0
EL2/TM 5	L732A (#10)	4.9 ± 0.06
EL2/TM 5	N735D (#3)	4.8
EL2/TM 5	V736A (#21)	4.9
TM 6	W773A (#11)	4.9 ± 0.12
TM 6	F776A (#12)	4.8
TM 6	F780A (#13)	4.9

It is of note that the differences in expression level were also evaluated with a modest set of saturation experiments using [3H]-LY341495; as can be seen from Table 6 and Figure 16, for the tested mutations (S688L (#1), G689V (#2), R636A (#6) and L639A (#7)), these studies furthermore confirmed that expression levels in the transiently transfected cell membranes could vary and that expression levels were lower than in the stably transfected cells. Nevertheless, the K_D of [3H]-LY341495 was unchanged, indicating that also orthosteric antagonists bind with similar affinity to the orthosteric binding site.
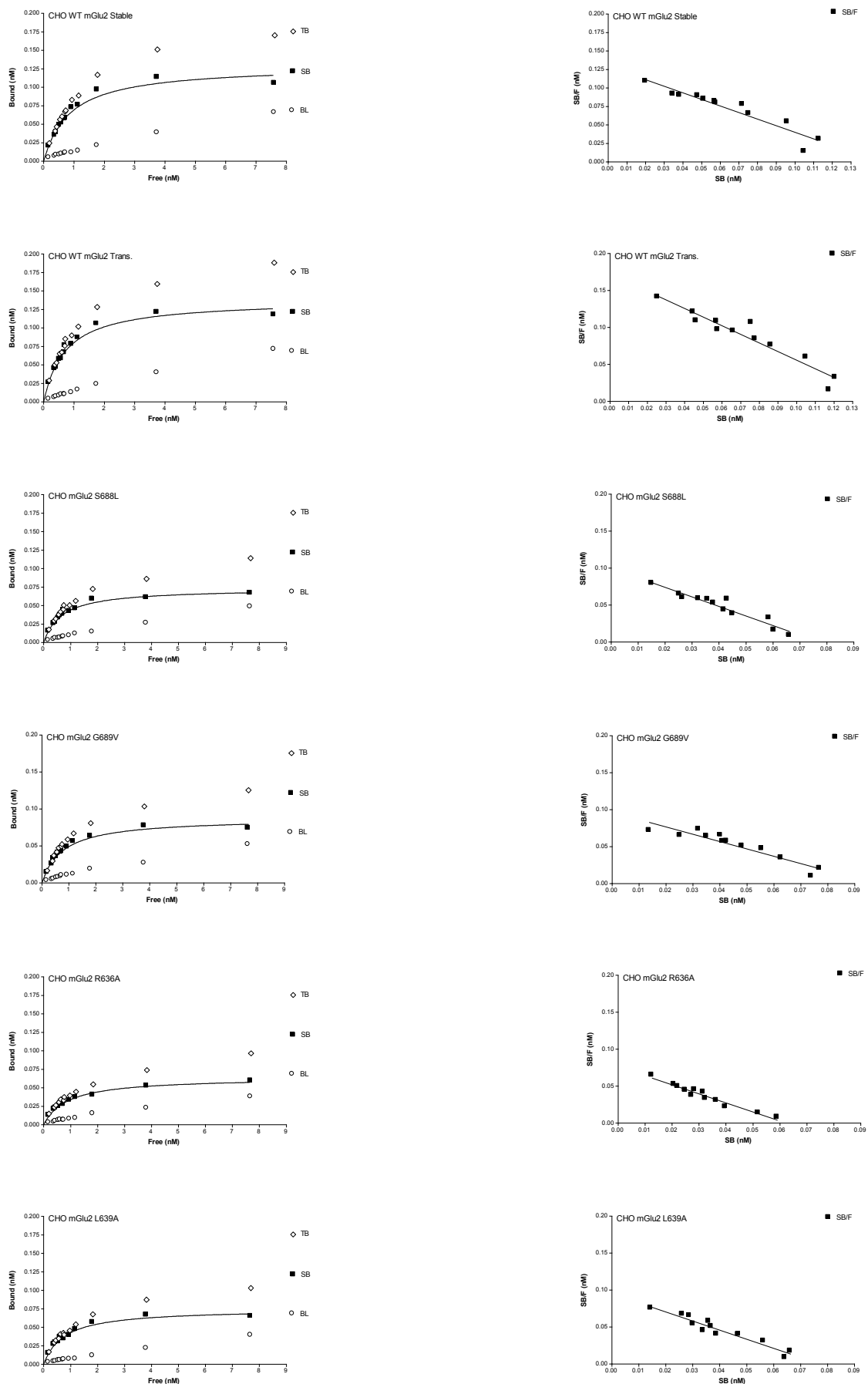
Figure 16: Saturation binding curves and correspondent Scatchard plots of [³H]-LY341495 binding to WT (stably and transiently transfected) and mutant mGlu2 receptors (S688L, G689V, R636A and L639SA) expressed in CHO-K1 membranes. Data are presented as nanomolar specifically bound. Data points were determined in triplicate (n=1).

Table 6: Equilibrium binding constants of [^{3}H]-LY341495 binding to CHO membranes expressing WT and mutant mGlu2 receptors (n=1).

	K_D (nM)	Bmax (fmol/mg of protein)
mGlu2 Stable WT	0.87	6480
mGlu2 Trans. WT	0.72	6900
S688L (#1)	0.67	3661
G689V (#2)	0.78	4379
R636A (#6)	0.88	3197
L639A (#7)	0.76	3748

3.2. Glutamate potency for WT and mutated mGlu2 receptors

In order to further verify the effect of the mutations on the glutamate binding pocket and on the signalling mechanisms of the receptor, the potency of glutamate in WT and mutant hmGlu2 was assessed through functional studies using a [^{35}S]GTPγS assay.

pEC$_{50}$ values were calculated from concentration-response curves of glutamate in membranes of CHO-K1 cells expressing the WT (stably and transiently transfected) and the mutated hmGlu2 receptors (Figure 16, Table 6). On the WT receptor, glutamate typically exerts effects with a pEC$_{50}$ of ~ 5.05 and EC$_{50}$ values of ~ 9 μM[1].
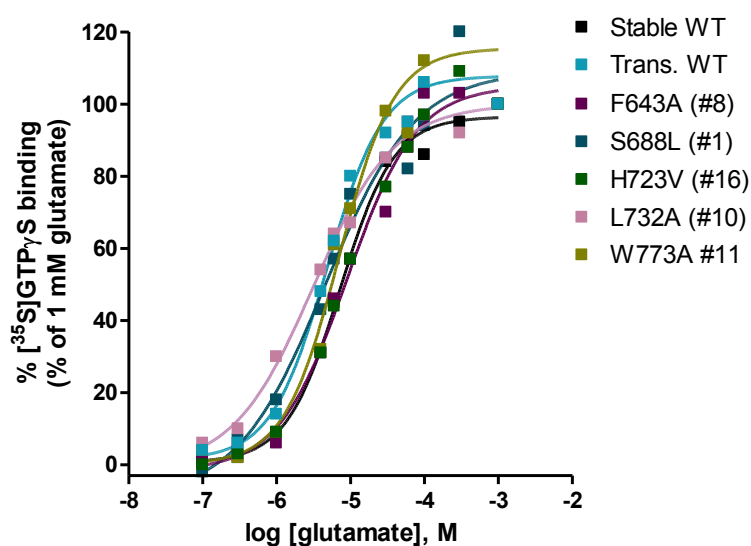
Figure 16: Representative graph of the effect of a set of mGlu2 receptor mutations on glutamate-induced [^{35}S]GTPγS binding. Data are from one experiment where glutamate concentration-response curves were determined on membranes from CHO-K1 cells expressing WT (stably and transiently transfected) and 5 mutant receptors (transiently transfected) in parallel. Results are expressed as a percentage of the response to 1 mM glutamate.

[1] Data from Janssen's central data warehouse.

Table 7: Effect of mGlu2 receptor mutations on glutamate-induced [^{35}S]GTPγS binding. Concentration-response curves were determined in the presence of increasing concentrations of glutamate on membranes from CHO-K1 cells expressing WT (stably and transiently transfected) and mutant receptors (transiently transfected). Mean pEC50±SD and mean percentage of glutamate stimulation (defined as the ratio between the response obtained under basal conditions i.e. only using assay buffer, corresponding to 100%, and the response obtained with 1 mM glutamate) ±SD are given. Data presented was calculated from at least 3 concentration-response measurements performed in triplicate; except for mutants R636A (#6) and M728A (#19) for which data was calculated from 2 concentration-response measurements performed in triplicate. Mutations are again sorted by receptor region.

Receptor Region	Mutation	pEC50	Response amplitude (%)
---	Stable WT	5.0 ± 0.12	637% ± 122%
---	Transient WT	5.3 ± 0.15	261% ± 73%
TM3	R635A (#18)	4.9 ± 0.24	217% ± 49%
	R636A (#6)	5.2 ± 0.15	196% ± 22%
	L639A (#7)	5.2 ± 0.12	280% ± 37%
	F643A (#8)	4.9 ± 0.22	222% ± 60%
	S644A (#14)	5.2 ± 0.12	237% ± 78%
TM4	S688L (#1)	5.2 ± 0.15	232% ± 64%
	G689V (#2)	5.2 ± 0.22	237% ± 89%
	S688L/G689V (#4)	5.2 ± 0.18	249% ± 76%
	S688L/G689V/N735D (#5)	5.3 ± 0.27	235% ± 69%
	V700L (#15)	5.2 ± 0.14	278% ± 32%
	S644A/V700L/H723V (#17)	5.0 ± 0.19	313% ± 55%
EL2/TM 5	H723V (#16)	5.1 ± 0.13	282% ± 58%
	D725A (#9)	5.3 ± 0.14	250% ± 19%
	M728A (#19)	5.1 ± 0.12	274% ± 132%
	S731A (#20)	5.2 ± 0.25	331% ± 61%
	L732A (#10)	5.4 ± 0.18	346% ± 103%
	N735D (#3)	5.1 ± 0.04	253% ± 69%
	V736A (#21)	5.1 ± 0.07	248% ± 19%
TM 6	W773A (#11)	5.0 ± 0.48	171% ± 30%
	F776A (#12)	5.3 ± 0.12	326% ± 82%
	F780A (#13)	5.2 ± 0.13	303% ± 43%

As shown in Figure 16 and Table 7, mutated receptors elicited concentration-response curves for glutamate with potencies that were similar to those of the WT receptor. The amplitude of the response to glutamate – i.e. the ratio between the response obtained under basal conditions (only using assay buffer), corresponding to 100%, and the response obtained with 1 mM glutamate – was considerably lower in transiently transfected CHO-K1 cells.

3.3. Effect of mGlu2 mutations on the activity of positive allosteric modulators

[^{35}S]GTPγS binding was used to assess the effect of each mutation on the activity of 14 mGlu2 receptor PAMs. The results of this analysis are summarized and presented as follows.

From each chemical series, one representative compound is chosen to be presented graphically: JNJ-40068782 (1,4-pyridones), JNJ-35814376 (1,5-pyridones), JNJ-42153605 (triazolopyridines) JNJ-40297036 (isoquinolones) and JNJ-41482012 (imidazopyridines). Results for the reference compounds JNJ-52149617 (LY2605740, acetophenone, Fell *et al*., 2011), JNJ-35815013 (BINA, Galici *et al.*, 2006) and JNJ-35814090 (LY487379, Schaffhauser *et al*., 2003) are also visualized in the graphs. Mutations are again sorted by receptor region. Hence, the results presented graphically represent a summary of the effects for a particular chemical structure, for a given receptor region. The results for the complete set of compounds are displayed in Table 8. This complete set of data will be discussed elsewhere (Section 4.3), and particular focus will be put on dissimilarities between compounds in a particular chemical series.

The [^{35}S]GTPγS experiments were set up as follows. In a first step, to preliminary assess the effect of each mutation on the PAM activity, only two concentrations of PAM were tested: a PAM concentration that produces 50% stimulation (EC$_{50}$) and a concentration that produces a maximal stimulation (EC$_{100}$)[2]. This two-concentration screening was considered necessary, as testing only the highest concentration (EC$_{100}$) could mask the true effect of the compound: as displayed in Figure 17, the maximal response (E_{max}) of the compound on the mutated receptor is the same as for the WT receptor, but the curve has shifted to the right. Although the E_{max} – which would be detected in case of using the compound's EC$_{100}$ – is the same, which would indicate that the compound has similar activity in WT and mutated receptor, the potency is lower.

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[2] These values were taken from previous experiments for which data is stored in Janssen's central database.
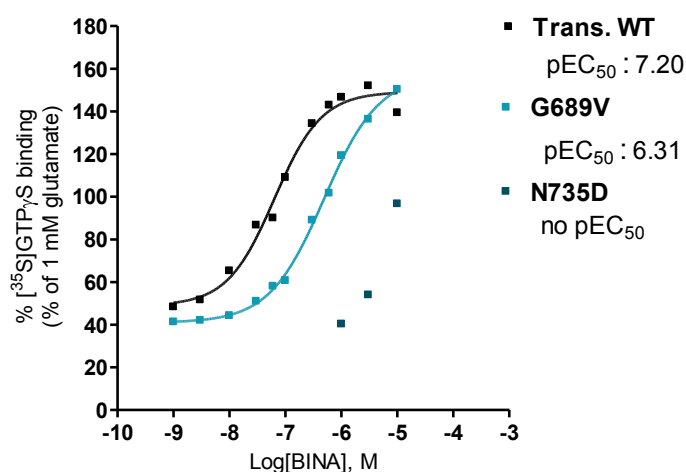
Figure 17: Effect of increasing concentrations of BINA (JNJ 35815013), a reference PAM, on glutamate-induced [^{35}S]GTPγS binding and the importance of performing a screening assay with EC50 and EC100: for EC100 the receptor with the mutation G689V exhibits similar effects as the WT (transiently transfected into CHO-K1); however, since the compound is less potent, this similarity is no longer observed with lower concentrations (e.g. EC$_{50}$).

As mentioned in the Methods section, PAMs were tested using 4 μM glutamate (correspondent to the EC$_{20}$). Per mutation, for each PAM compound, [^{35}S]GTPγS binding in the presence of the PAM was calculated as a percentage of the response to 1 mM glutamate (no PAM added). Effects lower than 75% of the effect seen on the transiently transfected WT receptor were considered significantly different, and hence these mutations were selected for further analysis.

Figure 18 displays the results obtained in the screening test for the representative compounds of each chemical series, comparing the effect of the different compounds between WT and mutant mGlu2. For a detailed overview table, showing the exact values obtained for the complete set of compounds and mutations, we refer to Appendix 3.

64
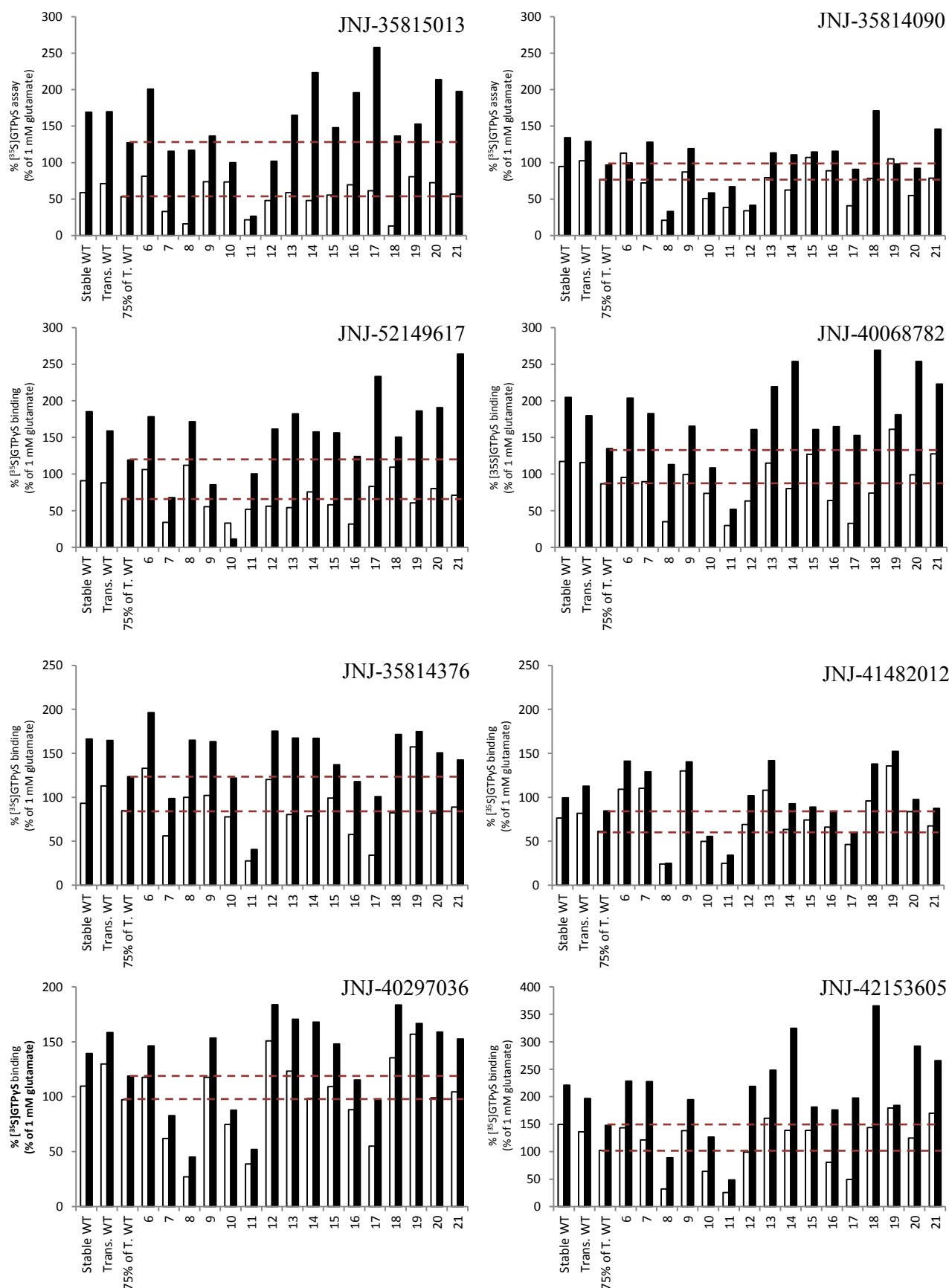
Figure 18: Screening of 8 mGlu2 receptor PAMs, representative of the 8 different chemical classes tested in this study, using the ☐ EC50 and ■ EC100 on WT (stably transfected "Stable WT" and transiently transfected "Trans. WT") and mutated hmGlu2 (mutations #6 to #21). For the mutations S688L (#1), G689V (#2), N735D (#3), S688L/G689V (#4) and S688L/G689V/N735D (#5) this screening was not performed. 75% of Trans. WT ("75% of T. WT") response is also displayed. Mutations for which the compound effect was lower than "75% of T. WT", for any of the concentrations (i.e. the mutations for which bars appear below the red dashed line) were further tested. See Appendix 3 for a complete summary of results.

65

As shown in Figure 18, this screening assay enabled an early exclusion of several mutations based on the criteria mentioned above: for all compounds, mutations R636A (#6), D725A (#9), V700L (#15), M728A (#19) and V736A (#21) showed an effect higher than 75% of the effect on WT, for both EC_{50} and EC_{100} of all compounds, and were therefore considered to have no effect on the activity of any of the compounds tested.

For the mutations showing an effect lower than 75% of the effect in WT for a given compound, a concentration-response curve of that compound on membranes of CHO-K1 cells expressing that mutation was generated. From this, potency (EC_{50}) and relative efficacy (E_{max}) values were calculated for the 14 PAMs on mGlu2 WT versus mutant receptors. Figures 19-22 graphically display the results obtained with the 8 representative compounds on the mutations localized in the different receptor regions. The potency (EC_{50}) and relative E_{max} calculated from the concentration-response curves for the 14 compounds on WT and mutant receptors are presented in Table 8. In this table, the difference in compound potency is also calculated as a ratio of EC_{50} between WT and mutant mGlu2 (for a more detailed table with additional information on individual experiments, see Appendix 4).

Figure 19 shows the results obtained from the concentration-dependent enhancement of glutamate-induced [^{35}S]GTPγS binding by the 8 representative compounds on WT mGlu2 and on mGlu2 with mutations localized in **transmembrane 3**. As seen in Figure 18, all the mutations seem to affect the activity of the tested compounds. Mutation F643A (#8) shows the most prominent results, as it not only causes a decrease in potency (right-ward shift of the concentration-response curve) but also a decrease in E_{max}. EC_{50} values were increased by 6.4 to 157-fold for the different compounds tested (Table 8). Mutation L639A (#7) also affects the activity of all compounds, albeit to a lower extent compared to mutation F643A (#8) (1.3 to 15-fold increase in EC_{50}, as shown in Table 8). Mutation S644A (#14) decreased the potency of all the compounds tested, with the exception of JNJ-39226421 (Figure 19, Table 8). Mutation R635A (#18) showed a decrease in potency of JNJ-35814376 (Figure 19E) and JNJ-40068782 (Figure 19H), but an increased maximal response. Additionally, this mutation caused a 5.6-fold increase in the potency of JNJ-35815013.
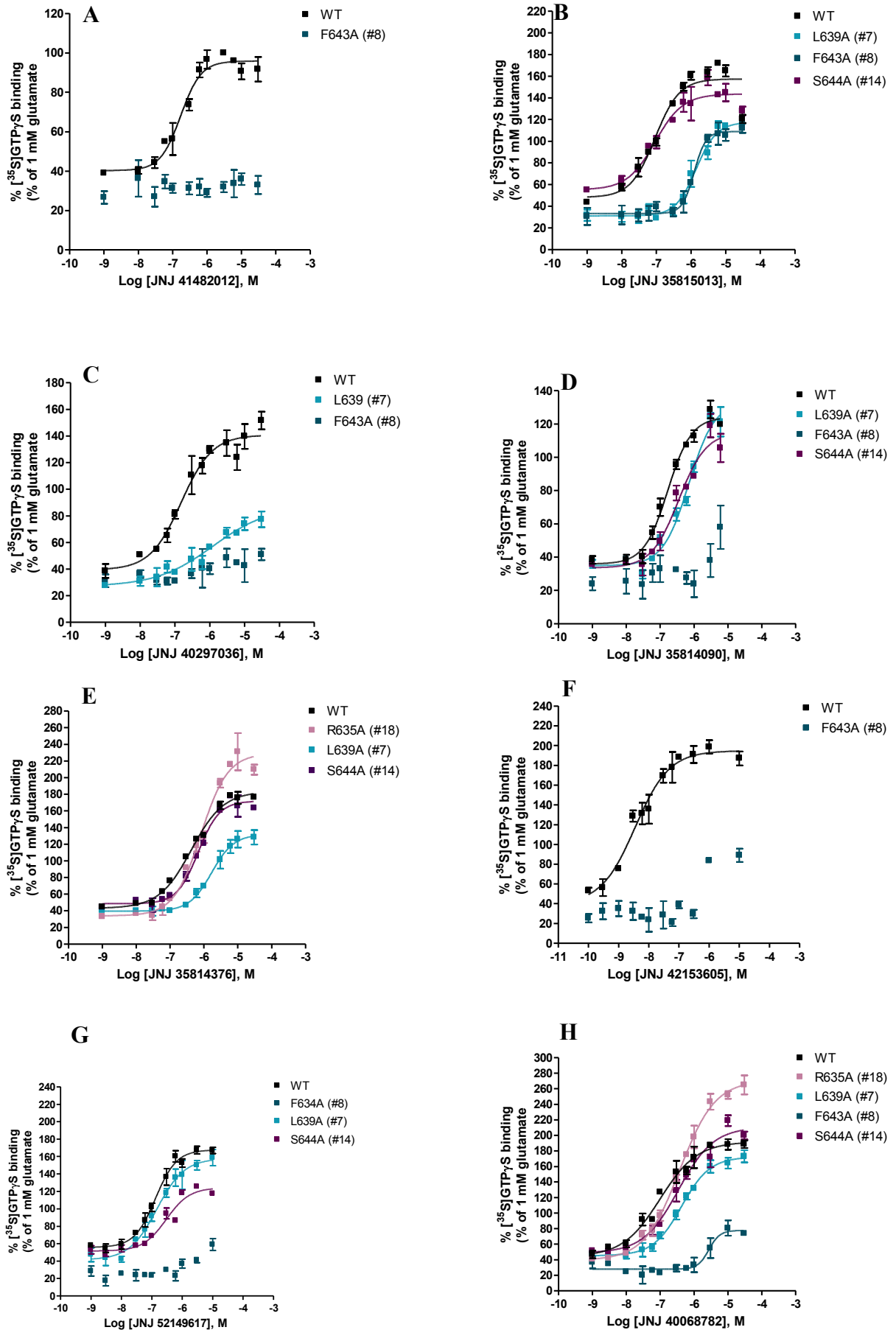
Figure 19: Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 3** (TM3). A to H, concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 8 PAMs, representative of the 8 chemical classes used in this study. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate. See Table 7 for a complete summary of results obtained for 14 PAMs on 21 receptor mutants.

67
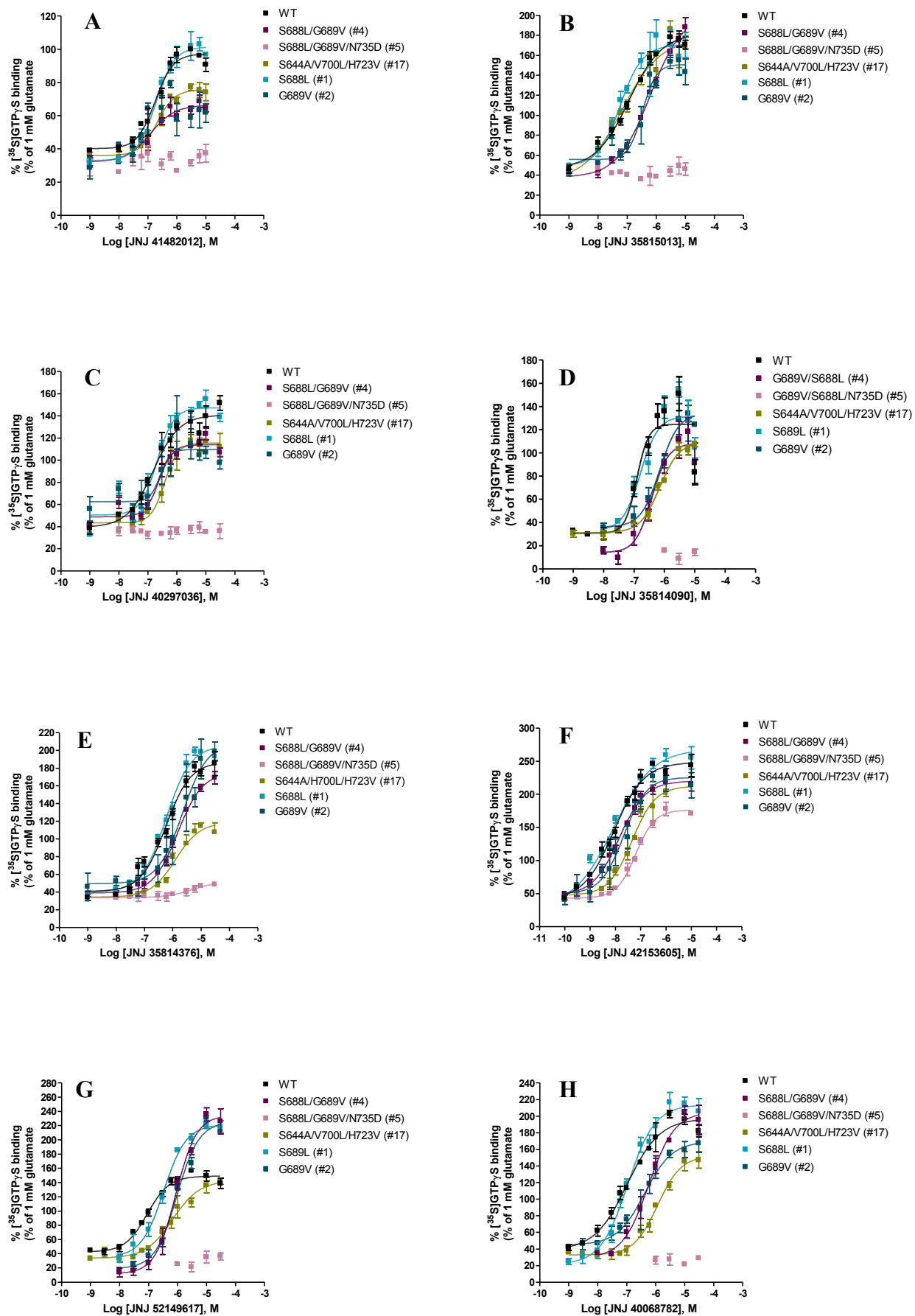
Figure 20: Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 4** (TM4). A to H, concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 8 PAMs, representative of the 8 chemical classes used in this study. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate. See Table 7 for a complete summary of results obtained for 14 PAMs on 21 receptor mutants.

68
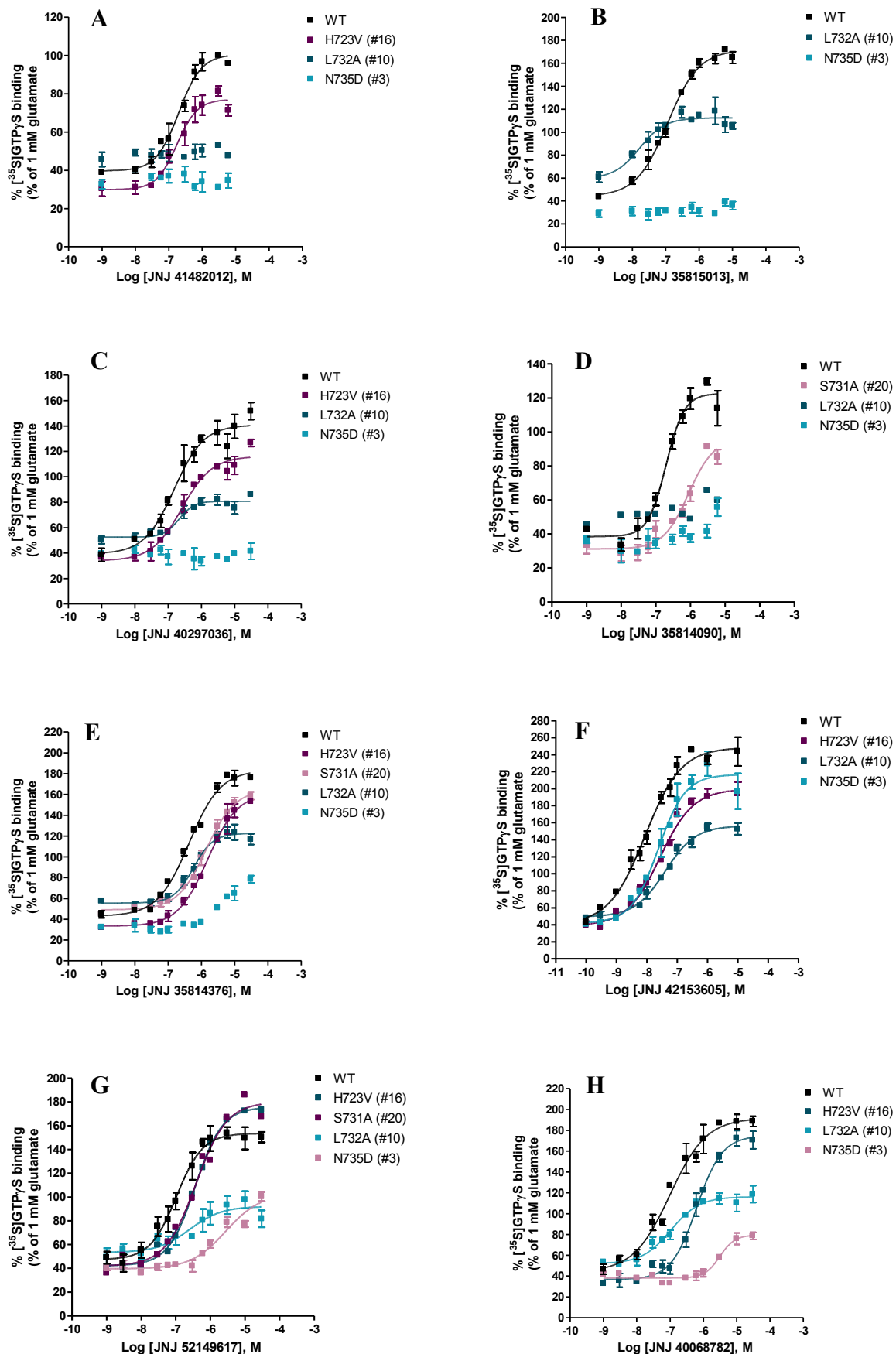
Figure 21: Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **extracellular loop 2 and transmembrane 5 (EL2/TM5)**. A to H, concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 8 PAMs, representative of the 8 chemical classes used in this study. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate. See Table 7 for a complete summary of results obtained for 14 PAMs on 21 receptor mutants.
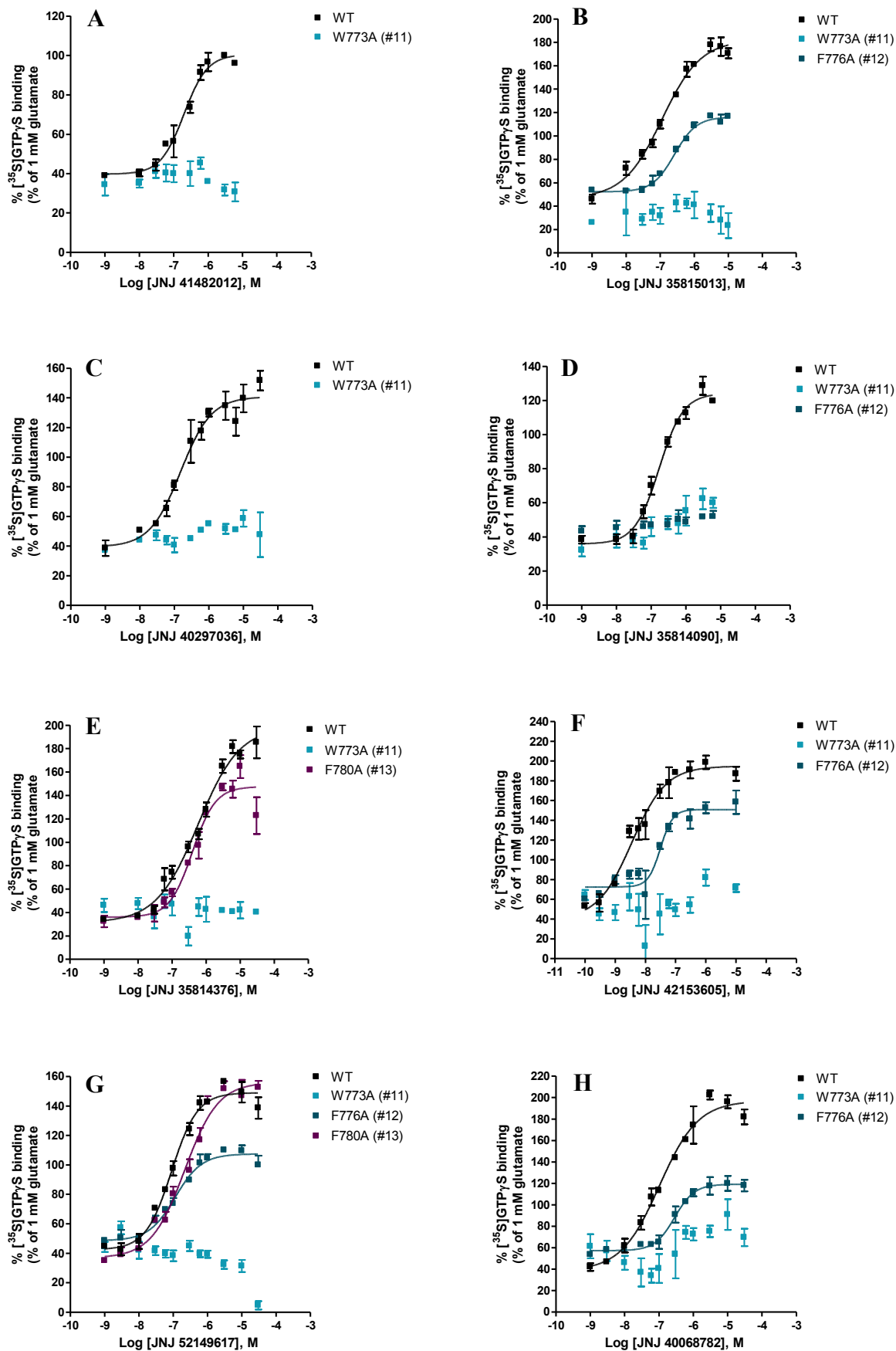
Figure 22: Effect of PAMs on glutamate-induced [^{35}S]GTPγS binding in WT mGlu2 and mGlu2 with mutations located in **transmembrane 6** (TM6). A to H, concentration-dependent enhancement of 4 μM glutamate-induced [^{35}S]GTPγS binding by 8 PAMs, representative of the 8 chemical classes used in this study. Results are expressed as a percentage of the response to 1 mM glutamate, and refer to one experiment performed in triplicate. See Table 7 for a complete summary of results obtained for 14 PAMs on 21 receptor mutants.

70

Figure 20 displays the results obtained with mutations localized in **transmembrane 4**. As shown in Figure 20 and Table 8, the entire set of mutations in this receptor region seems to affect the activity of all compounds, except for mutation S688L (#1) which for most cases yielded results similar as for WT mGlu2. Mutation G689V/S688L/N735D (#5) disrupted the concentration-response curve for all the compounds, and a prominent potency/E_{max} decrease was seen for the compound representing the triazolopyridines, namely JNJ-42153605.

Figure 21 displays the results from mutations localized in the regions of **extracellular loop 2** (mutation H723V) and **transmembrane 5**. All the mutations seem to affect the activity of the compounds, but mutations N735D (#3) and L732A (#10) elicited the biggest shifts in the activity of the tested compounds. Interestingly, the effect of the EL2 mutation, H723V (#16), seems consistent between compounds from different chemical series.

Finally, Figure 22 shows the results obtained for mutations localized in **transmembrane 6**. Mutation W773A (#11) affects the activity of all compounds, disrupting completely their ability to generate a concentration-response curve. This mutation caused a 4 to 55-fold increase in EC_{50} of the compounds tested (Table 8). Although to a smaller extent, mutation F776A (#12) also consistently affects the activity of the compounds belonging to different chemical series. For the compound JNJ-35814090 (Figure 22D), this mutation also led to the complete loss of the receptor's ability to respond to increasing concentrations of the compound.

Table 8: Potency (EC$_{50}$) and relative E_{max} of the enhancement of glutamate-induced [^{35}S]GTPγS binding by 14 PAMs on WT and mutant receptors after transient transfection into CHO-K1 cells. Compound potency is presented as a ratio of EC$_{50}$ between WT and mutant mGlu2.

NT – not tested; Sc – tested in screening assay; [pink] – ratio EC$_{50}$ mutant mGlu2 /EC$_{50}$ WT > 3 or n.v – no value (absence of curve)

Values shown as EC$_{50}$ / E$_{max}$; ratios (relative to WT) in parentheses below where applicable.

Compound class	Compound	Stable EC50	Stable Emax	WT EC50	WT Emax	(18) R635A EC50	R635A Emax	(6) R636A EC50	R636A Emax	(7) L639A EC50	L639A Emax	(8) F643A EC50	F643A Emax	(14) S644A EC50	S644A Emax
1,4-Pyridones	JNJ-40068782	139	206	87 (1.0)	201 (1.0)	378 (4.3)	246 (1.2)	Sc	Sc	572 (6.5)	186 (0.9)	2396 (27.4)	89 (0.4)	216 (2.5)	174 (0.9)
1,4-Pyridones	JNJ-41329782	68	215	57 (1.0)	202 (1.0)	Sc	Sc	Sc	Sc	109 (2.9)	204 (1.0)	1142 (20.0)	84 (0.4)	180 (3.2)	196 (1.0)
1,5-Pyridone	JNJ-35814376	637	196	523 (1.0)	186 (1.0)	853 (1.6)	225 (1.2)	Sc	Sc	1067 (2.0)	121 (0.7)	Sc	Sc	625 (1.2)	139 (0.7)
Triazolopyridines	JNJ-46281222	8	214	6 (1.0)	211 (1.0)	Sc	Sc	Sc	Sc	Sc	Sc	316 (55.6)	107 (0.5)	Sc	Sc
Triazolopyridines	JNJ-42153605	6	224	5 (1.0)	214 (1.0)	Sc	Sc	Sc	Sc	Sc	Sc	447 (84.3)	90 (0.4)	Sc	Sc
Triazolopyridines	JNJ-46356479	91	227	59 (1.0)	204 (1.0)	Sc	Sc	Sc	Sc	Sc	Sc	6783 (115.6)	86 (0.4)	Sc	Sc
Triazolopyridines	JNJ-42329001	49	218	37 (1.0)	200 (1.0)	Sc	Sc	Sc	Sc	Sc	Sc	5868 (156.8)	137 (0.7)	Sc	Sc
Triazolopyridines	JNJ-43245046	6	224	7 (1.0)	227 (1.0)	Sc	Sc	Sc	Sc	9 (1.4)	244 (1.1)	254 (38.7)	101 (0.4)	Sc	Sc
Isoquinolones	JNJ-39226421	178	130	172 (1.0)	141 (1.0)	117 (0.7)	92 (0.7)	Sc	1	227 (1.3)	74 (0.5)	n.v.	36 (0.3)	119 (0.7)	110 (0.8)
Isoquinolones	JNJ-40297036	143	139	148 (1.0)	147 (1.0)	(1)	(1)	Sc	1	1351 (9.1)	90 (0.6)	n.v.	49 (0.3)	Sc	Sc
Imidazopyridine	JNJ-41482012	172	97	175 (1.0)	101 (1.0)	Sc	Sc	Sc	Sc	Sc	Sc	n.v	36 (0.4)	Sc	Sc
THIIC	JNJ-52149617	113	166	114 (1.0)	159 (1.0)	Sc	Sc	Sc	Sc	161 (1.4)	171 (1.1)	724 (6.4)	62 (0.4)	305 (2.7)	131 (0.8)
BINA	JNJ-35815013	97	173	87 (1.0)	168 (1.0)	490 (5.6)	178 (1.1)	Sc	Sc	1320 (15.2)	121 (0.7)	1414.1 (16.3)	84 (0.5)	108 (1.2)	153 (0.8)
LY487379	JNJ-35814090	173	116	156 (1.0)	128 (1.0)	Sc	Sc	Sc	Sc	692 (4.4)	148 (1.2)	n.v.	53 (0.4)	276 (1.8)	105 (0.8)

72

Table 8 (Cont.)

Group	Compound	Stable EC50	Stable Emax	WT EC50	WT Emax	(1) S688L EC50	(1) S688L Emax	(2) G689V EC50	(2) G689V Emax	(4) G689V/S688L EC50	(4) G689V/S688L Emax	(5) N735D/G689V/S688L EC50	(5) N735D/G689V/S688L Emax	(15) V700L EC50	(15) V700L Emax	(17) S644A/V700L/H723V EC50	(17) S644A/V700L/H723V Emax
1,4-Pyridones	JNJ-40068782	139	206	87	201	160	201	603	187	635	201	n.v.	42	Sc	Sc	1262	163
				1.0	1.0	1.8	1.0	6.9	0.9	7.3	1.0		0.2			14.4	0.8
	JNJ-41329782	68	215	57	202	NT	NT	NT	NT	1349.0	124	NT	NT	Sc	Sc	621	186
				1.0	1.0					23.6	0.6					10.9	0.9
1,5-Pyridone	JNJ-35814376	637	196	523	186	520	206	2512	218	1445	125	n.v.	49	Sc	Sc	2225	135
				1.0	1.0	1.0	1.1	4.8	1.2	2.8	0.7		0.3			4.3	0.7
	JNJ-46281222	8	214	6	211	20	159	83	229	61	215	767.2	203	Sc	Sc	72	213
				1.0	1.0	3.6	0.8	14.6	1.1	10.8	1.0	134.9	1.0			12.7	1.0
	JNJ-42153605	6	224	5	214	7	250	25	215	22	223	212	180	Sc	Sc	47	209
				1.0	1.0	1.3	1.2	4.7	1.0	4.2	1.0	40.0	0.8			8.8	1.0
Triazolopyridines	JNJ-46356479	91	227	59	204	NT	NT	1349	269	309	197	3162.3	174	Sc	Sc	692	215
				1.0	1.0			23.0	1.3	5.3	1.0	53.9	0.9			11.8	1.1
	JNJ-42329001	49	218	37	200	NT	NT	170	178	577	186	489.8	208	Sc	Sc	115	230
				1.0	1.0			4.5	0.9	15.4	0.9	13.1	1.0			3.1	1.1
	JNJ-43245046	6	224	7	227	NT	NT	39	203	41	216	n.v.	142	Sc	Sc	42	289
				1.0	1.0			5.9	0.9	6.2	1.0		0.6			6.4	1.3
Isoquinolones	JNJ-39226421	178	130	172	141	NT	NT	NT	NT	n.v.	40	NT	NT	Sc	Sc	539	95
				1.0	1.0						0.3					3.1	0.7
	JNJ-40297036	143	139	148	147	209	147	537	117	264	114	n.v.	40	Sc	Sc	389	101
				1.0	1.0	1.4	1.0	3.6	0.8	1.8	0.8		0.3			2.6	0.7
Imidazopyridine	JNJ-41482012	172	97	175	101	130.0	104	~24	61	223	58	n.v	37	Sc	Sc	274	73
				1.0	1.0	0.7	1.0	-0.1	0.6	1.3	0.6		0.4			1.6	0.7
THIIC	JNJ-52149617	113	166	114	159	154	181	646	177	635	175	2896.3	56	Sc	Sc	620	151
				1.0	1.0	1.3	1.1	5.7	1.1	5.6	1.1	25.4	0.4			5.4	1.0
BINA	JNJ-35815013	97	173	87	168	145	167	832	171	575	186	21379.6	44	Sc	Sc	78	206
				1.0	1.0	1.7	1.0	9.6	1.0	6.6	1.1	246.6	0.3			0.9	1.2
LY487379	JNJ-35814090	173	116	156	128	114	133	327	132	345	110	n.v.	33	Sc	Sc	670	111
				1.0	1.0	0.7	1.0	2.1	1.0	2.2	0.9		0.3			4.3	0.9

Transmembrane 4 (spanning columns (1) S688L through (17) S644A/V700L/H723V)

Table 8 (Cont.)

| | | Stable | | WT | | Extracellular loop 2/Transmembrane 5 | | | | | | | | | | | | |
| | | | | | | (16) H723V | | (9) D725A | | (19) M728A | | (20) S731A | | (10) L732A | | (3) N735D | | (21) V736A | |
Class	Compound	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax
1,4-Pyridones	JNJ-40068782	139	206	87 / 1.0	201 / 1.0	549 / 6.3	189 / 0.9	Sc	Sc	Sc	Sc	1 / 1	1 / 1	95 / 1.1	130 / 0.6	3019.95 / 34.5	70 / 0.3	Sc	Sc
1,4-Pyridones	JNJ-41329782	68	215	57 / 1.0	202 / 1.0	260 / 4.6	217 / 1.1	Sc	Sc	Sc	Sc	245 / 4.3	247 / 1.2	63 / 1.1	140 / 0.7	NT	NT	Sc	Sc
1,5-Pyridone	JNJ-35814376	637	196	523 / 1.0	186 / 1.0	1625 / 3.1	152 / 0.8	Sc	Sc	Sc	Sc	1190 / 2.3	189 / 1.0	633 / 1.2	129 / 0.7	n.v.	81 / 0.4	Sc	Sc
Triazolopyridines	JNJ-46281222	8	214	6 / 1.0	211 / 1.0	Sc	Sc	Sc	Sc	Sc	Sc	Sc	Sc	58 / 10.3	142 / 0.7	~617 / 102.0	221 / 1.0	Sc	Sc
Triazolopyridines	JNJ-42153605	6	224	5 / 1.0	214 / 1.0	28 / 5.2	226 / 1.1	Sc	Sc	Sc	Sc	Sc	Sc	49 / 9.2	159 / 0.7	27 / 5.2	220 / 1.0	Sc	Sc
Triazolopyridines	JNJ-46356479	91	227	59 / 1.0	204 / 1.0	195 / 3.3	220 / 1.1	Sc	Sc	Sc	Sc	Sc	Sc	1015 / 17.3	131 / 0.6	NT	NT	Sc	Sc
Triazolopyridines	JNJ-42329001	49	218	37 / 1.0	200 / 1.0	Sc	Sc	Sc	Sc	Sc	Sc	Sc	Sc	576 / 15.4	141 / 0.7	NT	NT	Sc	Sc
Triazolopyridines	JNJ-43245046	6	224	7 / 1.0	227 / 1.0	15 / 2.3	281 / 1.2	Sc	Sc	Sc	Sc	Sc	Sc	82.5697 / 12.6	130 / 0.6	NT	NT	Sc	Sc
Isoquinolones	JNJ-39226421	178	130	172 / 1.0	141 / 1.0	253 / 1.5	115 / 0.8	Sc	Sc	Sc	Sc	Sc	Sc	57 / 0.3	73 / 0.5	NT	NT	Sc	Sc
Isoquinolones	JNJ-40297036	143	139	148 / 1.0	147 / 1.0	351 / 2.4	116 / 0.8	Sc	Sc	Sc	Sc	Sc	Sc	329 / 2.2	78 / 0.5	n.v.	41 / 0.3	Sc	Sc
Imidazopyridine	JNJ-41482012	172	97	175 / 1.0	101 / 1.0	175 / 1.0	78 / 0.8	Sc	Sc	Sc	Sc	Sc	Sc	n.v / 0.5	52 / 0.5	n.v / 0.4	39 / 0.4	Sc	Sc
THIIC	JNJ-52149617	113	166	114 / 1.0	159 / 1.0	281 / 2.5	174 / 1.1	Sc	Sc	Sc	Sc	285 / 2.5	169 / 1.1	357 / 3.1	91 / 0.6	2790.7 / 24.5	96 / 0.6	Sc	Sc
BINA	JNJ-35815013	97	173	87 / 1.0	168 / 1.0	Sc	Sc	Sc	Sc	Sc	Sc	Sc	Sc	15 / 0.2	110 / 0.7	7413.1 / 85.5	74 / 0.4	Sc	Sc
LY487379	JNJ-35814090	173	116	156 / 1.0	128 / 1.0	Sc	Sc	Sc	Sc	Sc	Sc	834 / 5.3	109 / 0.9	n.v. / 12.6	62 / 0.5	n.v.	40 / 0.3	Sc	Sc

Table 8 (Cont.)

| | | Stable | | WT | | Transmembrane 6 | | | | | |
| | | | | | | (11) W773A | | (12) F776A | | (13) F780A | |
Class	Compound	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax	EC50	Emax
1,4-Pyridones	JNJ-40068782	139	206	87 (1.0)	201 (1.0)	n.v.	89 (0.4)	440 (5.0)	132 (0.7)	Sc (0.8)	Sc (0.8)
	JNJ-41329782	68	215	57 (1.0)	202 (1.0)	1059	80 (0.4)	192 (3.4)	152 (0.8)	Sc	Sc
1,5-Pyridone	JNJ-35814376	637	196	523 (1.0)	186 (1.0)	n.v.	46 (0.2)	Sc	Sc	398.1 (0.8)	148 (0.8)
Triazolopyridines	JNJ-46281222	8	214	6 (1.0)	211 (1.0)	76 (13.3)	95 (0.4)	Sc	Sc	Sc	Sc
	JNJ-42153605	6	224	5 (1.0)	214 (1.0)	n.v.	76 (0.4)	24 (4.6)	178 (0.8)	Sc	Sc
	JNJ-46356479	91	227	59 (1.0)	204 (1.0)	2344.2 (40.0)	72 (0.4)	Sc	Sc	Sc	Sc
	JNJ-42329001	49	218	37 (1.0)	200 (1.0)	1258.9 (33.6)	82 (0.4)	70 (1.9)	163 (0.8)	Sc	Sc
	JNJ-43245046	6	224	7 (1.0)	227 (1.0)	361 (55.0)	92 (0.4)	7 (1.1)	158 (0.7)	Sc	Sc
Isoquinolones	JNJ-39226421	178	130	172 (1.0)	141 (1.0)	n.v.	49 (0.3)	Sc	Sc	Sc	Sc
	JNJ-40297036	143	139	148 (1.0)	147 (1.0)	n.v.	68 (0.5)	Sc	Sc	Sc	Sc
Imidazopyridine	JNJ-41482012	172	97	175 (1.0)	101 (1.0)	n.v	48 (0.5)	Sc	Sc	Sc	Sc
THIIC	JNJ-52149617	113	166	114 (1.0)	159 (1.0)	n.v.	51 (0.3)	125 (1.1)	118 (0.7)	218.8 (1.9)	156 (1.0)
BINA	JNJ-35815013	97	173	87 (1.0)	168 (1.0)	n.v.	44 (0.3)	199 (2.3)	106 (0.6)	Sc	Sc
LY487379	JNJ-35814090	173	116	156 (1.0)	128 (1.0)	631.0 (4.0)	63 (0.5)	n.v.	48 (0.4)	Sc	Sc

Chapter 4

Discussion

The pursuit for alternative therapeutic approaches devoid of the disadvantages associated with the use of orthosteric agonists has conferred prominence to allosteric modulators, namely PAMs. The recent emergence of PAMs as highly attractive subtype-selective mGlu2 receptor modulators makes it important to investigate the molecular factors mediating the interaction between these compounds and the receptor, as a way not only to understand how these compounds bind and activate the receptor, but also to enable the development of future more optimal compounds.

In the past decade, a number of studies have focused on the investigation of the binding mode of allosteric modulators on mGlu receptors. The different studies have suggested that residues in TM 3, 4, 5, 6 and 7, and also in the EL2, constitute the allosteric binding pocket for allosteric modulators. The reported mutations do not necessarily affect the binding of all allosteric ligands, although it has been shown that different compounds may share an overlapping binding site – even if they exhibit opposite effects towards the receptor (PAMs versus NAMs).

In the present study, the molecular interaction of 14 PAMs with the mGlu2 receptor was elucidated. For that, the activity of these compounds on WT and point-mutated mGlu2 receptors was compared in order to identify amino acids potentially important for the interaction between PAMs and the mGlu2 receptor. In previous work, the compounds tested in this study were shown not to displace [^{3}H]-LY341495 binding, confirming their binding to an allosteric binding site (data not shown); additionally, previous studies revealed that these compounds exhibit selective positive allosteric modulation at recombinant mGlu2 receptors (data not shown).

4.1. Expression of WT and mutant mGlu2 receptors

Western blot analysis revealed a similar pattern in the expression of WT and mutant receptors, although membranes of CHO-K1 cells stably transfected with WT mGlu2 receptor revealed apparent higher expression levels. In addition to Western blot analysis, saturation binding assays were performed for mutations S688L, G689V, N735D, S688L/G689V and S688L/G689V/N735D which enabled the quantification of the total amount of receptors. Although this quantification was not carried out for all the mutations, it clearly shows that mutant receptors have variable expression levels, displaying lower expression levels when compared to the stable transfection.

78

4.2. Comparison between the affinity and potency for glutamate between WT and mutant mGlu2

Despite the confirmation that mutant receptors are being expressed by CHO-K1 cells, their ability to bind the agonist, glutamate, also needed to be confirmed to assure that the receptor could achieve the conformation required for agonist binding. In fact, glutamate was able to displace [^{3}H]-LY341495 in a concentration-dependent manner, both from the WT receptor stably expressed in CHO-K1 cells and from transiently transfected WT as well as mutant receptors. Accordingly, the IC$_{50}$ values were overall similar. These results confirm that mutant receptors are not only being expressed by CHO-K1 cells, but they also are able to bind glutamate in a similar way as WT, indicating that they are still able to acquire the conformation needed for agonist binding.

Further studies were carried out to assess the functional properties of mutant receptors. Concentration-response curves for glutamate-induced [^{35}S]GTPγS binding were performed in order to compare the functional activity of WT and mutant receptors. Even though glutamate response amplitudes varied (likely as a result of differences in expression levels and/or transfection efficiency), mutated receptors elicited glutamate concentration-response curves with resulting potency values that were similar to those of WT receptor. This result suggests that the mutations did not have a significant effect on the potency of glutamate towards the receptor and confirmed that the mutant receptors were functionally active.

4.3. Effect of mGlu2 mutations on the activity of positive allosteric modulators

A brief overview of the results presented in Figures 19-22 and Table 4 makes it possible to infer that different mutations have different effects on the activity of the PAMs tested in this study, which suggests that the different compounds may not share the exact same binding mode as expected. Given the diversity of the chemical structures of the compounds, they will form subtly different interactions. Several studies, as the ones performed by Hemstapat *et al.* (2006, 2007), showed indeed evidence of different allosteric binding pockets for different allosteric modulators.

Effect of mGlu2 mutations on the different chemical classes

When analyzing the effect of mutant mGlu2 receptors on the activity of 1,4-Pyridones, mutations F643A, S688L/G689V/N735D, N735D and W773A were shown to decrease the enhancing activity of this compound, with high increases in EC_{50} values (18.5 to 34.7-fold increase) and a reduction in the efficacy of the compounds. Interestingly, mutations H723V, S644A/V700L/H723V and F776A (and also the mutation G689V/S688L in JNJ 41329782) caused a considerable increase in EC50 values (3.4 to 14.4-fold increase), but the efficacy was not severely affected, which can indicate that this mutation affected the affinity of the compound towards the receptor. Figure 23 illustrates the 3D position of the mutated amino acids in the mGlu2 receptor.

Figure 23: 3D representation of the receptor and the mutations that seem to affect the binding of 1,4-Pyridones (the two images represent different angles).

80

For the 1,5-pyridones tested on this study, mutations G689V, G689V/S688L, H723V and S644A/V700L/H723V led to an increase in the EC_{50} (2.76 to 4.8-fold increase) but to small effects on the relative efficacy, whereas mutations N735D, N735D/G689V/S688L and W773A completely disrupted the activity of this compound.

For triazolopyridines, the most potent of the compounds tested (EC_{50} vary between 6 and 91 nM), the effects of the mutations in the different compounds were overall consistent. Mutations S688L/G689V/N735D (and, even though with smaller effects, each one of the correspondent single mutations), F643A, L732A, W773A and S644A/V700L/H723V show a prominent increase in EC_{50} values for the different compounds. The mutations F643A and W773A caused major decreases in the relative efficacy of this class of compounds. Interestingly, mutation L732A consistently and somewhat preferably affects this class of compounds. Figure 24 illustrates the 3D position of the mutations that seem to be important for the activity of this class of compounds. Compounds belonging to triazalopyridine class were, by far, the most affected by the mutations tested: an overview of the ratios between the EC_{50} of the compounds on mutant and WT mGlu2 clearly demonstrates that the mutations have a greater impact on the activity of these compounds. Interestingly, this is accompanied by the high potency of this class of molecules at WT receptor. This increased potency arises due to a more optimal interaction with the protein target. Therefore given this highly optimized fit with the mGlu2 receptor it is natural to expect they would be most sensitive to changes in the binding site. Consequently, L732 seems to be important to render that additional interaction, or indeed increase compound's potencies.
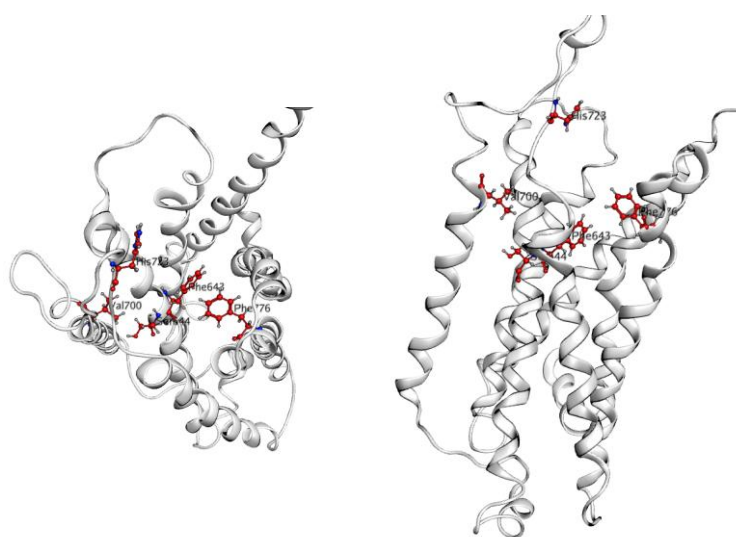

Figure 24: 3D representation of the receptor and the mutations that seem to affect the binding of triazalopyridines (the two images represent different angles).

For isoquinolones, mutations F643A and W773A completely abolished the PAMs activity. The EC50 was impossible to calculate due to a completely disruption of a concentration-response curve. Mutation S644A/V700L/H723V caused a 2.6 to 3.1-fold increase in EC_{50} and a reduction in the relative efficacy. Particularly for the compound JNJ 40297036, mutation L639A caused a 9.1-fold increase in EC50 and a relative efficacy of 90%, against the 141% observed for the WT. The lack of data for some mutations of the transmembrane 4 does not enable the assessment of their effect on compounds activity. Nevertheless, mutation G689V and the triple mutation S688L/G689V/N735D seem to affect the binding of JNJ-40297036. Interestingly, mutant L732A increased five times the activity of the isoquinolone compound, JNJ-39226421.

The enhancing property of the imidazopyridine was abolished in mutants N735D, N735D/G689V/S688L, F643A, L732A and W773A. These mutations completely abolished the receptor capacity of responding to increasing PAM concentrations.

For the reference compounds acetophenone, BINA and LY487379, mutations N735D, N735D/G689V/S688L (Figure 25), F643A and W773A show relatively consistent results. These mutations caused an increase in EC_{50} values (4 to 246.6-fold increase), or a complete obliteration of the PAM effect. BINA showed a ~ 250-fold increase in EC_{50} for the mutation N735D/G689V/S688L, suggesting the importance of this binding pocket for this compound. As for the isoquinone compound JNJ-39226421, also for BINA the mutation L732A increased its activity three times. Interestingly, mutations G689V, G689V/S688L, L639A, L732A, F776A, S644A/V700L/H723V, R635A and S731A differently affected these three compounds, causing an increase in the EC_{50} for one of them, but having no effect on the others.
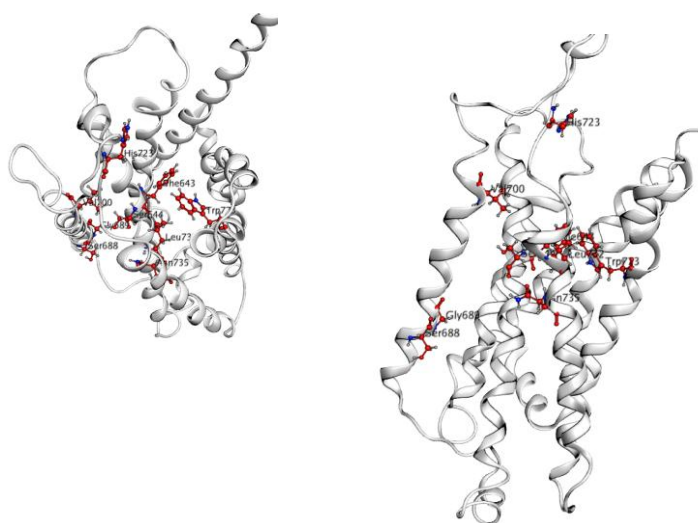

Figure 25: 3D representation of the receptor and the mutations that seem to affect the binding of reference compounds (the two images represent different angles).

Mutation W773A consistently affected the binding off all the compounds, without exception. W773 is located on transmembrane 6 and is conserved in class C GPCRs. Previous mutagenesis studies have shown that the amino acid located at the same position at other mGlu receptors (namely, mGlu1 and mGlu5) is important for the binding of allosteric modulators (Malherbe *et al.*, 2003a,b; Malherbe *et al.*, 2006; Muhlemann *et al.*, 2006). Accordingly, the modeling studies performed for this work suggest that this amino acid is part of a hydrophobic cluster within the expected transmembrane allosteric binding site, formed also by the amino acids F643, L732, W773, and F776. This hydrophobic cluster interacts with the ligand in its predicted binding mode, as Figure 26 displays. Therefore, the high impact of this mutation on the binding of all the compounds tested is consistent with the predictions of the homology model.

As mentioned above, mutation L732A increased the potency of two compounds: JNJ-35815013 (BINA) and JNJ-39226421 (isoquinolone). Although the reasons of this potency increase are unknown, it is interesting to notice that this effect was seen for only one of the compounds belonging to isoquinolone chemical class.
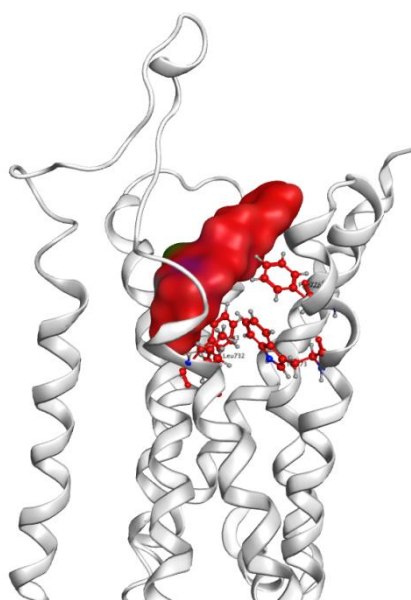
Figure 26: Modelled mGlu2 receptor structure showing bound pose for Janssen mGlu2 PAM in surface representation and the cluster of hydrophobic amino acids, Phe643, Leu732, Trp773, and Phe776.

mGlu2/3 sequences comparison

When assessing the impact of the mutations selected for this study based on the differences between mGlu2 and mGlu3 receptor sequences (S644A, V700L and H723V), only the triple mutation caused major shifts in the activity of PAMs. This large effect did not occur when using the single amino acid mutations (only the mutation H723V led to a loss of effect in several compounds when applied alone) (Table 8). This suggests that these three amino acids may form an important binding pocket for the activity of PAMs and may be responsible for their subtype-specificity. However, these mutations represent only a small part of the mutations that were selected based on this criteria (Table 4, Methods section). Therefore, the testing of the other mutations is needed in order to have more conclusive results. The amino acids S688, Gly689 and Asn735, previously identified in literature, were also selected for mutagenesis based on the sequence divergence between mGlu2 and mGlu3 receptor (Rowe *et al*., 2008) and the high impact of G689V, N735D and the double and triple mutations of these three amino acids confirms their importance in the mGlu2 subtype-selective properties of PAMs.

Overall important amino acids

The overall observation of the ratios between the EC50 of mutant and WT mGlu2 receptors clearly highlights some trends in the effects of the different mutation on PAM performance: mutations F643A, G689V, W773A, S688L/G689V, S688L/G689V/N735D (but not the individual mutations) and S644A/V700L/H723V (but not the individual mutations) have a general impact on the activity of all the structurally distinct compounds (Figure 27). Therefore, these amino acids seem to participate in the structure of a common binding pocket for PAM. The differences in the effects observed for other mutations are possibly a consequence of the features specific for each structure.
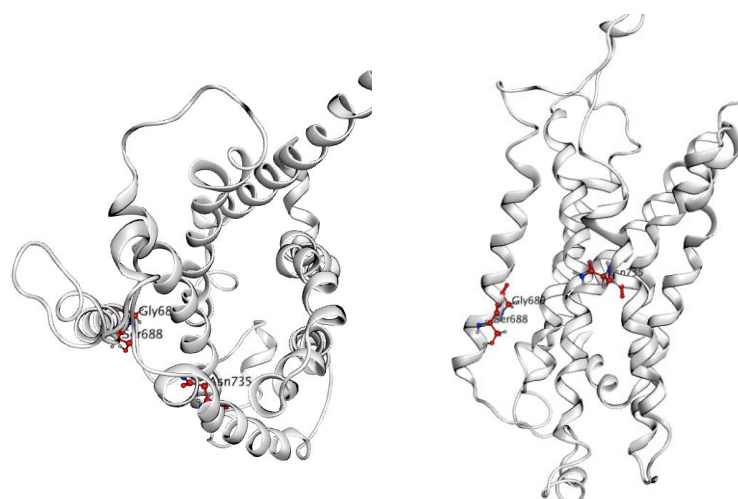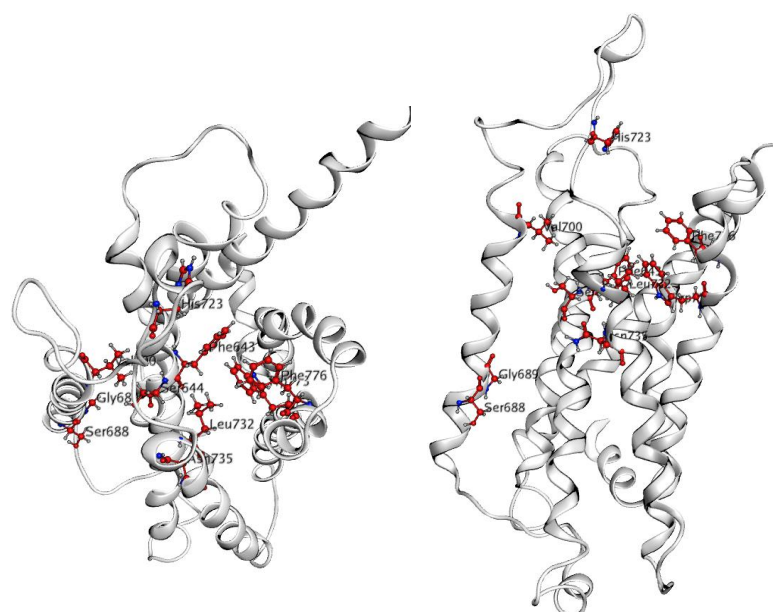
Figure 27: 3D representation of the receptor and the mutations that seem to affect the binding of the PAMs tested in these study (the two images represent different angles).

When assessing the effects of mutations on PAMs activity (in our case using [^{35}S]GTPγS binding assay), it is important to clarify the possible effects that a mutation can have in the receptor function: 1) the mutation can prevent the receptor to acquire a functionally active conformation in the membrane, regardless of the activity of the compound (it can cause, for example, an increase in the internalization of the receptor, disabling the receptor to localize in the membrane); 2) the mutations may enable the binding of the compound, but lead to a conformation that does not allow the interaction with G protein and, therefore, no functional response is detected; 3) the mutated receptor does not allow the binding of the compound or decreases the affinity of the compound for binding to the receptor. In the first and second situations, we hypothesize that the receptor would not be able to be activated even by glutamate, in which case this would be picked up in the glutamate concentration-response curve. Still, the fact that not only potency but also efficacy (E_{max}) for some compounds is decreased, may suggest that certain mutations affect the receptor signaling machinery in some way. In the last situation, although the effects of each mutation are assessed through the impact on a functional response (efficacy), this is in fact the result of an upstream event – the binding of the compound (affinity). As a next step, it would hence be important to assure that the compound is actually able to bind the mutant receptor which could be investigated through radioligand binding studies using a tritiated form of an allosteric modulator or using a tritiated agonist and assessing the effects of the PAM on its binding properties.

85

Chapter 5

Conclusions

The proven success of GPCRs as drug targets for the treatment of CNS disorders renders them attractive targets of research. Specifically, the mGlu2 receptor is currently being pursued as the target for the treatment of several neurological and psychiatric diseases. The quest for increasingly potent and selective ligands for this receptor is leading to the identification of novel ligands, with improved physicochemical and pharmacokinetic properties. Accordingly, mGlu2 positive allosteric modulators constitute an attractive approach for the selective activation of this receptor. Therefore, the identification of the molecular determinants of mGlu2 receptor-PAMs binding is highly attractive.

The present study provides further additional insight on the interactions that drive the activity of PAMs towards mGlu2 receptor. Surprisingly, not only did double or triple mutations affect the activity of the PAMs tested, but a strong and consistent effect was also observed for single amino acid mutations, which enabled the identification of specific molecular determinants that might be important for the activity of these compounds.

Mutations F643A, G689V, W773A, S688L/G689V, S688L/G689V/N735D and S644A/V700L/H723V were shown to have a general impact on the activity of all the structurally distinct compounds. Interestingly, the mutations found by Lundström *et al* (2011) to be important for NAMs binding show different results for the PAMs tested in this study: R636A and F780A, two mutations with a high effect on the binding of NAMs, have no effect on the activity of the PAMs tested. Still, mutations F643A, H723V, L732A, N735D and R635A were shown by Lundström *et al.* to have an effect on NAMs, also seem to affect the activity of PAMs which may suggest common binding sites for NAMs and PAMs.

The knowledge obtained from the mapping of new allosteric modulators is helping to understand the regions of the mGlu receptors that are critical for modulation of positive allosteric modulators activity. Ultimately, this study will contribute to guide research towards the achievement of more selective and potent drugs.

Appendix

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

Abbreviation	Name
(+)-ACPT-III	(3RS,4RS)-1-aminocyclopentane-1,3,4-tricarboxylic acid
(1S, 3R)-ACPD	1S,3R-1-aminocyclopentane-trans-1,3-dicarboxylic acid
(2*R*, 4*R*)-APDC	(2R, 4R)-4-Aminopyrrolidine-2,4-dicarboxylic acid
(RS)-PPG	(RS)-4-Phosphonophenylglycine
(S)-3,4-DCPG	(S)-3,4-Dicarboxyphenylglycine
(*S*)-3,5-DHPG	3,5-Dihydroxyphenylglycine
1S,3S-ACPD	1S,3S-1-aminocyclopentane-1,3-dicarboxylic acid
2S,4S-4MG	2S,4S-4-methylglutamic acid
ABH x D-I	(1S,2S,4S,5S)-2-aminobicyclo[2.1.1]hexane-2,5-dicarboxylic acid
ACPT-I	1-aminocyclopentane-1,3,4-tricarboxylic acid
ADX47273	(S)-(4-fluorophenyl)-(3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]piperidin-1-yl)methanone
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
Bay 36-7620	(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'-biphenyl]-4-carboxylic acid
CDPPB	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide
CHPG	2-chloro-5-hydroxyphenylglycine
CPCCOEt	7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester
CPPG	(RS)-a-cyclopropyl-4-phosphonophenylglycine
CPPHA	N-[4-Chloro-2-(phthalimidomethyl)phenyl]salicylamide N-[4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide
CTZ	Cyclothiazide
DCG-IV	(2S,2' R,3'R)-2-(2',3')-Dicarboxycyclopropyl glycine
DFB	3,3'-Difluorobenzaldazine

E-CBQA	1-amino-3-[2'-3',5'-dioxo-1',2',4'-oxadiazo-lidinyl)] cyclobutane-1-carboxylic acidcyclobutane-1-carboxylic acid
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
FTIDC	4-[1-(2-fluoropyridin-3-yl)-5-methyl-1H-1,2, 3-triazol-4-yl]-N-isopropyl-N-methyl-3,6-dihydropyridine-1(2H)-carboxamide
HYDIA	(1S,2R,3R,5R,6S)-2-amino-3-hydroxybicyclo[3.1.0]hexane-2,6-dicarboxylic acid
JNJ16259685	(3,4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone
L-AP4	(2S)-2-amino-4-phosphonobutanoic acid
L-CBG-I	(2S,1'S,2'S)-2-(2-carboxycyclobutyl)glycine
LCCG-I	(2S,1'S,2'S)-2-(carboxycyclopropyl)glycine
L-F2CCG-I	(2S,1'S,2'S)-3'3'-difluoro-2-(carboxycyclopropyl)glycine
LY341495	2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)propionic acid
LY354740	(1S,2S,5R,6S)-(+)-2-aminobicylco[3.1.0]hexane-2,6-dicarboxylic acid
LY367385	(S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid
LY379268	(−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid
LY38979	(−)-2-thia-4-aminobicylco[3.1.0]hexane-4,6-dicarboxylic acid
LY487379	2,2,2-Trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(3-pyridinylmethyl)ethanesulfonamidehydrochloride
MAP4	(S)-a-methyl-2-amino-4-phosphonobutanoic acid
MCPG	α-Methyl-4-carboxyphenylglycine
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-(3,4-dichlorobenzyloxy)-6-fluorobicyclo[3.1.0] hexane-2,6-dicarboxylic acid
MMPIP	6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazolo[4,5-c]pyridin-4(5H)-one
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
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
NAAG	N-acetylaspartylglutamate
NPS 2390	2-quinoxaline-carboxamide-N-adamantan-1-yl
PHCCC	N-phenyl-7-(hydroxyhydroxy-imino) cyclopropa[b]chromen-1a-carboxamide
R214127	[3H]1-(3,4-dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-2-phenyl-1-ethanone
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
S-4MeGlu	S-4-methyleneglutamic acid
S-AP4	S-4-Phosphono-2-aminobutyric acid
S-Homo-AMPA	2-Amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butyric acid
SIB-1757	6-methyl-2-(phenylazo)pyridin-3-ol
SIB-1893	(E)-2-methyl-6-styrylpyridine
S-SOP	S-serine-O-phosphate
VU0080421	pyrazolo[3,4-d]pyrimidine
VU0155041	*cis*-2-[[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid
VU29	*N*-(1,3-Diphenyl-1*H*-pyrazolo-5-yl)-4-nitrobenzamide
VU71	4-nitro-N-(1,4-diphenyl-1Hpyrazol-5-yl)benzamide
YM-298198	6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide
Z-CBQA	(Z)-1-Amino-3-[2'-(3',5'-dioxo-1',2',4'-oxadiazolidinyl-cyclobutane-1-carboxylic acid
Z-cyclopentyl AP4	*cis*-(±)-1-Amino-3-phosphonocyclopentane carboxylic acid

Appendix 2: mGlu2 receptor positive allosteric modulators tested in this study

1,4-Pyridones	JNJ-40068782
	JNJ-41329782
1,5-Pyridones	JNJ-35814376
Triazolopyridines	JNJ-46281222
	JNJ-42153605
	JNJ-46356479
	JNJ-42329001
	JNJ-43245046
Isoquinolones	JNJ-39226421
	JNJ-40297036
Imidazopyridine	JNJ-41482012
THIIC	JNJ-52149617
BINA	JNJ-35815013
LY487379	JNJ-35814090

Appendix 3: Results from the screening assay performed for all the mutations and PAMs. The EC_{50} and EC_{100} of each PAM are indicated.

PAM	JNJ 40068782-AAA			JNJ 46281222 - AAA			JNJ 42153605 - AAA			JNJ 46356479-AAA			JNJ 42329001-AAA			JNJ 39226421 - AAA			JNJ 35814376 - AAA		
[PAM] (M)	1.E-07	1.E-05	n	3.E-07	3.E-06	n	3.E-08	3.E-06	n	1.E-07	3.E-06	n	3.E-08	3.E-06	n	1.E-07	3.E-06	n	3.E-07	3.E-06	n
Stable	117%	205%	10	207%	223%	10	149%	221%	11	138%	212%	11	122%	206%	11	62%	122%	10	122%	206%	11
WT	116%	180%	8	179%	194%	8	136%	197%	8	140%	187%	8	114%	181%	8	87%	153%	8	114%	181%	8
75% of WT	87%	135%		134%	146%		102%	148%		105%	141%		85%	136%		66%	114%		85%	136%	
R636A	96%	204%	1	222%	286%	1	143%	228%	1	146%	252%	1	184%	237%	1	93%	160%	1	184%	237%	1
L639A	90%	183%	1	173%	163%	1	121%	227%	1	118%	195%	1	168%	220%	1	50%	93%	1	168%	220%	1
F643A	35%	113%	1	59%	121%	1	32%	89%	1	24%	68%	1	24%	80%	1	31%	32%	1	24%	80%	1
D725A	100%	166%	1	166%	207%	1	138%	195%	1	119%	200%	1	164%	183%	1	93%	143%	1	164%	183%	1
L732A	74%	109%	1	88%	111%	1	64%	127%	1	59%	78%	1	47%	106%	1	65%	79%	1	47%	106%	1
W773A	30%	52%	1	37%	65%	1	25%	48%	1	41%	85%	1	30%	62%	1	14%	23%	1	30%	62%	1
F776A	63%	161%	1	191%	218%	1	99%	219%	1	146%	237%	1	83%	178%	1	98%	141%	1	83%	178%	1
F780A	115%	219%	1	230%	228%	1	161%	248%	1	172%	243%	1	155%	211%	1	83%	147%	1	155%	211%	1
S644A	80%	254%	1	261%	350%	1	138%	325%	1	151%	319%	1	98%	307%	1	49%	129%	1	98%	307%	1
V700L	127%	161%	1	192%	193%	1	139%	181%	1	133%	198%	1	127%	182%	1	81%	133%	1	127%	182%	1
H732V	64%	165%	1	156%	202%	1	81%	176%	1	93%	179%	1	88%	204%	1	56%	120%	1	88%	204%	1
S644A/V700L/H723V	33%	153%	1	111%	196%	1	50%	197%	1	48%	175%	1	69%	232%	1	38%	109%	1	69%	232%	1
R635A	74%	269%	1	267%	331%	1	144%	365%	1	130%	277%	1	131%	370%	1	56%	142%	1	131%	370%	1
M728A	161%	181%	1	173%	170%	1	179%	184%	1	153%	172%	1	144%	197%	1	123%	123%	1	144%	197%	1
S731A	99%	254%	1	245%	315%	1	125%	292%	1	152%	257%	1	132%	271%	1	72%	143%	1	132%	271%	1
V736A	128%	223%	1	237%	273%	1	170%	266%	1	143%	259%	1	129%	241%	1	70%	135%	1	129%	241%	1

PAM	JNJ 41482012 - AAA			JNJ 40297036 - AAA			JNJ 35815013 - AAA			JNJ 35814090 - AAC			JNJ 52149617 - AAC			JNJ 41329782 - AAA			JNJ 43245046- AAA		
[PAM] (M)	3.E-07	3.E-06	n	3.E-07	3.E-06	n	3.E-08	3.E-06	n	3.E-07	3.E-06	n	1.E-07	3.E-06	n	1.E-07	3.E-06	n	3.E-09	3.E-07	n
Stable	76%	99%	10	110%	139%	10	59%	169%	10	95%	134%	9	91%	185%	9	142%	225%	9	104%	226%	9
WT	82%	113%	8	130%	158%	8	71%	170%	8	102%	129%	7	88%	159%	7	142%	190%	7	107%	204%	7
75% of WT	61%	84%		97%	119%		53%	127%		77%	97%		66%	119%		107%	143%		80%	153%	
R636A	109%	141%	1	118%	146%	1	81%	201%	1	113%	100%	1	106%	178%	1	130%	231%	1	163%	258%	1
L639A	110%	129%	1	62%	83%	1	33%	116%	1	72%	128%	1			1			1			1
F643A	24%	25%	1	27%	45%	1	16%	117%	1	21%	33%	1	34%	68%	1	35%	84%	1	46%	119%	1
D725A	130%	140%	1	118%	153%	1	74%	137%	1	87%	119%	1	112%	171%	1	117%	159%	1			1
L732A	50%	56%	1	75%	88%	1	73%	100%	1	51%	59%	1	55%	86%	1	92%	145%	1	47%	111%	1
W773A	25%	34%	1	39%	52%	1	22%	26%	1	39%	67%	1	33%	11%	1	21%	76%	1	42%	77%	1
F776A	69%	102%	1	151%	184%	1	48%	102%	1	34%	42%	1	52%	100%	1	88%	177%	1	80%	203%	1
F780A	108%	141%	1	123%	171%	1	59%	165%	1	80%	113%	1	56%	162%	1	119%	199%	1	113%	228%	1
S644A	63%	93%	1	98%	168%	1	48%	223%	1	63%	111%	1	54%	182%	1	83%	277%	1	86%	304%	1
V700L	74%	89%	1	109%	148%	1	56%	148%	1	107%	115%	1	76%	158%	1	156%	192%	1	119%	195%	1
H732V	66%	84%	1	88%	115%	1	70%	196%	1	89%	116%	1	58%	156%	1	92%	141%	1	75%	186%	1
S644A/V700L/H723V	46%	60%	1	55%	98%	1	61%	258%	1	41%	91%	1	32%	124%	1	40%	164%	1	61%	266%	1
R635A	96%	138%	1	135%	184%	1	13%	137%	1	78%	171%	1	83%	233%	1	145%	371%	1	166%	444%	1
M728A	136%	152%	1	157%	167%	1	81%	153%	1	105%	98%	1	109%	151%	1	184%	185%	1	161%	212%	1
S731A	84%	98%	1	99%	159%	1	72%	214%	1	55%	92%	1	61%	186%	1	97%	242%	1	91%	240%	1
V736A	67%	87%	1	104%	153%	1	57%	197%	1	79%	146%	1	80%	191%	1	188%	252%	1	99%	225%	1

Appendix 4: Potency (EC$_{50}$) and relative Emax of the enhancement of glutamate-induced [^{35}S]GTPγS binding by 14 PAMs on WT and mutant receptors after transient transfection into CHO-K1 cells. Compound potency is presented as a ratio of EC$_{50}$ between WT and mutant mGlu2. (SD: standard deviation)

Top table — Transmembrane 3

	Stable						WT						(18) R635A					(6) R636A			(7) L639A			(8) F643A			(14) S644A		
	EC50	SD	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	mean	mean	mean	mean	mean	mean	mean	mean	SD	n	mean	SD	n
JNJ-40068782	139	47	15	206	13	15	87 (1.0)	23	7	201	27		378 (4.3)	27	3	246 (1.2) Sc	Sc	572 (6.5) Sc	Sc	225	Sc	2395.6 (27.4)	862	3	89 (0.4)	25	3	216 (2.5)	111	3
JNJ-41329782	68	15	10	215	14	10	57 (1.0)	15	4	202 (1.0)	9	4	Sc			Sc	Sc	109 (2.9) Sc		16	Sc	1142 (20.0)	256	2	84 (0.4)	10	3	180 (3.2)	144	3
JNJ-35814376	637	147	12	196	16	12	523 (1.0)	163	5	186 (1.0)	14	5	853 (1.6)	83	2	225 (1.2) Sc	Sc	1067 (2.0) Sc	Sc	822	Sc	Sc		3	Sc			625 (1.2)	165	3
JNJ-46281222	8	4	6	214	6	6	6 (1.0)	5	3	211 (1.0)	25	3	Sc			Sc	Sc	Sc	Sc		Sc	316 (55.6)	—	2	107 (0.5)	12	2	Sc		
JNJ-42153605	6	4	9	224	12	9	5 (1.0)	3	3	214 (1.0)	30	3	Sc			Sc	Sc	Sc	Sc		Sc	447 (84.3)	323	2	90 (0.4)	1	2	Sc		
JNJ-46356479	91	28	9	227	10	9	59 (1.0)	27	4	204 (1.0)	13	4	Sc			Sc	Sc	Sc	Sc		Sc	6782.9 (115.6)	2171	3	86 (0.4)	9	2	Sc		
JNJ-42329001	49	18	9	218	16	9	37 (1.0)	9	4	200 (1.0)	30	4	Sc			Sc	Sc	Sc	Sc		Sc	5867.8 (156.8)	1045	3	137 (0.7)	33	3	Sc		
JNJ-43245046	6	1	9	224	11	9	7 (1.0)	1	3	227 (1.0)	11	3	Sc			Sc	Sc	9 (1.4) Sc	Sc	1	2	254 (38.7)	49	2	101 (0.4)	4	3	Sc		
JNJ-39226421	178	36	9	130	16	9	172 (1.0)	37	4	141 (1.0)	20	4	117 (0.7)	37	1	92 (0.7) Sc	Sc	227 (1.3) Sc	Sc	97	2	n.v.		2	36 (0.3)	11	2	119 (0.7)	10	2
JNJ-40297036	143	31	10	139	8	10	148 (1.0)	25	4	147 (1.0)	9	4	Sc			Sc	Sc	1351 (9.1) Sc	Sc	1073	3	n.v.		3	49 (0.3)		3	Sc		
JNJ-41482012	172	27	8	97	7	8	175 (1.0)	28	3	101 (1.0)	2	3	Sc			Sc	Sc	Sc				n.v		2	36 (0.4)		2	Sc		
JNJ-52149617	113	23	17	166	16	17	114 (1.0)	42	8	159 (1.0)	26	8	Sc			Sc	Sc	161 (1.4) Sc	Sc	20	4	724 (6.4)	—	1	62 (0.4)	10	3	305 (2.7)	69	3
JNJ-35815013	97	34	12	173	9	12	87 (1.0)	35	7	168 (1.0)	22	7	490 (5.6)		1	178 (1.1) Sc	Sc	1320 (15.2) Sc	Sc	86	2	1414.1 (16.3)	231	3	84 (0.5)	42	4	108 (1.2)	39	3
JNJ-35814090	173	65	16	116	9	16	156 (1.0)	102	8	128 (1.0)	12	8	Sc			Sc	Sc	692 (4.4) Sc	Sc	—	1	n.v.		2	53 (0.4)	19	3	276 (1.8)	88	3

Bottom table — Transmembrane 4

	(1) S688L			(2) G669V			(4) G689V / S688L			(5) N735D / G689V / S688L			(15) V700L		(17) S644A/V700L/H723V		
	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n	mean	mean	mean	SD	n
JNJ-40068782	160 (1.8) NT	145	3	603 (6.9) NT	212	4	635 (7.3) 1349	416	3	n.v.	0	2	Sc	Sc	1262 (14.4)	123	3
JNJ-41329782	201 (1.0) NT			187 (0.9) NT			201 (1.0) 124	16	3	NT			Sc	Sc	163 (0.8) 621	42	2
JNJ-35814376	520 (1.0) NT	59	2	2512 (6.9)	—	1	23.6 (0.6) 1445	—	1	n.v.	—	1	Sc	Sc	186 (0.9) 2225 (10.9)	170	2
JNJ-46281222	20 (1.0)	—	1	218 (1.2) 4.8	—	1	125 (0.7) 61	71	2	767.17 (134.9) 695	49	1	Sc	Sc	135 (0.7) 573 (4.3)	23	3
JNJ-42153605	3.6 7 (1.3)	2	2	83 (1.1) 14.6	69	2	215 (1.0) 10.8	52	2	212 (40.0) 240	203	2	Sc	Sc	213 (1.0) 72 (4.3)	43	2
JNJ-46356479	25 (1.2) NT	25	2	25 (1.0) 4.7	4	2	223 (1.0) 4.2	11	2	3162.3 (53.9) —	180	3	Sc	Sc	209 (1.0) 47 (12.7)	4	3
JNJ-42329001	NT			1349 (23.0)	—	1	309 (1.0) 5.3	—	1	489.78 (13.1) 0	174	1	Sc	Sc	215 (1.1) 692 (8.8)	0	1
JNJ-43245046	NT			170 (0.9) 4.5	—	1	577 (1.0) 15.4	598	2	41 (0.9) —	208	1	Sc	Sc	230 (1.1) 115 (11.8)	42	2
JNJ-39226421	NT			39 (0.9) 5.9	—	1	186 (1.0) 6.2	35	1	NT	216	1	Sc	Sc	289 (1.3) 3.1	34	2
JNJ-40297036	209 (1.4)	—	1	537 (3.6)	—	1	40 (1.0) NT	—	1	n.v.	142	2	Sc	Sc	95 (0.7) 42 (6.4)	74	2
JNJ-41482012	130 (0.7)	46	2	−24 (−0.1)	—	2	264 (0.8) 1.8	34	2	n.v	40	2	Sc	Sc	539 (3.1) 389 (2.6)	130	1
JNJ-52149617	154 (1.3)	120	3	646 (5.7)	324	3	223 (0.6) 1.3	142	2	2896.3 (25.4) 376	37	2	Sc	Sc	101 (0.7) 274 (1.6)	16	3
JNJ-35815013	145 (1.7)	118	3	832 (9.6)	720	3	635 (1.1) 5.6	280	3	21380 (246.6) —	56	3	Sc	Sc	73 (0.7) 620 (5.4)	50	3
JNJ-35814090	114 (0.7)	19	3	327 (2.1)	257	3	575 (1.0) 6.6	270	3	n.v.	44	1	Sc	Sc	151 (1.0) 78 (0.9)	78	2

Legend:

NT	Not tested
Sc	Tested in screening assay
(red)	If ratio > 3 or n.v
n.v.	No value (absence of curve)

Extracellular loop 2/Transmembrane 5

	(16) H723V				(9) D725A	(19) M728A	(20) S731A				(10) L732A			(3) N735D			(21) V736A	
	mean	SD	n		mean	mean	mean	SD	n	mean	mean	SD	n	mean	SD	n	mean	mean
JNJ-40068782	549 / 6.3	77	3	189 / 0.9 / 25 / 3	Sc	Sc	Sc			95 / 1.1	130 / 0.6	12	3	3019.95	70 / 0.3	1 / 3	Sc	Sc
JNJ-41329782	260 / 4.6	135.6	3	217 / 1.1 / 72 / 3	247 / 1.2	245 / —	Sc	19	1 / 2	63 / 1.1	140 / 0.7	7 / 5	3	NT	NT	15	Sc	Sc
JNJ-35814376	1625 / 3.1	368	3	152 / 0.8 / 31 / 3	189 / 1.0	1190 / 2.3	Sc	12	2 / 2	633 / 1.2	129 / 0.7	229 / 9	3	n.v.	81 / 0.4	0 / 1	Sc	Sc
JNJ-46281222	1			1	Sc	Sc	Sc			58 / 0.7	142 / 0.7	47 / 7	3	~617	221 / 1.0	1 / 1	Sc	Sc
JNJ-42153605	28 / 5.2	1	2	226 / 1.1 / 36 / 2	Sc	Sc	Sc			10.3	159 / 0.7	20 / 16	3	102.0	220 / 1.0	2 / 2	Sc	Sc
JNJ-46356479	195 / 3.3	—	1	220 / 1.1 / 25 / 2	Sc	Sc	Sc			49 / 9.2	131 / 0.6	387 / 24	4	27 / 5.2	NT	5	Sc	Sc
JNJ-42329001	1			1	Sc	Sc	Sc			1015 / 17.3	141 / 0.7	131 / 7	3	NT	NT		Sc	Sc
JNJ-43245046	15 / 2.3	6	3	281 / 1.2 / 80 / 3	Sc	Sc	Sc			576 / 15.4	130 / 0.6	21 / 6	3	NT	NT		Sc	Sc
JNJ-39226421	253 / 1.5	100	2	115 / 0.8 / 15 / 2	Sc	Sc	Sc			82.57 / 12.6	73 / 0.5	10 / 4	2	NT	NT		Sc	Sc
JNJ-40297036	351 / 2.4	84	3	116 / 0.8 / 8 / 3	Sc	Sc	Sc			57 / 0.3	78 / 0.5	136 / 3	3	n.v.	41 / 0.3	1 / 1	Sc	Sc
JNJ-41482012	175 / 1.0	77.89	3	78 / 0.8 / 2 / 3	Sc	Sc	Sc			329 / 2.2	52 / 0.5	6	3	n.v	39 / 0.4	2 / 2	Sc	Sc
JNJ-52149617	281 / 2.5	71	3	174 / 1.1 / 17 / 3	169 / 1.1	285 / 2.5	Sc	11	3 / 3	357 / 3.1	91 / 0.6	225 / 6	3	2790.74 / 24.5	96 / 0.6	227 / 2	6 / 3	Sc	Sc
JNJ-35815013	Sc			Sc	Sc	Sc	Sc			15 / 0.2	110 / 0.7	2 / 5	2	7413.1 / 85.5	74 / 0.4	— / 1	31 / 3	Sc	Sc
JNJ-35814090	Sc			Sc	109 / 0.9	834 / 5.3	Sc	13	3 / 3	n.v.	62 / 0.5	4 / 4	_n.v.	40 / 0.3	3 / 3	Sc	Sc

Transmembrane 6

	(11) W773A			(12) F776A			(13) F780A		
	mean	SD	n	mean	SD	n	mean	SD	n
JNJ-40068782	n.v.		3	440 / 5.0	233	3	Sc		
JNJ-41329782	1059 / 18.5	17.2	2	192 / 3.4	34	3	Sc		
JNJ-35814376	n.v.		3	Sc			398.1 / 0.8	148 / 0.8	1
JNJ-46281222	76 / 13.3	—	1	Sc			Sc		
JNJ-42153605	n.v.		3	24 / 4.6	7	3	Sc		
JNJ-46356479	2344 / 40.0	—	1	Sc			Sc		
JNJ-42329001	1259 / 33.6	—	1	70 / 1.9	16	2	Sc		
JNJ-43245046	361 / 55.0	93	2	7 / 1.1	1	3	Sc		
JNJ-39226421	n.v.		2	Sc			Sc		
JNJ-40297036	n.v.		3	Sc			Sc		
JNJ-41482012	n.v		3	Sc			Sc		
JNJ-52149617	n.v.		3	125 / 1.1	34	4	218.8 / 1.9	156 / 1.0	1
JNJ-35815013	n.v.		4	199 / 2.3	26	3	Sc		
JNJ-35814090	631 / 4.0	6	3	n.v.		2	Sc		

NT	Not tested
Sc	Tested in screening assay
(pink)	If ratio > 3 or n.v
n.v.	No value (absence of curve)

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