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Metabotropic glutamate receptors: mechanism of activation

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Abreviations

AA: arachidonic acid

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate

AR: adrenergic receptor

AC: adenylate cyclase

Acc: Active closed-closed conformation of ECDs

Aco: Active closed-open conformation of ECDs

AM: allosteric modulator

BRET: bioluminiscence resonance energy transfer

cAMP: cyclic adenosin-monophosphate

CaSR: calcium-sensing receptor

CFP: cyan fluorescent protein

CRD: cystein-rich domain

CT: C-terminus

DAG: diacylglycerol

DFB: 3,3´-difluorobenzaldehyde

e1, e2, e3: first, second and third extracellular loop

ECD: extracellular domain

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FRET: fluorescence resonance energy transfer

GABA: γ-aminobutyric acid

$GABA_{B1}$: GABAB receptor subunit 1

$GABA_{B2}$: GABAB receptor subunit 2

GFP: green fluorescent protein

GPCR: G-protein coupled receptors

G-protein: GTP-binding protein

GDP: guanosine-diphosphate

GTP: guanosin-tris-phosphate

HA: hemagglutinin

HBSS: Hank's balanced salt solution

HD: heptahelical domain

HEK 293: human embryonic kidney cells

i1, i2, i3: first, second and third intracellular loop

IP3: inositol-1,4,5-trisphosphate

LB1/2: lobe 1/lobe 2

LBD: ligand binding domain

LIVBP: leucin/isoleucin/valin binding protein

LTD: long-term depression

mGluR: metabotropic glutamate receptor

MPEP: 2-methyl-6-(phenylethynyl)-pyridine

NMDA: N-methyl-D-aspartate

OR: opioid receptor

PBP: periplasmic binding protein

PIP2: phosphoinositol-4,5-bis-phosphate

PKA: cAMP-dependent protein kinase

PKC: protein kinase C

PLC: phospholipase C

Roo: Resting open-open conformation of ECDs

SDS: sodium dodecyl sulphate

TM: transmembrane

TMD: transmembrane domain

TR-FRET: time resolved FRET

YFP: yellow fluorescent protein

Abstract

Any living organism receives constantly many signals that have to be evaluated and weighted to respond in an appropriate way. To perform all functions needed for precise control of homeostasis and for communication with the surrounding environment, signals coming from the outside are recognized and transferred into modulation of intracellular signaling cascades. These mediate response to the extracellular stimulus as well as intercellular communication.

Cell communication is mediated by several types of receptors, located either intracellularly (including nuclear receptors) that modulate gene transcription and receptors localized on plasma membrane. Cell membrane receptors are transmembrane proteins that are divided into three superfamilies according to their structure and principles of signal transduction. These are ion channel-linked receptors, enzyme-linked receptors and G-protein-coupled receptors (GPCRs).

GPCRs comprise the biggest family of membrane receptors and are one of the largest gene families in general. They are encoded by about 1% of genes in mammals. Many of them bind sensory ligands (rhodopsin, taste and olfactory receptors), but others also recognize ions, amino acids, nucleotides, peptides and large glycoproteins (1). They play a crucial role in such distant physiological functions as from chemotaxis in yeasts to neurotransmission in mammals. More than 50% of therapeutic compounds on the market act via some GPCR. Therefore it is not surprising that these receptors are intensively studied.

Metabotropic glutamate receptor 1 plays fundamental role in neuronal signaling in several brain regions that control moving, processes of memory and higher cortical analyzing functions, last but not least also neuronal survival. Impairment of the mGluR1-mediated signaling could markedly contribute or cause severe neurological disasters. MGluR1 is able to activate distinct types of G-proteins and thus triggers different signaling pathways, having different output effects. MGluR1-mediated signaling is influenced by several protein-protein interactions. Different functions of metabotropic glutamate receptors are intensively studied in broader consequences of distinct brain regions and cellular demands and compositions. Investigation of mechanisms of mGlu receptor activation could markedly contribute to disclosure of their functioning.

The main structural motif of all GPCRs is a heptahelical domain with the extracellular N-terminus constituted of seven transmembrane alpha helices that are

linked by three extracellular and three intracellular loops, C-terminus being located inside of the cell (1).

Class C GPCRs possesses in addition a large extracellular N-terminal domain, which is composed of two lobes that close upon ligand binding (2). The conformational change of the extracellular domain is transmitted on the heptahelical domain upon activation of the receptor and this activates G-protein(s) on the intracellular side. This conformational change is fundamental for transfer of the signal into the cell and involves structural rearrangement of transmembrane helices and intracellular loops that is important for G-protein coupling.

For many GPCRs it has been recently reported that they form dimers or higher-order oligomers (3). Some of them are composed of two identical subunits (metabotropic glutamate receptors) and are called homodimers, others are heterodimers ($GABA_B$ receptor). It has been shown that dimerization of GPCRs is crucial for activation of some receptors and transfer of the signal. For example, in heteromeric $GABA_B$ receptor the existence of two different subunits, one binding GABA (γ-aminobutyric acid) and the other activating G-proteins, is pivotal. But what is the reason for homodimeric receptors to exist?

In our studies we adressed and partially explained principle of GPCR activation in respect to their dimeric nature.

I. Theoretical part

1. Introduction

Cells within an organism communicate in a complex way to perform all physiological functions and to respond to external conditions. In other words, cellular communication enables interactions of the organism with an external environment and to react to many stimuli to maintain homeostasis.

Cells are constantly exposed to hundreds of different signals. By different sets of signals, cells are programmed for survival, differentiation or death (apoptosis).

The broad spectrum of these molecules act only on a few types of membrane signal transducers - ion channel-linked receptors, enzyme-linked receptors and receptors that are linked to heterotrimeric guanosine triphosphate-binding proteins (G-proteins), so-called GPCRs (4, 5). Activation of these receptors modulate activity of associated intracellular proteins. This leads to various cellular events.

2. G-protein coupled receptors

Among cell surface receptors, more than 1000 GPCRs are encoded in mammalian genomes and thus constitute the largest family of receptors. They mediate very divergent functions in mammals. Most of them are sensory receptors like taste, olfactory receptors or rhodopsin. Approximately 4-5 hundreds of them discern and transduce a message of nonsensory ligands such as ions, neurotransmitters (amino acids and their derivatives), nucleotides, fatty-acid derivatives, peptides and large proteins including hormones. Only for more than 1/5 of GPCRs, the physiological ligands are known. Receptors that are known to exist but their endogenous ligands have not been identified yet are called „orphan“ receptors.

The broad spectrum of endogenous binding molecules evokes a broad spectrum of responses in the organism through different receptors. As an example, GPCRs influence action of cardiovascular system (β-adrenergic receptor (AR), muscarinic acetylcholine receptor 2, angiotensin and endothelin receptors, tromboxan A_2 or purinergic receptors), endocrine system and metabolism (receptors for corticotropin-releasing hormone, growth hormone-releasing hormone, gonadotropin-releasing hormone etc.). They also modulate many immune functions such as chemotaxis, proliferation, differentiation, mediator release and phagocytosis (chemokine, tromboxan A_2 receptors) and are involved in development and cell growth (protease-activated receptor, endothelin receptors, some muscarinic and serotonin receptors etc.). Finally they have multiple roles in nervous system from modulation of synaptic neurotransmisson to transduction of sensory stimuli (α2-adrenergic receptors, μ- and δ-opioid receptors, $GABA_B$, adenosin type 1, cannabinoid receptor type 1, serotonin and dopamine receptors, metabotropic glutamate receptors (mGluRs**),** rhodopsin, olfactory and taste receptors) (6).

Interestingly, the same ligand can activate many different receptors, e.g. at least 9 distinct GPCRs are activated by epinephrine and 14 different serotonin receptor subtypes exist. Thus one ligand can activate distinct receptor subtypes specific for certain tissues and physiological functions. For example, muscarinic acetylcholine receptors are of 5 types being present in heart, endothelial and neuronal cells. In contrast, mGluRs are of 8 types and have multiple functions in nervous system.

For a long time, GPCRs have been known to modulate ion-linked channels via G-proteins. Recently, there is an increasing evidence that GPCRs act also to regulate other membrane or intracellular proteins through direct protein-protein interactions.

2.1. Structure and diversity of GPCRs

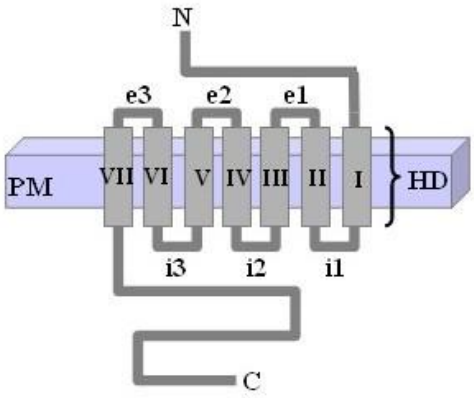


Fig. 1: Structure of heptahelical receptors

Despite the diversity of their sequences, chemical and functional diversity of their signal molecules, all GPCRs display a similar structure. They consist of a polypeptide chain starting extracellularly crossing seven-times the plasma membrane to form seven transmembrane (I-VII) α-helices, so called heptahelical domain (HD). The helices are linked together by 3 extracellular (e1, e2, e3) and 3 intracellular loops (i1, i2, i3). The C-terminus (CT) is located on the intracellular site of the membrane (**Fig. 1**). The three intracellular loops form intracellular face for interaction with many partner proteins, not only G-proteins but also kinases (G-protein receptor kinases), arrestins and others. The TM helices of heptahelical receptors are clustered together to form a functional unit (**Fig. 2**).

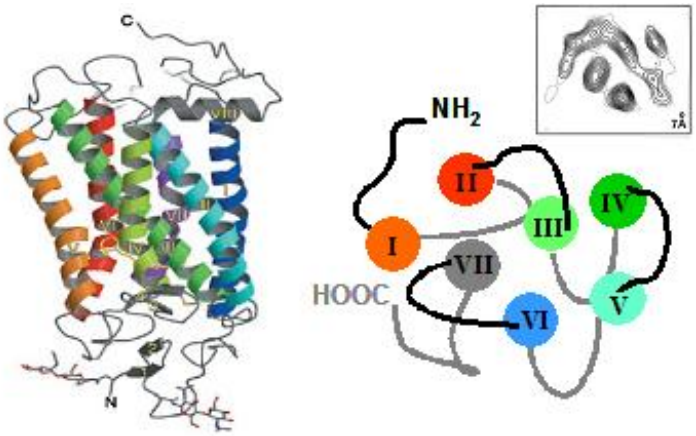


Fig. 2: Structure of rhodopsin and organization of the transmembrane helices
(left) Structure of rhodopsin paralel to the plane of the membrane as determined by X-ray crystallography (7). (right) The arrangement of the seven transmembrane helices of rhodopsin based on the density map obtained from electron cryomicroscopy according to G. Schelter (Cambridge, UK) (inset). Colours correspond to illustration on the left.

Although receptors from different families share no sequence homology (1), the similar serpentine structure of the HD and activation of common downstream cascades through G-proteins make them related.

As mentioned, GPCRs are divided according to sequence similarity and way of the ligand binding into 4 classes (after very recent IUPHAR (International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification) classification) (1, 8) (**Table 1**). GPCR domains involved in ligand binding are nearly as diverse as the chemical structures of the known agonists (9, 10).

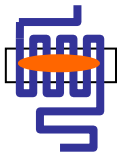
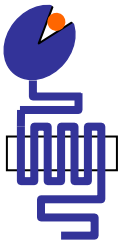
Class A GPCRs (rhodopsin-like receptors) is activated by small ligands like biogenic amines (catecholamines, acetylcholine, dopamine, histamin and serotonin), nucleotides, opiates, lipids, short peptides, cytokines, hormones (folicle stimulating hormone, luteinizing hormone etc.) and proteases (e.g. thrombin). These small molecular weight ligands bind within the hydrophobic core of the heptahelical domain or to extracellular site of the receptor into N-terminal domain and extracellular loops, mainly e1 and e2 (**Table 1**). They also encompasses a large group of "orphan" receptors (8).

Class B (calcitonin receptor-like receptors) of GPCRs has relatively long N-terminal domain that plays role in ligand binding. As the binding site for peptides and proteins include the N-terminus and extracellular hydrophilic loops. The agonists are e.g. calcitonine, secretin, gonadotropin-releasing hormone, corticotropin-releasing factor or vasoactive intestinal polypeptide.

Members of class C GPCRs (metabotropic glutamate receptor-like receptors) possess a very large extracellular domain. This domain binds small ligands such as amino acids, ions, peptides and sugars. Class C comprises of receptors for main excitatory (glutamate) and inhibitory (GABA) transmitter in the NS – metabotropic glutamate receptors and $GABA_B$ receptors. Other receptors are calcium-sensing receptor (CaSR), pheromone, taste receptors and group of „orphan" receptors (1, 8).

Last class of receptors, so-called Frizzled receptors, encompasses seven TM receptors that specifically bind small glycoproteins called Wnts and are involved mainly in devolopment, specification of tissues etc. (8, 11).

Table1: Diversification of G-protein coupled receptors (GPCRs) (1, 8).
GPCRs are divided into three families according to their sequence similarities and way of ligand binding. Schematic representation of membrane receptors (blue) belonging to each class with illustration of bound ligand (orange) (in the middle). Examples of ligands are on the right. ATP: adenosine-trisphosphate, CRF: corticotropin-releasing factor , fMLP: N-formyl-Met-Leu-Phe, FSH: follicle stimulating hormone, GABA: γ-aminobutyric acid, GnRH: gonadotropin-releasing hormone, LH: luteinizing hormone, PACAP: pituitary adenylate cyclase activating polypeptide, PAF: platelet-activating factor, PTH: parathyroid hormone, TSH: thyroid-stimulating hormone, VIP: vasoactive intestinal polypeptide.

GPCR-family	Schematic illustration	Known ligands
Class A Rhodopsin-like receptors		Retinal, odorants, catecholamines, adenosine, ATP, opiates enkephalins anandamide
		Peptides, cytokines, chemokines, fMLP, PAF-acether, thrombin
		Glycoprotein hormones (LH, TSH, FSH,...)
Class B Calcitonin receptor-like receptors		Calcitonin, secretine, PTH, VIP, PACAP, GnRH, CRF
Class C Metabotropic glutamate receptor-like receptors		Glutamate, GABA, Ca^{2+}, pheromones, sweet, umami
Frizzled receptors		

2.2. Dimerization of GPCRs

Originaly, GPCRs were assumed to exist and function as monomers with the dogma that one ligand activates one receptor that in turn activates one G-protein. There is growing evidence, that many GPCRs form dimers or higher-order oligomers (3). Oligomerization opens new possibilities to explain functioning of GPCRs in terms of their activation, transduction of the signal and inter-receptor interference with its physiological impacts, but also requires more difficult approaches for prooving the existence of oligomers *in vivo*. Various inter-GPCR interactions were demonstrated either *in situ* (12-19) or *in vitro* (17, 19-21).

The question is whether all GPCRs form dimers? Within the rhodopsin-like receptors, rhodopsin was shown to exist as a dimer *in situ* and as such can be further arranged into oligomeric, so-called para-crystaline arrays (22, 23). But rhodopsin can form a functional unit also as a monomer, although signaling through this kind of interaction is less efficient in comparison to dimeric form (24).

Oligomerization has been reported for other members of class A GPCRs. Opioid receptors (ORs) were shown to form oligomers within their subfamily (25, 26) or with other receptors from the class A - β-ARs or chemokine receptors (27-31). α_{1b}-AR also forms quaternary structures, possibly chain-like structures involving either symmetric or asymmetric inter-helical interactions (32). However, some chemokine receptors were observed to exist as constitutive dimers and oligomers (CXCR4, CCR2, CXCR2) or heterodimers (CCR2:CCR5, CCR2:CXCR4), others were indicated to form monomers (CCR5, CXCR1) (30, 33-37). Similarly, five somatostatin receptors can assemble in functional homo- or heterodimers and/or hetero-oligomers with dopamine receptors (17, 38).

In contrast to marked flexibility of these receptors to form mono-, di- or oligomers, members of the class C GPCRs form constitutive homo- (19, 39-41) or heterodimers (21, 42-45), although formation of functional complexes with other GPCRs or ion-channels in sense of bilateral mediation was also observed (12, 14, 18, 20, 46).

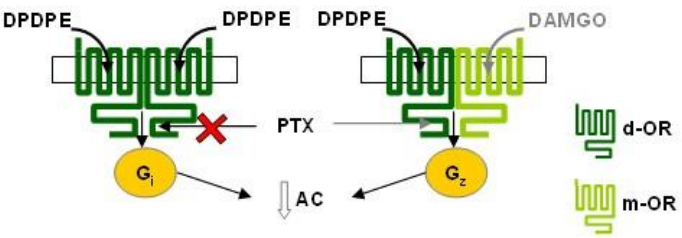
The phenomenon of heterodimerization was described between different types of receptors, e.g. dopamine, adenosine, angiotensin, bradykinin, chemokine, $GABA_B$, taste, olfactory, muscarinic, opioid, serotonin and somatostatin receptors (17, 19, 21, 26, 30, 38, 39, 43, 46-54).

2.2.1. Physiological consequences of dimerization

We could ask what is the physiological reason for phenomenon of dimerization and oligomerization? Oligomeric structures in general appear to be essential for biosynthesis, cellular transport, diversification and degradation (55). In some cases, receptor dimerization is essential for receptor function e.g. of the $GABA_B$ receptor (56), metabotropic glutamate receptors (57), taste receptors (58), calcium sensing receptor (59), rhodopsin (24, 60), opioid and chemokine receptors (25, 26, 58, 61) and others.

Dimer formation alters GPCR binding properties and/or G-protein coupling

Dimerization of ORs has been shown to alter opioid ligand properties (25, 62) and affect receptor trafficking (27, 61). δ-OR forms heterodimers either with μ-OR (25) or κ-OR (26). In both cases, functional dimers are formed with unique binding properties that function synergistically. Heterodimerization probably enables formation of unique binding site between different subtypes of opioid receptors either for ligand or for intracellularly associated G-protein. Opioid receptors usually trigger G_i-activation cascade, but in case of μ-OR:δ-OR heterodimer, they inhibit adenylyl cyclase (AC) by pertussis toxin-insensitive way, probably through $G_z\alpha$-protein (25) (**Fig. 3**). Further δ-OR agonist can substitute the bound μ-OR agonist and vice versa (62). These results confirmed that heterodimerization leads to formation of the different binding site, to changes in pharmacological properties and to coupling to different Gα-protein and signaling pathways. Dimerization thus enables to generate greater diversity of opioid signaling.



Receptor dimer	[^{3}H]-naloxone	+ DPDPE	[^{3}H]-naloxone	+ DAMGO
	inhibition	competition	no effect	competition
	inhibition	no effect	inhibition	competition

Fig. 3: Heterodimerization alters pharmacological properties of receptors
Maximal effect of selective agonist treatment on competition for [^{3}H]-naloxone binding by DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephaline) and by DPDPE ([D-Pen^2,D-Pen^5]enkephaline) in membranes from cells expressing δ-OR or μ-OR alone or co-expressing both receptors (25). AC: adenylyl cyclase, PTX: pertussis toxin, G: G-protein, δ-OR, μ-OR: δ- and μ-opioid receptor, respectively.

Dimers appear to modulate physiological response

Chemokine receptor can form homodimers as well as heterodimers and their clustering can be influenced by the composition of chemoattractant soup in the neighbourhood of leukocytes. For example simoultaneous co-activation of chemokine heterodimers CCR2:CCR5 facilitates the chemokine receptor sensitivity and triggers different type of signaling cascade. In addition, the activation of this heterodimer does not induce down-regulation, but triggers cell adhesion mechanims (30).

Chemotaxis is further influenced by ORs in a negative manner. μ-OR or δ-OR induce phosphorylation of CXCR1 and CXCR2, that is blocked by preincubation with opioid antagonist, but neither induce their internalization nor restrict the chemokine binding. Thus heterodimerization of opioid and chemokine receptors interfere with chemokine-induced directional migration of immune cells (61). This study suggests a mechanism by which opiates function as antiinflammatory agents and foreshadows that heterodimerization markedly mediates receptor functioning that may have essential effects on physiological functions.

Dimer formation is necessary for receptor function: $GABA_B$ receptoras an example

The importance of dimerization for the receptor function can be demonstrated on the $GABA_B$ receptor. This receptor is composed of two distinct subunits, $GABA_{B1}$ and $GABA_{B2}$ (21, 43, 57). Both proteins in this receptor have very distinct functions.

$GABA_{B1}$ subunit has been cloned relatively late using radio-labeled high affinity antagonists (63). This is also because $GABA_{B1}$ does not reach the cell surface alone (64, 65) and exhibits low affinity for agonists compared with the endogenous receptor on brain membranes (63). The inability of $GABA_{B1}$ to reach the cell surface lies in the presence of the retention signal (Arg-Ser-Arg-Arg or RSRR) in the C-terminus of this subunit (66). Mutation of the four amino acids RSRR into ASAR enables this subunit to reach the cell surface, however it is not functional (57, 67) (**Fig. 4**).

The $GABA_{B2}$ subunit, which is required for the formation of a functional receptor, has been identified a year after $GABA_{B1}$, interestingly independently in three laboratories, using co-immunoprecipitation, yeast-two-hybrid screening approach and co-localization (21, 42, 43). Co-expression of both subunits produced functional receptor with agonist affinities and other properties comparable to natural receptor.

The role of $GABA_{B2}$ subunit does not only lie in the proper trafficking and enhancing the agonist affinity of $GABA_{B1}$ subunit, but mainly in the mechanism of

receptor activation. The study of chimeric receptors $GABA_{B1/2}$ composed of ligand binding domain (LBD) of $GABA_{B1}$ and HD of $GABA_{B2}$ and reverse chimera $GABA_{B2/1}$ demonstrated that they were neither expressed nor functional when expressed alone, although co-expression of both led to the formation of a functional receptor (67). Furthermore, chimeric dimers that contained two HDs of $GABA_{B1}$ subunit with mutated retention signal reached the cell surface but were nonfunctional, whereas dimers with two HDs of $GABA_{B2}$ subunit were expressed and functional (67). These data led to conclusion that the $GABA_{B2}$ subunit possesses an important molecular determinants for the G-protein activation.

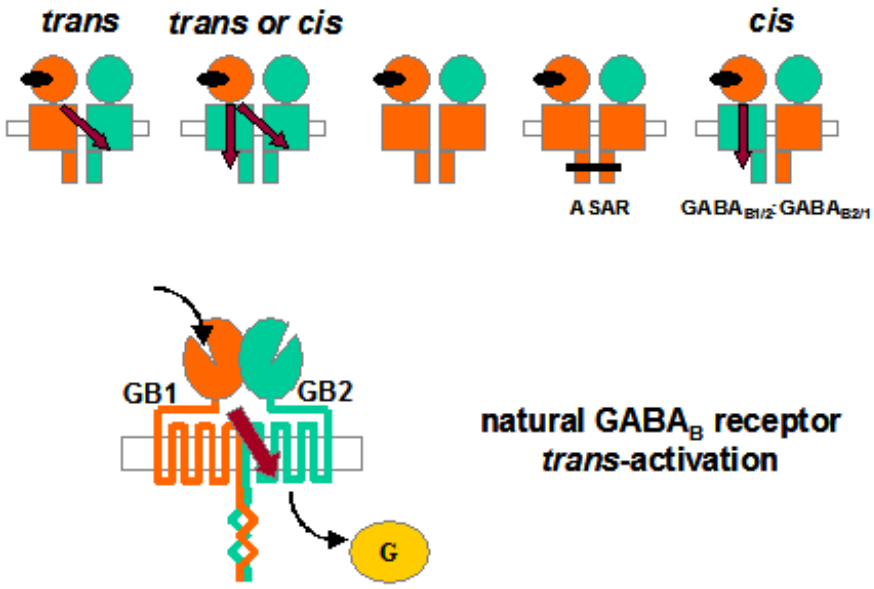


Fig. 4: Allosteric interaction of $GABA_B$ receptor subunits
In natural $GABA_B$ receptor, $GABA_{B1}$ subunit binds GABA, whereas $GABA_{B2}$ subunit activates a G-protein (trans-activation). $GABA_B$ receptor composed of two TMDs of $GABA_{B2}$ subunit still activates G-proteins although less efficiently, whereas reverse chimera containing both TMDs of $GABA_{B1}$ subunit is incapable to activate G-proteins even if it is expressed (using mutation of the ER retention signal RSRR into ASAR). Interestingly, receptor composed of $GABA_{B1/2}$ and $GABA_{B2/1}$ subunits is capable to activate G-proteins by cis-activation as indicated by brown arrow. Second and third intracellular loops also play major role in coupling and activation of a G-protein (67).

Trans-activation was reported for some other receptors from class A GPCRs, e.g. luteinizing hormone receptor (68) or histamin H1 receptor and α_{1b}-AR heterodimers, where one of the receptors was fused with functional G-protein but was not able to activate it, whereas the second receptor was fused with impaired G-protein. Coexpression of both mutants rescued function of the heterodimer, whereas

homodimers were non-functional (69). In these cases *trans*-activation seems to occur ocassionally, under artificial conditions (**Fig. 5**).

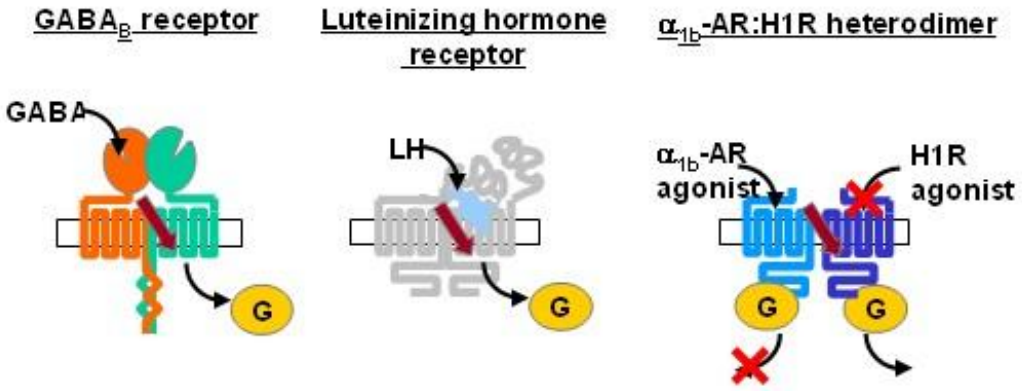


Fig. 5: Dimerization is necessary for receptor function
Schematic representation of trans-activation (dark red aroow) in GABA$_B$ receptor (left) luteinizing hormone receptor (middle) and heterodimer consisting of chimeric α1b-adrenergic receptor fused with non-functional G-protein and histamin H1 receptor (H1R) impaired in agonist binding fused with functional G-protein (right) (67-69).

In contrast, homodimeric receptors are composed of exactly the same subunits that can bind and transfer signal in the same way. What is the reason for receptor homomerization then? As was shown for rhodopsin, in the dimeric state rhodopsin activates transducine more efficiently (24, 70), although one receptor is capable of activating transducine. In case of metabotropic glutamate receptors, the binding of a single agonist to receptor dimer activates the receptor only partially (57), therefore assembling of the functional dimer is necessary for full activation of the receptor and formation of appropriate intracellular signal.

2.2.2. Constitutive *vs.* conditional dimerization

Whether the dimerization is a permanent or transient feature of a particular GPCR may depend on the entire receptor life cycle and may be also different between distinct receptors. There is evidence that formation of some GPCR dimers occurs in early biosynthesis of the receptor. The fact, that dimerization takes place in endoplasmic reticulum (ER) (**Fig. 6**) was well demonstrated on members of all classes of GPCRs (19, 21, 71). The fact, that the GABA$_{B1}$ subunit of GABA$_B$ receptor is retained in the ER when expressed by itself (64) due to retention signal RSRR (Arg-Ser-Arg-Arg) in its C-terminus (66) and that the co-expression of GABA$_{B2}$ subunit masks this signal and thus allows the heterodimer to reach the cell surface (72), supports this idea. Other

members of class C GPCRs (mGluRs, CaSR) also form dimers in ER (19, 40). Immature forms of oxytocin and vasopressin receptors were shown to be present as dimers in ER as well (71).

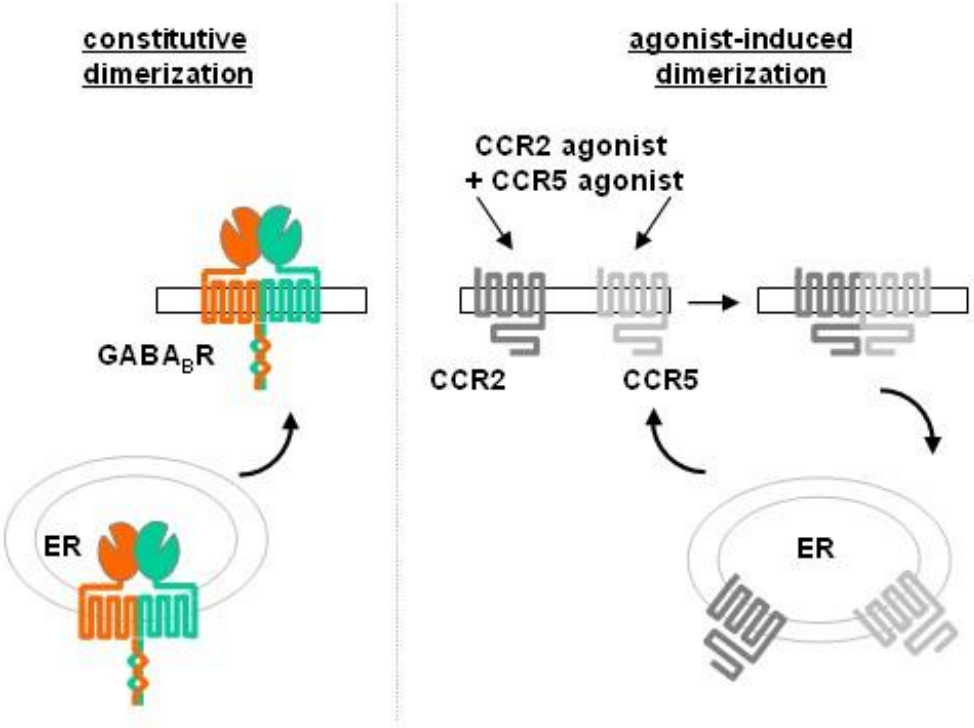


Fig. 6: Principles of GPCR dimerization
Many receptors form constitutive dimers, e.g. $GABA_B$ receptors (left) form dimers in ER and as such is trafficked to the cell surface (66, 72). In the contrary, some receptors, such as chemokine receptors CCR2 and CCR5 (right), form heterodimers under simultaneous stimulation with their specific agonists (30).

These observations suggest, that it is unlikely to induce receptor dimerization upon agonist stimulation of the receptor. It was further proved by study of Ramsay et al. (31), who showed that addition of agonist or antagonist does not alter the bioluminiscence signal determining homo-oligomerization of opioid receptors. Similar results were obtained with β_2-AR and chemokine receptors (33, 73).

On the other hand, there is an evidence for aggregation of some receptors from class A GPCRs in response to ligand binding (30, 35, 74) (**Fig. 6**). Up to date, many receptor-receptor interactions have been demonstrated, e.g. hetero-oligomerization of dopamine and somatostatin receptors or assembling of mGluR1 with A_{1A} receptors into functional complexes. It is possible that depending on composition of dimers or oligomers different pharmacological (25, 62) or trafficking (26) properties can occur. In contrast, recent studies using bioluminiscence resonance energy transfer (BRET)

approach, that allowed us for the first time to study the association of molecules *in vivo* under physiological conditions, show no or small effect of agonist-induced changes in monomer/dimer constitution of chemokine CXC4 receptors (33). The effect of ligands could rather reside in conformational changes of receptor dimers, like in case of melatonine receptors (75).

On the other hand, whether the receptor dimer undergoes dissociation into monomers upon agonist stimulation is another question. Previous studies demonstrated that δ-OR is separated into monomers after receptor activation and as such is internalized (76). Some other receptors of class A dissociate under agonist receptor activation although they form constitutive dimers (77-79). This dissociation could prevent β-arrestin-dependent receptor internalization. On contrary, it is unlikely that metabotropic glutamate or calcium sensing receptors would dissociate into monomers to be internalized, especially because their ECDs are linked by a covalent interaction.

2.2.3. Molecular determinants for receptor dimerization

Two structural models for dimer formation have been suggested: contact dimers and domain-swapped dimers (**Fig. 7 and 8**). In contact dimers, domains of individual receptors interact mostly through hydrophobic interactions, while maintaining their respective LBDs (23, 32, 80). The domain-swapping model is described as exchange of TM helices from both receptors (81, 82).

There is a strong evidence for participation of hydrophobic interactions between transmembrane helices in receptor dimer interface. However, early studies suggested that both N- and C-terminal portions of receptors from class A GPCRs are involved in formation of homodimers (76, 83). The role of N-terminus was also mentioned in more recent studies with yeast α–factor receptor (84). Interestingly, heterodimerization of adrenergic receptors can be regulated by N-glycosylation has taken into account also the role of post-translational modifications in formation of GPCR dimers (85).

It is likely that all TM domains can be involved in dimer formation depending on their hydrophobic properties and thus on type of the receptor (23, 32, 80, 86-90). For example, TMIV and TMV helices are involved in intradimer contact in crystallized rhodopsin dimers (23). In dopamine D2 receptor it is also TMIV helix that is involved in formation of homodimer interface as shown in **Fig. 7** (80, 86). In contrast, in α_{1b}AR formation of interprotomer interactions is made between TMI helices (32). Other

members from class A form dimers through TMVI helix. For example, a peptide corresponding to the sequence of this TM domaindisrupted dimer formation in leukotriene B4 receptors (90).

Fig.7: A model of contact dimer
Proposed interprotomer interactions in dopamine D2 receptor homodimers predicted by correlated mutational analysis (86).

In comparison to class A and B, class C GPCRs differ from the previous two by the large extracellular binding domain. This suggests that the molecular determinants for receptor dimerization could be different and that ECD in receptors of class C GPCRs could play a critical role in their dimerization.

While metabotropic glutamate and calcium sensing receptors form dimers also by formation of intramolecular disulfide bridges between the two extracellular domains (39, 91-93), $GABA_B$ receptor heterodimerization was reported to stabilized by coiled-coil interaction between leucine zipper peptides in C-termini of $GABA_{B1}$ and $GABA_{B2}$ (94). Removal of $GABA_{B1}$ C-terminus does not prevent formation of functional receptor heterodimers (72) suggesting a role of other domains in heteromeric assembly of $GABA_B$ receptor.

The domain swapping-model suggested an efficient way of formation of dimerization interfaces (81) and was supported mainly by experiments with adrenergic-muscarinic receptor chimeras. α_2/M3 and M3/α_2, composed of the first five transmembrane domains of one receptor and the last two transmembrane domains of the other **(Fig. 8)**. Each of the chimera was naturally inactive, when expressed alone, being unable to neither bind a ligand nor activate G-proteins. However co-expression of the two chimeras restored binding and signaling to both muscarinic and adrenergic agonists (82).

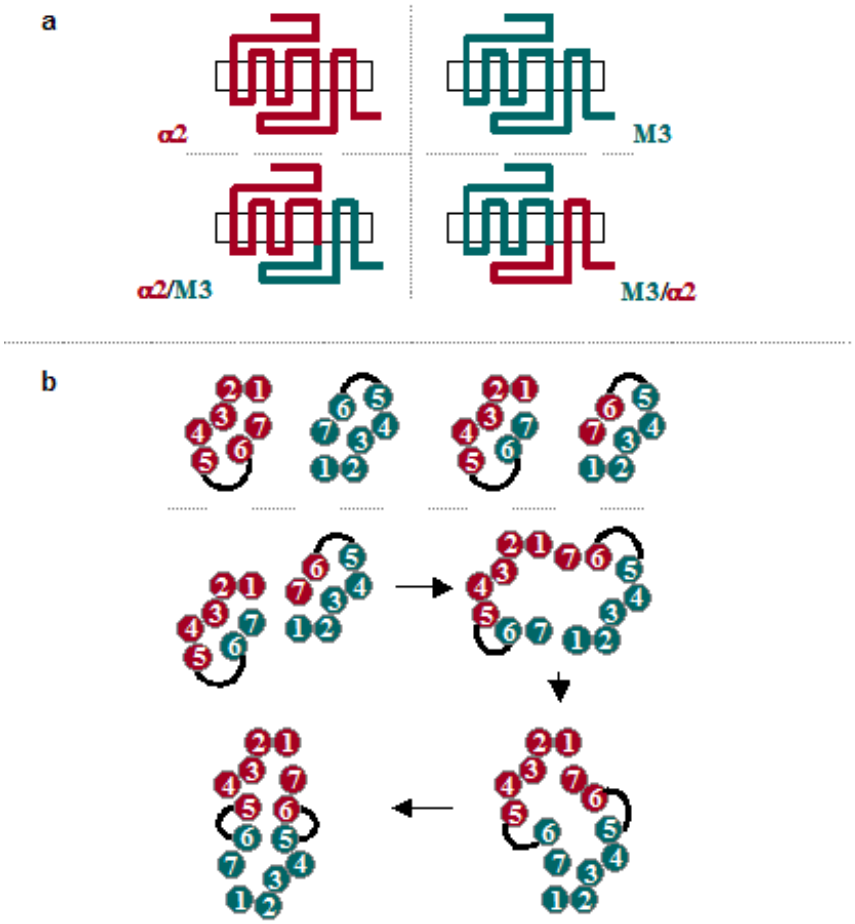


Fig. 8: Domain swapping model
(a) Schematic representation of wild-type α2-adrenergic (α2) and muscarinic 3 (M3) receptors and both mutual chimeras α2/M3 and M3/α2 according to 95. (b) Proposed domain swapping was expected between M3 and α2 receptor chimeras (81, 82).

2.3. Receptor activation

Mechanisms of receptor activation were studied by either molecular or biochemical approaches. Structural reorganization can be also predicted by computational analysis (95) but the most valuable data could be obtained recently by using resonance energy transfer methods, FRET (fluorescence resonance energy transfer) and BRET. Ayoub et al. (75) have reported that melatonine receptor dimers undergo conformational changes upon ligand binding that enable to unmask structuraly important domains for G-proteins. Moreover, FRET enables to measure even the rate constants and thus determines how fast are these receptors activated (96).

It was previously reported that mutation of fourth amino acids within C-terminal part of i3 loop of β_2-AR lead to permanently activated receptor (97) confirming, that intracellular parts also play role in the receptor activation. In rhodopsin family, the conserved D(E)RY motif in the cytoplasmic site of TMIII is involved in receptor activation. Protonation of aspartate residue causes shift of arginine residue out of polar pocket formed by hydrophilic residues in TMI, II and VII leading to exposure of i2 and i3 to intracellular space (98). The movement of TMIII and TMVI has been confirmed by different methods including Fourier transform infrared resonance spectroscopy (99), surface plasmon resonance spectroscopy (100, 101) or tryptophan UV absorbance spectroscopy (102). Both molecular and spectroscopic approaches prooved that intraprotomer movement is important for class A receptor activation. The same rearrengement is expected in class C GPCRs supported by finding that ECD of mGluRs adopts different conformational stages (103). The change in conformation is believed to be transferred onto TM helices. But what is exact movement inside of a certain protomer and how does this proposed movement mediate G-protein coupling/activation is not recently known.

2.4. Signalization mediated by G-proteins

GPCRs modulate numerous cellular cascades. The heterotrimeric G-protein pathway is the most pronounced and was the first GPCR pathway described.

2.4.1. Heterotrimeric G-proteins

Inside the cell, heterotrimeric G-proteins transfer information from activated heptahelical receptor to effector molecules. The heterotrimeric G-protein consists of an α-subunit that binds and hydrolyzes guanosine triphosphate (GTP) and of a β- and a γ-subunit that form an undissociable $\beta\gamma$-complex (104-106) (**Fig. 9).**

According to the intracellular functions, G-protein α-subunits are divided into four families $G_s\alpha$, $G_{i/o}\alpha$, $G_q\alpha$ and $G_{12/13}\alpha$ (107). $G_s\alpha$ family of G-proteins stimulates ACs and this stimulation leads to accumulation of cyclic adenosine monophosphate (cAMP). $G_{olf}\alpha$ proteins, that belong to this family, transduce signal coming through a variety of olfactory receptors. In contrast, second family of $G_{i/o}\alpha$ proteins inhibits various types of ACs. Third family of $G_{q/11}$-proteins couples receptors to β-isoforms of

phospholipase C (PLC) (108), whose activation leads to production of inositol-phosphates and to release of calcium from intracellular stores. Finally, the fourth family of G-proteins – G_{12} and G_{13} that is often activated by receptors coupling to $G_{q/11}$, activates various downstream effectors including phospholipase A, D or small GTPase RhoA (109-111).

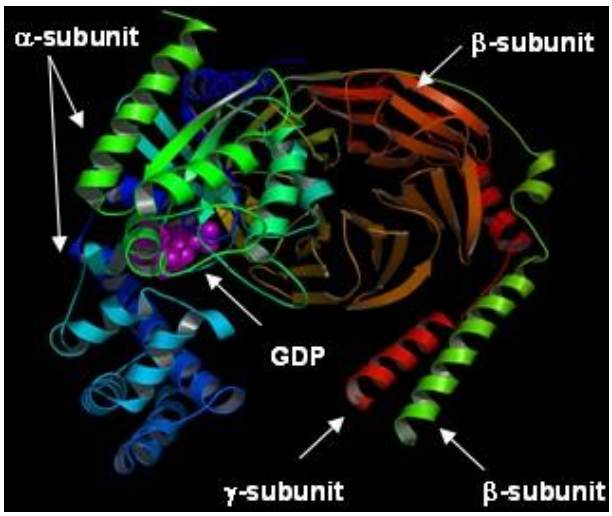
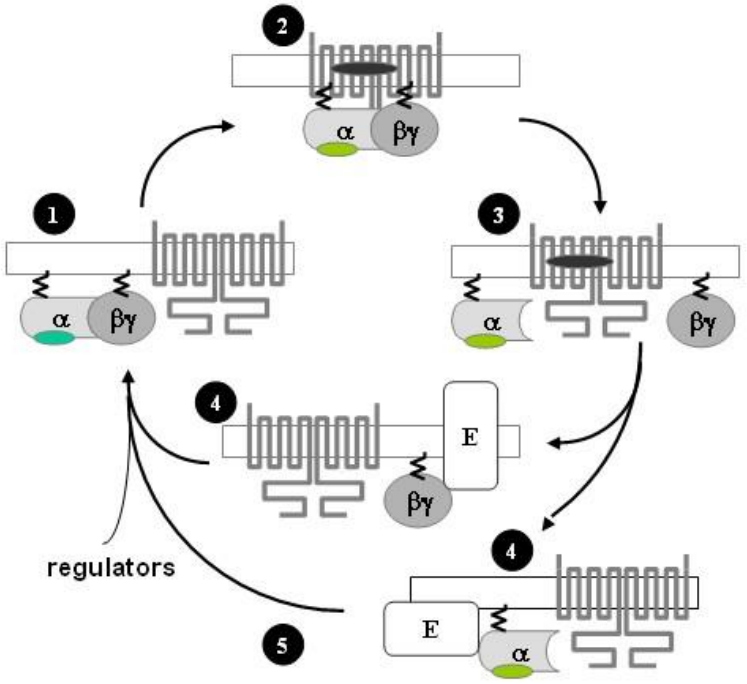


Fig. 9: Structure of heterotrimeric G-proteins *Gα-subunit (blue to green), GTPase domain (green), helical domain (blue) with bound GDP (magenta), β-subunit (red to green), γ-subunit (red).*
(*H. Hamm, Vanderbilt University, Nashville, USA, http://pharmacology.mc.vanderbilt.edu/Faculty/Hamm_Lab*)

The generally assumed life cycle of G-proteins typically divide into few steps. Interaction of activated GPCR with a G-protein catalyses the exchange of GTP to GDP (guanosine diphosphate), which in turn leads to dissociation of heterotrimeric G-protein into α-subunit and βγ-complex, enabling each of them to activate their intracellular effector cascades (6). Signaling is terminated by the hydrolysis of GTP by the GTPase activity of the α-subunit that is influenced by several regulatory proteins. The resulting GDP-bound α-subunit reassociates with the βγ-complex to enter a new cycle (107) (**Fig. 10**).

Fig. 10: Activation-inactivation cycle of heterotrimeric G-protein *1. Inactive state: Gα-subunit with bound GDP is in a close proximity to the receptor. 2. Activated receptor promotes the exchange of GDP to GTP 3. and dissociation of heterotrimeric G-protein complex. 4. Each, Gα-subunit and βγ-complex, modulate effector (E) functions. 5. Spontaneous hydrolysis of GTP by GTPase activity of the Gα-subunit can be influenced by several regulators.*



2.4.2. Receptor-G-protein coupling

How do GPCRs activate G-proteins? Various regions in Gα subunit as well as regions within Gβ and Gγ subunits are compatible and this drives selectivity between GPCRs and G-proteins (112). Previous doubts about the receptor:G-protein ratio have been adressed recently. The proposal of the ratio of a receptor dimer to a G-protein being 1:1 originating from predicted structure, size of binding interfaces and mapping of the contact sites between receptor and its G-protein seem to be confirmed recently (90). Thus it is unlikely that each of the two subunits within the receptor dimer would interact with its own G-protein simply because of the lack of enough space.

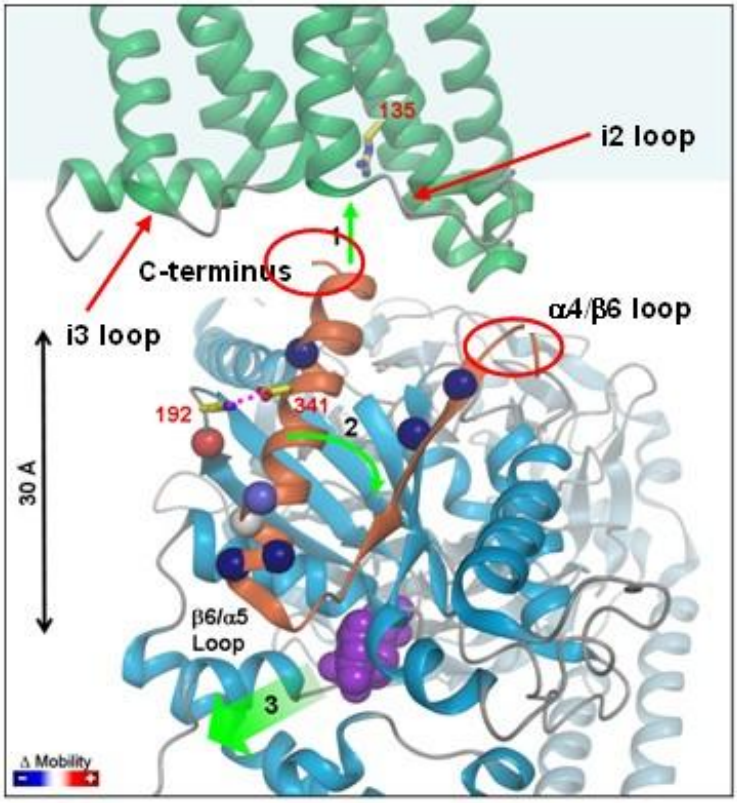


Fig. 11: Molecular determinants of receptor-G-protein coupling
Regions participating in G-protein-receptor contact include extreme C-terminus (Cys –4) and α4-β6 loop domain on the side of G-protein and second (i2) and third (i3) intracellular loops of the receptor on the side of the receptor. Green arrows illustrate movement of the most flexible parts in a G-protein upon the activation. In the inactive stat GTPase domain binds GDP (magenta). (H. Hamm, Vanderbilt University, Nashville, USA, http://pharmacology.mc.vanderbilt.edu/Faculty/Hamm_Lab).

Within G-protein, the decissive role in G-protein-receptor interaction is encoded within the extreme C-terminus of the Gα protein, mainly residues on positions -3 and –4 (113-115). For $G_{i/o}$ family is the residue at the position –4 a cystein. Importance of this residue was proved in a study, where mutation of cystein –4 in $G_o\alpha$ into isoleucin was sufficient to suppress its coupling to mGluR2 (113). On the other hand in two members of this family - $G_i\alpha2$ and $G_t\alpha1$ - the last 8 C-terminal amino acids are the same and nevertheless they couple to different receptors (116). Another regions participating in the interaction are N-terminal domain (117), α2-helix and α2-β4 loop

(118, 119), α4-helix and α4-β6 loop domains α5-helix (120), α3-β5 region and the small segment that links αN-terminal helix to β1-strand (121). The extreme N- and C-terminus, αN-β1 loop, α4-β6 loop domain and the α5-helix contribute to the specifity of the Gα-GPCR interaction (**Fig. 11**).

On the receptor side, there are several regions that mediate the interaction with a G-protein. The way of activation of the G-protein seems to be different between classes A and B GPCRs on one hand and class C GPCRs on the other, since the i3 loop is very short in class C GPCRs (122), whereas it is the longest in class A and B and on the other hand very short i2 loop in families 1 and 2 in contrast to long i2 loop in the class C GPCRs.

The tripeptide sequence D(E)RY (Asp (Glu), Arg, Tyr) in the N-terminal end of the i2 loop is highly conserved in class A and play a critical role in G-protein coupling and activation. Mutation of these aminoacids dramatically decreases G-protein activation by the receptor as well as activity of the receptor (98, 123-125). The importance of next regions - i3 loop and C-terminal domain – in the recognition and coupling to G-proteins on different receptors from class A and B GPCRs was proved several times (115, 126-128).

The importance of both i2 and i3 loops as well as C-terminus of class C GPCRs in recognition and binding of G-proteins was studied in heterologous systems (129-132). Chimeric mGlu3 receptors possessing at least i2 loop and CT of mGlu1 receptor were able to strongly activate the chloride currents in *Xenopus* oocytes and to produce stronger signal if they possess another intracellular loop (i1 or i3), too (129). These data shows that the i2 loop cooperate with other intracellular domains to control G-protein coupling. It was further shown that i2 loop is also involved in coupling of $GABA_B$ receptor to the G-protein (131).

Francesconi and Duvoisin (130) proved that both i2 and i3 loops are critical for coupling to PLC and AC of mGlu1a receptor. Using site-directed mutagenesis they found 3 residues in i2 loop responsible for selective interaction with $G_q\alpha$ and another 3 residues responsible for selective interaction with $G_s\alpha$. Mutation of Lys690 to Ala altered mGlu1 receptor signaling properties through G_i protein. Within i3 loop, there is Phe781 crucial for coupling of both G-protein pathways (130). The central part of i2 loop of mGluRs is responsible for selective recognition of the C-terminal end of the Gα-subunit, especially the residue in –4 position of Gα C-terminus (132). These results

support the idea that the C-terminus of Gα-subunit is recognized and bound by i2/i3 loop cavity of the receptor in concert with other intracellular part - C-terminus and i1 loop - of the receptor. Taking into an account the receptor:G-protein ratio, it is likely, that one of the subunits will bind Gα subunit, whereas the other will contact βγ-dimer (**Fig. 11).**

3. Metabotropic glutamate receptors (mGluRs)

Glutamate mediates majority of the excitatory neurotransmission in the brain and is known to play also an important role in neuronal plasticity, neural development, neurodegeneration and neuropathologies (133-136). Glutamate receptors are divided into two distinct groups. Ionotropic receptors are channels that upon agonist binding opens and allow ions to pass through the membrane and thus cause change in the membrane potential. Metabotropic glutamate receptors (mGluRs) are on the other hand coupled to intracellular signal transduction via G-proteins (4, 137).

The group of metabotropic receptors are of eight subtypes mGluR1-mGluR8. They are further classified into three groups according to their sequence similarities, signal transduction mechanisms and agonist selectivities (122). Receptors of the same group share about 70% sequence identity but only 45% between the groups (122). Group I contains mGluR1 and mGluR5 subtypes that positively regulate PLC through $G_q\alpha$ – protein. Their activation leads to accumulation of inositol-triphosphates (IP_3) and intracellular Ca^{2+} in heterologous expression system as well as cultured neuronal or glial cells (138-140). Other six receptors couple to $G_{i/o}$-protein and thus negatively regulate AC. These are further divided into two groups – group II (mGluR2 and mGluR3) and group III (mGlu4, mGluR7 and mGluR8) depending on their agonist selectivities.

First metabotropic glutamate receptor (mGluR1a) was independently cloned in two laboratories (141, 142). Based on mGluR1a sequence, probes for hybridization or primers for PCR cloning were designed and seven other related mGluRs were cloned (143-149). Expression in CNS and subcellular localization of different receptors and their splice variants vary.

3.1. Localization and physiology

All three groups of mGluRs are expressed in hippocampus with a different expression pattern (150). While group I receptors are expressed in all hippocampal neurons, group II receptors predominate in principal cells of CA2, CA3 and CA4 regions and fail to be expressed in pyramidal cells of CA1. Group III mGluRs is confined to the mossy fiber projection field in CA3 stratum lucidum (150). Under these observations the role of mGluRs and glutamate-dependent synaptic contacts in establishing memories was proposed. Another type of memory mediated through

mGluRs (long-term potenciation) is established in CA1 region of hippocampus (151). Expression, although in a lesser extend, of mGluR1 on striatal cholinergic interneurons (152), dopaminergic neurons in substantia nigra (138), somatostatin-positive neurons (153) and in neocortex, amygdala, hypothalamus and medulla indicates its role in almost all brain functions. The same receptors mediate voltage-insensitive inhibition of calcium channels in sympathetic neurons (154).

Lack of the mGlu1 receptor in mGluR1–deficient mice causes severe motor coordination and spatial learning deficits (155). The abundant expression of group I mGluRs in cerebellum, ventral tegmental area and nucleus accumbens sustained the role of these receptors in the regulation of motor activity (156). Furthermore correct development of cerebellar neurons might be under the control of group I mGluRs (157). Increase in the dendritic calcium concentration is important for the induction of long-term depression (LTD) at paralel fiber-Purkinje cell synapses. LTD is believed to be one of the mechanisms of cerebellar motor learning (151, 158).

Interestingly receptors coupled to adenylate cyclase have special roles in sensory system, e.g. mGluR6 was uniquely found on ON bipolar cells (144, 159, 160) and mGluR4 was proposed to be one of the umami taste receptor, because of the fact that activation of mGluR4 by L-4-phosphono-2-aminobutyric acid mimics the taste of monosodium glutamate (161).

MGluRs modulate many neuronal functions including release of the neurotransmitter from presynaptic terminal or induction of long-term changes at postsynaptic terminals (162-167). They also modulate other neurotransmitter receptors including ionotropic and metabotropic glutamate receptors (14, 168) as well as several types of ion-channels (18, 169).

For example, mGluRs activate potassium channels by calcium-dependent mechanism in cerrebellum (170). Moreover, mGluR1a inhibits P/Q-type Ca^{2+} channels and this action seems to be mediated by direct interaction between C-termini of both proteins (18). Interestingly, activation of mGluR1 or 5 in cultured hippocampal neurons induces internalization of NMDA (N-methyl-D-aspartate) receptors and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors on excitatory synapses (171). The connection between ionotropic and metabotropic glutamate receptors represented by intracellular scaffolding proteins (172) could be responsible for involvement of mGluRs in modulation of processes of memory and learning (166).

Their multiple functions in the central nervous system foreshadow also their crucial role in several neurological disorders such as pain, epilepsy, Alzheimer's and Parkinson's disease, pathology associated with ischemia, schizophrenia, anxiety and drug addiction (166, 173-187). Growing number of selective compounds for mGluRs make possible the study of the physiological as well as pathological roles of these receptors in the nervous system and simultaneously represent promising instruments for treatment of neurological and psychiatric disorders in which glutamatergic neurotransmission is abnormally regulated (188-191).

3.2. Structure of mGluRs

The existence of mGluRs has been known since 1970s. MGluR1 was cloned using strategy of functional expression screening procedure in *Xenopus* oocytes (141, 142). The mRNA synthesized in vitro prepared from a rat cerebellum cDNA library was injected in *Xenopus* oocytes where the second messenger pathway links G-protein activation with chloride channel currents that are detected electrophysiologically in response to an agonist. Application of L-glutamate, L-quisqualate, ibotenate and *t*-ACPD (*trans*-1-aminocyclopentane-1,3-dicarboxylate) to an oocyte injected with mRNA of mGluR clone evoked IP_3 formation and intracellular Ca^{2+} mobilization, whereas kainate and NMDA had no effect (141). MGluR clone was sequenced and a hydrophobicity analysis proved the existence of at least eight hydrophobic segments, each consisting of approximately 20 amino acids and two large hydrophilic regions upstream and downstream from this hydrophobic cluster, respectively (141).

The topology deduced from the primary sequence shows four structural characteristics. The hydrophilic amino terminus is preceeded by about 20 hydrophobic amino acid residues that may serve as a signal peptide. The N-terminal or extracellular domain is followed (ECD) by a cystein-rich domain (CRD), seven hydrophobic transmembrane domains (TMD) and an intracellularly located C-terminus (**Fig. 12**). Five N-glycosylation sites (Asn 98, Asn 223, Asn 397, Asn 515 and Asn 747) within the N-terminal domain were pointed out proposing that N-terminus is located extracellularly. Later, it was shown, that postranslational modification such as N-glycosylation is fundamental for protein expression and function (118). Receptor phosphorylation has important role in receptor desensitization and internalization (192).

Existence of several serines and threonines, potential phosphorylation sites, within C-terminal hydrophilic domain suggested its intracellular localization (141).

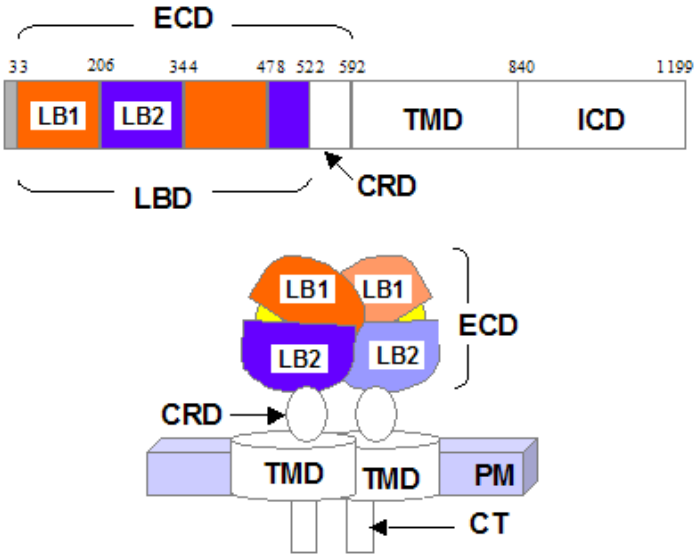


Fig. 12: Structure of mGluRs
(top) Box-scheme of domain order in mGluR1. LB1 (lobe 1) and LB2 (lobe 2) constitute a ligand binding domain (LBD) are illustrated in red and blue, respectively. (bottom) Spatial model of dimeric mGluR1 with bound glutamate (yellow) in the plasma membrane (PM) (103). ECD: extracellular domain, CRD: cystein-rich domain, TMD: transmembrane domain, CT: C-terminus.

Because of the same natural ligand for all eight mGluRs, N-terminal domain share high sequence homology. On contrary, the C-tails vary in the length and the amino acid sequence. The C-termini specifically bind different intracellular proteins which assemble different intracellular pathways and in some cases this is regulated by alternative splicing between receptor variants arising from one gene.

3.2.1. Extracellular domain and glutamate-binding site

Most of the receptors from class C GPCRs possess a large (approximately 600 residues) ECD that makes them unique. First evidence that the ligand-binding site is localized in the N-terminal domain was given by examining the agonist selectivities of the mGlu1 and 2 chimeric receptors (193). Both receptors have similar affinity to glutamate but vary in affinities to t-ACPD and L-quisqualate. Replacement of the N-terminal portion of the mGluR1 by that of mGluR2 enhances its affinity to t-ACPD whereas decrases affinity to L-quisqualate. Conversely, exchange of the mGluR2 N-terminal domain by that of mGluR1 created mutant with affinities to the agonists similar to mGlu1 wild-type receptor (193). It was confirmed by Okamoto et al. (194), who constructed a soluble mGlu1 receptor without the membrane-anchored domain, that the

soluble receptor constisting of the LBD and CRD is sufficient to confer the affinity and selectivity of ligand binding and that this affinity and specificity is comparable to the full-lenght receptor.

The ECD is related to bacterial leucin/isoleucin/valin binding protein (LIVBP), glutamine-binding protein or leucine-binding protein (195, 196) (**Fig. 13).** Bacterial periplasmic binding proteins (PBPs) serve as initial receptors to active transport for a variety of amino acids, sugars, peptides and other nutrients. Although the sequence similarity between certain PBPs is very low, they share similar bilobal tertiary structure that was found by using X-ray crystallography analysis (197, 198). Despite the weak sequence similarity between ECD of mGluRs and LIVBP, it was used to predict a model of a bilobar tertiary structure of ligand-binding domain of the mGlu1 receptor. The clear evidence of the predicted tertiary structure was confirmed by X-ray crystallography using mGlu1 receptor ligand binding domain either in absence or presence of glutamate and furthermore in the presence of competitive antagonists and gadolinium cations (103, 199).

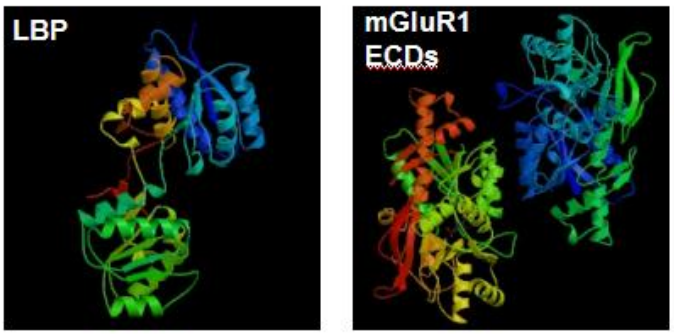
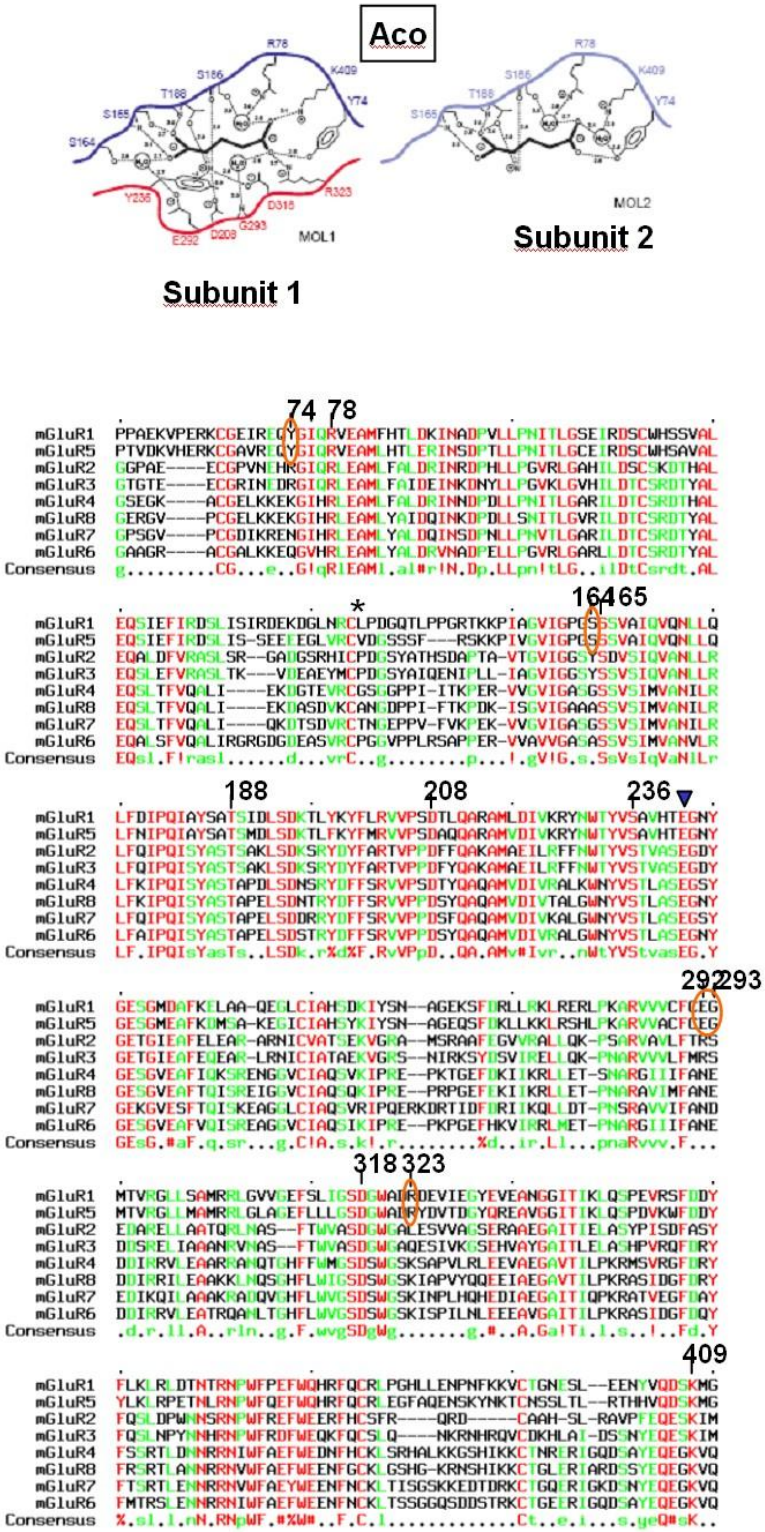


Fig. 13: Amino acid-binding proteins have common bilobar structure
Structural similarity between a single leucine-binding protein (LBP, left) (198) and dimeric extracellular domain (ECD, right) or glutamate-binding domain of mGluR1 stabilized in its inactive conformation by group I selective antagonist (103) as determined by X-ray crystallography (RCSB PDB = The Research Collaboratory for Structural Bioinformatics, Protein Data Bank).

Ligand binding site of mGluR1 was well documented (103) and authors indicated Tyr 74 as the most important for ligand binding. Seven other polar residues, among them mainly Ser 165, Thr 188, Tyr 236 and Asp 318 that contribute to the ligand recognition are conserved among all members of mGluR subfamily (**Fig. 14**). Next, five polar residues (Tyr 74, Ser 164, Glu 292, Gly 293 and Arg 323) are conserved only in the group I mGluRs (mGluR1 and mGluR5) and may be responsible for the ligand preference of the group I receptors (103). Alanine-screening analysis showed that a single mutation of these residues **(Fig. 14**) was responsible for complete loss of quisqualate binding in mGluR1 and that T188A, D208A, Y236A and D318A mutants did not show any intracellular response, although they were expressed (200).



Corresponding residues in mGluR5 Ser 151 and Thr 174 when mutated into alanine are not able to participate in agonist binding anymore (57). Similarly, mutation of Tyr 64 and Thr 174 or two other residues - Tyr 222 or and Asp 304 - led to the same loss of quisqualate binding (57). Homological residues in mGluR8 responsible for agonist binding are Tyr 227, Asp 309 (201).

Fig. 14: Ligand binding domain (LBD) of mGlu1 receptor

(top) Schematic representation of the glutamate binding sites within a Aco dimer of mGluR1 (103). Polar interactions: dotted lines, domain colouring asi in Fig. 13. (bottom) Representative sequence alignment of LBDs of all rat mGluR subtypes (Multalin, Blosum62-12-2, http://prodes.toulouse.inra.fr/multalin/multalin.html). Amino acids highly homological (90%) are highlighted in red, amino acids with low consensus value (50%) are highlighted in green and residues with lower than 50% homology are ilustrated in black colour. Crucial residues involved in glutamate binding are numbered. Cysteine 140 (star) and glutamate 238 (blue arrowhead) are conserved among all members of mGluRs.

3.2.2. Cystein-rich domain

The CRD consists of four β-strands that are linked by turns and are held in a compact structure by three disulfide bridges. The CRD might contain two motives (C-X_2-G-X-Y-X-X_{4-9}-Cys; Y is amino acid with large side-chain). Between these two sequential motives is a region of two conserved hydrophobic amino acids and conserved cysteins. All of them could have potential to form hydrophobic interactions or intermolecular disulfide bridges between two receptors in a homodimer (202) (**Fig. 15**).

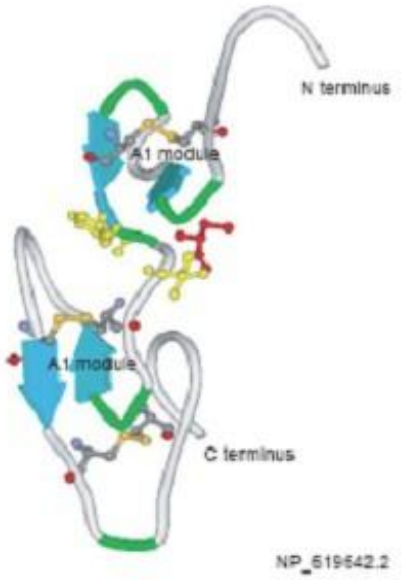


Fig. 15: Structural model of cysteine-rich domain (CRD)

Liu et al. (202) proposed formation of 3 disulfide bridges in CRD of class C GPCRs (dark yellow). The side chains and surface of the two conserved hydrophobic residues (Phe and Val) between upper and lower module are coloured in yellow, the cystein between the hydrophobic residues is highlighted in red.

On contrary, incubation of the mGlu5 receptor with trypsin led to only 17 kDa decrease in the molecular weight of this receptor, suggesting the dimerization determinant within first 155 amino acids (39). In mGluR1 there are 3 cysteins within these 155 amino acids, Cys 67, 109 and 140, respectively. Among them Cys 140 has been proposed to function as S-S crosslinker between the two ECDs in the mGluR1 dimer because mutant C140A receptor run much faster on the SDS (sodium dodecyl sulaphate) gels with a position corresponding to the monomer band (92).

3.2.3. Heptahelical domain and signal transduction

Up to date the predicted seven-spanning transmembrane region was confirmed by X-ray crystalography only in case of bovine rhodopsin receptor (203). Structure of mGluRs was also predicted on the basis of hydrophobicity analysis and confirmed by complicated molecular mutagenetic approaches (141, 204) or computational analysis (95).

It was observed that truncated mGlu5 receptor lacking the ECD and C-terminus, thus bearing only HD, behaves like rhodopsin-like receptor in terms of G-protein coupling and regulation by ligands (205). Interestingly, 2-methyl-6-(phenylethynyl)-pyridine hydrochloride (MPEP), a non-competitive antagonist for full-lenght mGluR5, acts as an inverse agonist, and 3,3′-difluorobenzaldehyde (DFB), a positive allosteric modulator for wild-type receptor, acts as a full agonist. These data suggest an intrinsic activity in mGluI receptors that can be activated by modifying of the TMD. In the case of rhodopsin, the potential intrinsic activity is suppressed by the dimer formation. Change in conformation of ECD upon ligand binding can cause a release of the suppression and following activation of the receptor (206).

It was previously revealed that all intracellular domains of mGluRs play a role in G-protein activation (129). Later data showed that i2 loop is necessary but not sufficient for activation of PLC and that both i2 and i3 loops and C-terminus are important for efficient coupling to G-protein (130). Furthermore it was reported that the role of i2 loop is consistent in recognition of the C-terminus of Gα-subunit (132).

3.2.4. C-terminal domain determines intracellular signaling properties

There are several splice variants that have been found for mGluR1 (148), mGluR5 (207, 208), mGluR6 (209), mGluR7 (210-212) and mGluR8 (210) within the C-termini. Splice variants of mGlu1 receptor are called -1a, -1b, -1c, -1d, -1e and –1E55 (**Fig. 16)**. The first sequenced mGluR1 (mGluR1a) has long C-terminus (318 amino acids). A shorter variant, mGluR1b, lacks last 292 amino acids (213). The long C-terminus of mGluR1a is replaced by 11 or 26 amino acids in mGluR1c and mGluR1d, respectively (148, 214). The additional splice variants mGluR1e and –1E55 are only composed of the extracellular domain and thus, if expressed it could be secreted or attached to the membrane by a lipid modification (122, 215).

What is the physiological consequence for the alternative splicing of mGluR mRNA? The splice variants might be targeted differently in the cell (216). More recently, it was observed that targeting of mGluR1 to dendrites and axons of transfected retinal neurons is controlled by alternative splicing. The mGlu1a receptor colocalized with a dendritic marker, whereas its shorter variant –1b was expressed in soma and axons and overlapped with an axonal marker (217).

It was also shown, that agonist potecies may differ to distinct splice variants of mGluR1 (218, 219). It was later reported that different length of C-terminus can influence the sensitivity to agonists (211). One hypothesis suggests that the C-terminal domain may influence either the stability of the protein or its membrane targeting and receptor density on the cell surface. Second hypothesis is that the C-terminal domain influences the coupling efficacy to the G-protein.

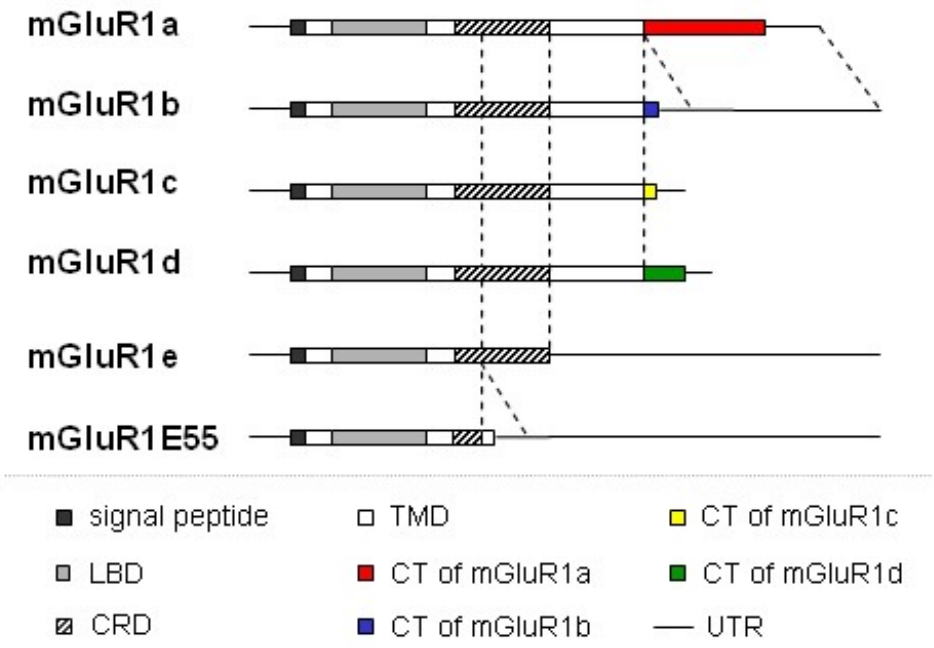


Fig. 16: Schematic comparison of the C-termini (CT) of splice variants of mGluR1
Identical sequences are joined by dashed lines. Coding sequences (boxes) for signal peptide (SP), ligand binding domain (LBD), cystein-rich domain (CRD), transmembrane domain (TMD) and C-terminus (CT) of different splice variants and their colour representations are illustrated below. UTR: untranslated region.

Distinct splice variants vary in the distribution throughout the brain. For example the long C-terminal variant is restricted to non-principal neurons, whereas mGluR1b expressed strongest labeling in principal cells in hippocampus (220). RNA blot analysis showed that mGluR1a is highly expressed in cerebellum and olfactory bulb. In situ hybridization revealed mRNA expression of this variant in many cell groups in the CNS, in granule cells and dentate gyrus, CA2 and CA3 pyramidal cells, Purkinje cells in cerebellum, in olfactory bulb and in thalamic nuclei (141). Furthermore, mGluR1a is also abundantly expressed in hippocampal region CA1 as well as in ventral tegmental area and nucleus accumbens (156, 221). Interestingly mGluR1a is preferentially localized perisynaptically and extrasynaptically (222-224),

whereas mGluR1b was reported to be expressed rather in soma and dendritic spines (225).

But what are the reasons for existence of almost the same receptors within the certain region of CNS like mGluR1a and –1b? Some data suggests that the C-terminus may play significant role in receptor trafficking or at least differently affects interactions with intracellular proteins (226). Furthermore, group I mGluRs is mediated by intracellular scaffolding proteins called Homer proteins (227) that serve as a link between group I receptors and other scaffolding and several target proteins (172). Homer proteins affect cell surface expression of these receptors as well as subcellular distribution and trafficking (228-230). The particular distribution of mGluR1b (225), its lower coupling efficacy (211, 231) and Homer-independent cell surface targeting indicate that this splice variant may be responsible for activation under conditions of high glutamate release when the long C-terminal receptor is already internalized.

3.3. Mechanisms of the mGlu receptor activation

3.3.1. Conformational changes within extracellular domain

MGluRs exist as intermolecular disulfide-linked dimers within ECD (19, 39, 194). They form dimers already in ER (19) and as such they are trafficked to the cell surface and required for the receptor function (57).

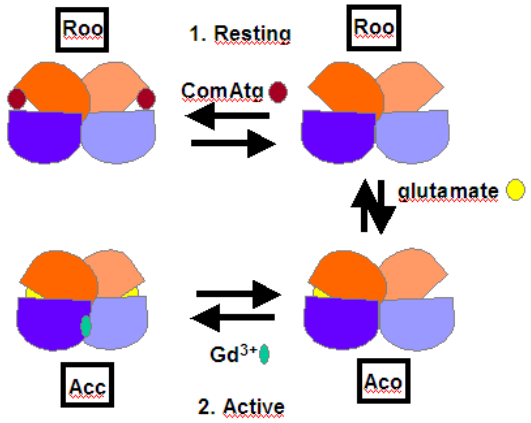


Fig. 17: Different conformational stages of the extracellular domain of mGluR1 functioning in a dimeric form
Ligand-binding domain (LBD) of mGluR1 can gather three different conformations that exist in equilibrium. Roo (resting open open) represents inactive stage, both LBD within a dimer are open and can be further stabilized by competitive antagonist (brown sphere). Active conformation is represented by Aco (Active close open) and Acc (Active close close) forms that are stabilized upon glutamate (yellow sphere) and Gd3+ (green sphere) binding, respectively. Colouring is the same like in Fig. 12 (103, 199). ComAtg: competitive antagonist.

Configuration of the ligand-binding domain can be defined by describing open (o) and closed (c) intra-protomer conformations and two different states – resting (R) and active (A) (**Fig. 17**). R and A conformations are modulated through the dimer interface (103). The bilobal protomer is composed of two domains, lobe 1 (LB1) and

lobe 2 (LB2), and flexibly changes to form open or closed states. Structure of three crystals of the mGluR1 ligand-binding domain was identified by X-ray crystallography (103). These are structures called resting open-open (Roo), active closed-open (Aco) and active closed-closed (Acc) (**Fig. 17**). First two conformations can occur even in the absence of the ligand, the latter may be responsible for the basal activity of the receptor, the dynamic equilibrium between each other exists and is shiffted in favor of the second structure in presence of the glutamate. Roo conformation can be further stabilized by competitive antagonist (199) (**Fig. 17**).

Upon glutamate binding, the two lobes (LB1 and LB2) of an "attacked" protomer come together enclosing the bound glutamate that stabilizes this state. It was reported that closure of at least one ECD is necessary to allow the dimer of ECDs to reach the active state (201). Furthermore, crystals of mGluR1 ligand-binding domain containing glutamate and gadolinium trivalent cations (Gd^{3+}) were shown to be symmetrical and represent Acc conformation. Both protomers and thus both receptor subunits get closer to each other due to neutralizing a negative LB2-LB2 interface by Gd^{3+} interacting with a cluster of negatively charged amino acids, mainly Glu 238 (199, 206) (**Fig. 18**). Neutralization of this glutamate by His or Ala substitution caused an increase in the basal activity and therefore facilitated the activation of mGluR1 receptor (232). Recent publication of Kniazeff et al. (57) has demonstrated that closing of both protomers (Acc) is required for full activation of mGlu receptors. Closure of only one protomer (Aco), as was published earlier to be sufficient for receptor activation (201), represents just partially active receptor conformation (57).

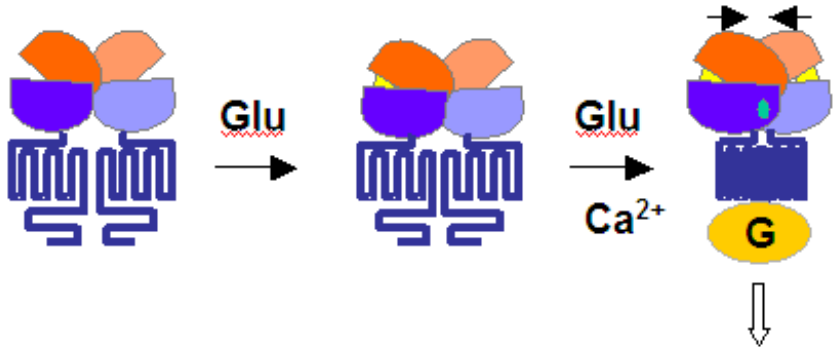


Fig. 18: Proposed mechanism of the activation of dimeric metabotropic glutamate receptors
Glutamate (Glu) causes closure of the ligand-binding domain where binds. High concentration of calcium (Ca^{2+}) can facilitate glutamate binding by the adjacent subunit. Large conformational change in the extracellular portions (arrows) of the receptor leads to rearrangement of the transmembrane helices as well as uncovering of the intracellular loops that couple G-protein (G).

Physiological concentrations of the extracellular calcium (0,8-1,5mM) can enhance the glutamate sensitivity of mGlu1 receptor (233). In cerebellar Purkinje cells extracellular calcium causes increase of the sensitivity of mGluR1 to glutamate (234) (**Fig. 18**). Recently it was shown that the effect is mediated by $GABA_B$ receptor which co-immunoprecipitated with mGluR1 from cerebellar neurons and in the absense of GABA was able to promote the action of mGluRs (235). This could explain the need for $GABA_B$ receptor on excitatory synapses.

3.3.2. Rearrangement of the transmembrane helices

How is the information transferred from ECD into intracellular part of the receptor is not known. The structural rearrengements of the cytoplasmic regions crucial for the activation of a G-protein was given recently using FRET approach (236). Transfer of the energy between CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) that were fused to i1 or i2 and to truncated C-terminus of mGluR1 was measured. The authors observed significantly reduced FRET ratio in the mGluR1-i1-CFP/mGluR1-i1-YFP heterodimer, whereas significanty increased FRET in mGluR1-i2-CFP/mGluR1-i2-YFP dimer (**Fig. 19**). According to these data they proposed a model of rearrengement of transmembrane helices and intracellular loops in which the i2 loops of both subunits are getting closer to each other, whereas the distance between i1 loops is increasing.

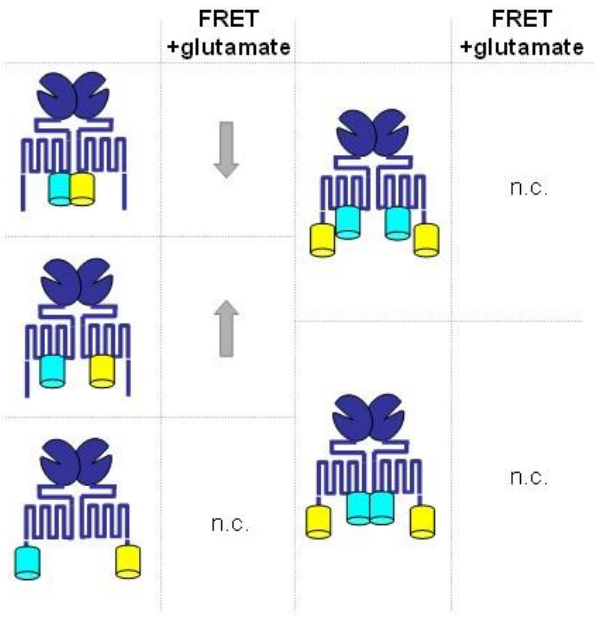


Fig. 19: Effect of the glutamate on the FRET efficiency of each mGluR1 heterodimer fused with CFP and YFP
Schematic illustrations of mGluR1 receptor fused with CFP or YFP in its i1, i2 loops or short C-terminus and change in FRET signal upon glutamate binding (after 236). n.c.: no change

Taken together, large conformational changes within dimeric ligand binding domain upon agonist binding involving either LB1-LB1 or LB2-LB2

domain movement lead to receptor activation. It strongly depends on extracellular environment, amounts of glutamate released from presynaptic terminals and presence of polyvalent cations, whether receptor reaches its full active conformation or not. Furthermore, these changes are probably responsible for additional intradomain moving and rearrengement of the heptahelical domain as well as uncovering of G-protein-recognition residues in intracellular loops and finally G-protein activation (**Fig. 18**). The sensitivity of the receptors can be further influenced by other proteins and receptors, like in case of $GABA_B$ and mGluR1 receptor complex in cerebellar Purkinje cells (235).

3.4. Signaling through mGlu receptors

As mentioned above, mGlu receptors are divided into three groups. Group I mGluRs is coupled to G_q-protein that stimulates PLC. In the case of mGluR1a, the receptor has been shown to induce IP_3 accumulation and release of calcium from intracellular stores (237). Besides of $G_{q/11}$-triggered responses, mGluR1 is able to induce G_s and $G_{i/o}$ signallization pathways (130, 207, 237-240). MGluR1 also triggers activation of several kinase pathways including cAMP-dependent protein kinase (PKA) and protein kinase C (PKC**)** (241, 242), as well as ERK and Src kinases (239, 243-247). Moreover mGluR1a appears to regulate opening of K^+ (248, 249) and Ca^{2+} channels (250) and activity of NMDA and AMPA receptors (251, 252).

Group II and III mGluRs are coupled to inhibitory $G_{i/o}$-proteins that negatively regulate AC. In addition, glutamate is able to trigger a variety of direct and indirect mechanisms in various types of cells, including stimulation of cyclic AMP or cyclic guanosine monophosphate responses, activation of other types of phospholipases (D and A_2) and release of arachidonic acid (AA) (237, 253-256). MGluRs are able to up- or down-regulate several types of cation channels either those voltage-sensitive or ligand-activated (14, 18, 122, 169, 249) and regulate gene expression by activation of various types of extracellular signal-regulated kinases (ERKs) (212, 239, 247, 254, 257).

Various mGlu receptor types are expressed in both, neuronal and glial cells. Peavy and Conn (254) showed abundant protein expression of the group I mGluRs in cortical glial cells, where its activation induces phosphorylation of ERK2 probably through mGluR5-mediated signal transduction pathway (254). Besides activation of ERK, group I mGluRs was shown to stimulate PLD and release of arachidonate in cultured astrocytes (255, 256). In striatal astrocytes, AA supresses glutamate reuptake

into astrocytes and could be thus involved in regulation of neuronal signaling. These results suggest that the same receptor can induce different transduction mechanism in distinct cells and environment. What does this determine? Stella et al. (256) suggested that in striatal astrocytes adenosine-triphosphate released from cholinergic terminals potentiates the glutamate-evoked AA release. Co-activation of mGluRs and β-adrenergic receptors that are localized on glia, results in increase of cAMP accumulation (258). Receptor-receptor interactions either direct or indirect could probably contribute to determination of the signal transduction pathway.

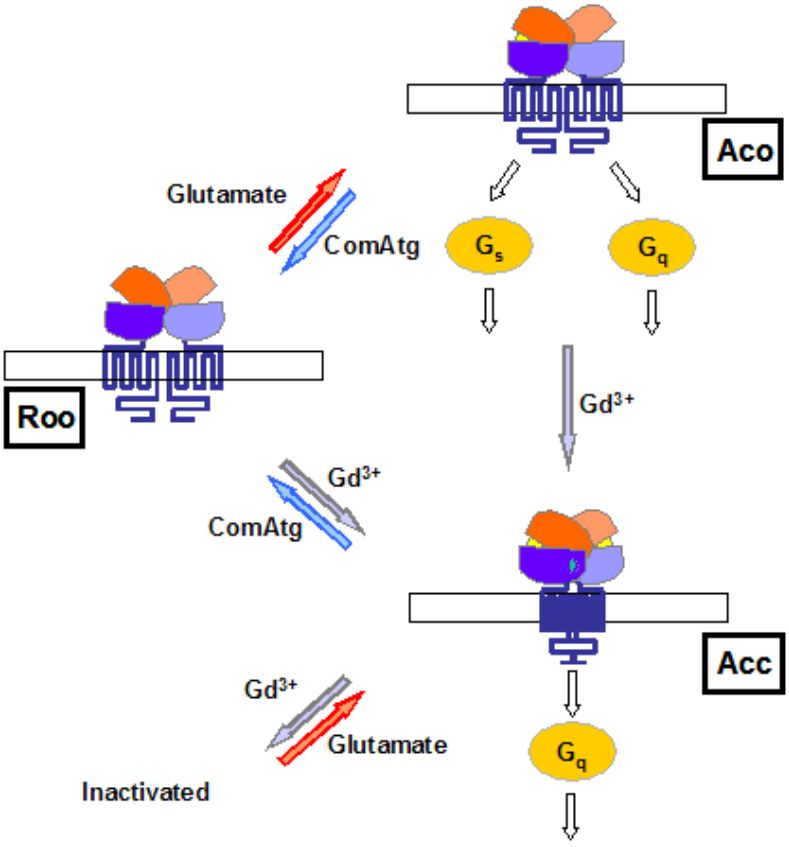


Fig. 20: Relation between different ligand-induced conformational stages of the mGluR1a in respect to specificity of G-protein activation
Schematic representation of functional differences between Aco and Acc conformations of mGluR1 and their preferential coupling to G-proteins. Glutamate-induced Aco state activates both Gs and Gq pathways, whereas Gd^{3+} leads to Gq but not Gs coupling. High concentration of Gd^{3+} induces a nonfunctional inactivated state. Thus mGluR1a serves as a multiple regulator of the signaling depending on conformational states (259).

Tateyama and Kubo (259) reported that mGluR1 expressed in CHO cells activates either G_q or G_s-protein depending on distinct conformation of N-terminal as well as intracellular domains under specific receptor activation. As discussed above, receptors spontaneously change their conformations and agonists stabilize their active conformation. Polyvalent cations such as Gd^{3+} stabilize Acc conformation of mGluR1 by strong interaction with positively charged amino acids between LB2 lobes (206), whereas glutamate can induce change from Roo to Aco conformation only. Recently, the authors have shown that receptor which was activated by glutamate and reached Aco conformation can activate both G_s and G_q proteins, whereas receptor in fully active conformation (Acc) can stimulate only

G_q protein itself (**Fig. 20**) in the heterologous expression system. They further suggest, that whether receptor activates G_q or G_s protein depends on the organization of the intracellular domains, although they were not able to demonstrate the rearrangement of the intracellular parts experimentaly by FRET approach (259).

Activation of PLC and its downstream effectors

Synaptically released glutamate acting on mGluRs evokes inositol phosphate-mediated mobilization of calcium ions in cerebellar Purkinje cells and hippocampal pyramidal neurons (260-263). Stimulation of group I mGluRs leads to activation of $G_q\alpha$ type of G-protein that activates PLC (122, 264) which in turn catalyzes conversion of phosphoinositol-4,5-bis-phosphate (PIP_2) into IP_3 and diacylglycerol (DAG) (**Fig. 21**). These second messengers further activate distinct intracellular cascades.

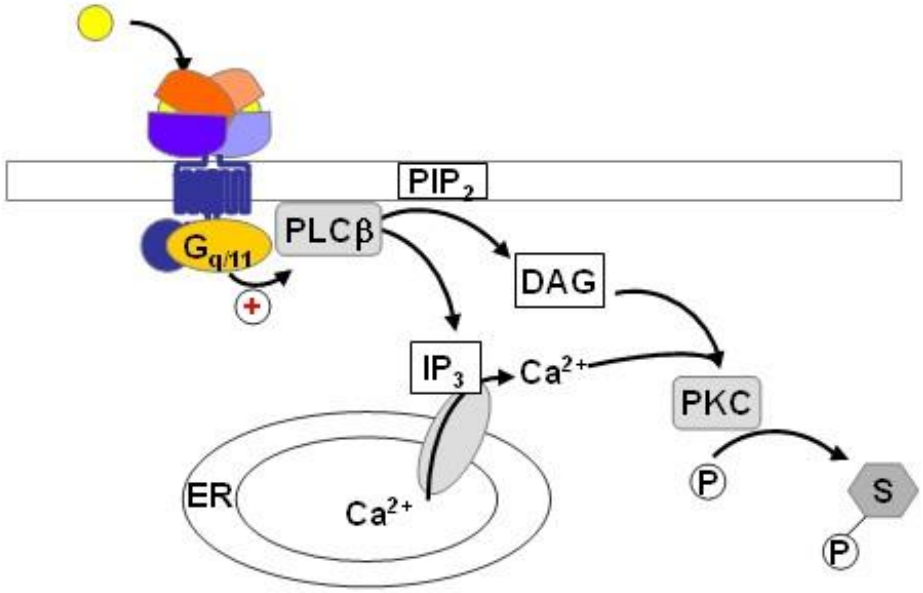


Fig. 21: Gq-mediated signaling
PLC: phospholipase C, PIP_2: 1-phosphatidyl-inositol-4,5-bis-phosphate, DAG: diacylglycerol, IP_3: inositol-1,4,5-triphosphate, PKC: protein kinase C S: substrate, ER: endoplasmic reticulum, P: phosphate. Colouring the same like in previous figures.

The first messenger, IP_3, opens IP_3-receptors in the membrane of ER that transfer calcium from the intracellular stores to the cytosol. Calcium ions then serve as a next messenger activating many intracellular proteins such as calmodulin or several kinases. The second messenger, DAG, activates PKC in dependence of increased calcium concentration (**Fig. 21**). This kinase can activate or inactivate distinct proteins, other enzymes, receptors or channels (255, 265-267). In cerebellar granule cells, both

PLC activation and calcium signaling were found to be mediated exclusively by mGluR1 subtype, although both group I mGluRs, mGluR1a and mGluR5, have been detected in these cells. It was demonstrated, that the activation of PLC strongly depends on the presence of extracellular Ca^{2+} (268) which is in agreement with observations demonstrating the binding of extracellular calcium into N-terminal binding domain of mGluR1a and showing that extracellular Ca^{2+} facilitates affinity of this receptor to glutamate (234, 240, 269).

Regulation of adenylyl cyclase and downstream effectors

Group II and group III mGluRs commonly inhibit AC by activation of pertusis toxin-sensitive $G_{i/o}\alpha$ subunit in Chinese hamster ovary cells, in brain slices, cultured neurons and astrocytes (213, 270-273). Their presynaptic localization enables to participate in the negative feedback control mechanism of glutamate release (163).

AC-mediated signaling pathway starts with activation of AC by stimulatory G-protein (G_s). AC is one of the membrane integral proteins that catalyze conversion of ATP to cAMP that in addition to Ca^{2+} serves as another diffusible second messenger. Main target for cAMP is PKA that phosphorylates various substrates from mGluRs to nuclear transcription factors. In constrast, $G_{i/o}$-proteins inhibit enzymatic activity of AC and affect other proteins by using the second branch of $G_{i/o}$-mediated responses, $\beta\gamma$-subunit. This complex further triggers cascade of GDP/GTP exchange events between several small GTPases starting with regulation proteins Grb2 and SOS/Ras that trigger a kinase cascade, which in turn lead to metabolic regulation or phosphorylation of transcription factors.

In summary, activity, synaptic distribution, expression and down-regulation of group I mGluRs are likely to be dynamically regulated in response to neuronal activity and their signalization leads to various cellular responses depending on extracellular environment and protein composition in the individual type of a cell. Signaling triggered through mGluRs can regulate long-term plastic changes at synapses and thus contribute to processes of memory. These can be modulated on various levels of signaling pathway going from the receptors to regulation of gene transcription.

3.5. Pharmacological properties of mGluRs

Pharmacological ligands for the mGlu receptors are competitive ligands, acting within the N-terminal ECD and non-competitive ligands that bind to the HD.

Orthosteric ligands are all amino acids or their analogs with selectivity to the natural binding site, which probably reflects evolutionary conserved structure over the mGluR family. Moreover, these compounds are often also agonists for ionotropic glutamate receptors, e.g. the most potent group I mGluR agonist identified to date is L-quisqualate **(Fig. 22**), but it is also a potent AMPA receptor agonist (274) and thus can be used only in heterologous system. Therefore, it is not surprising that several pharmaceutical companies such as BAYER AG, Novartis Pharma AG, SIBIA Neurosciences, La Roche AG, Eli Lilly focus on development of new non-competitive agonists and antagonists.

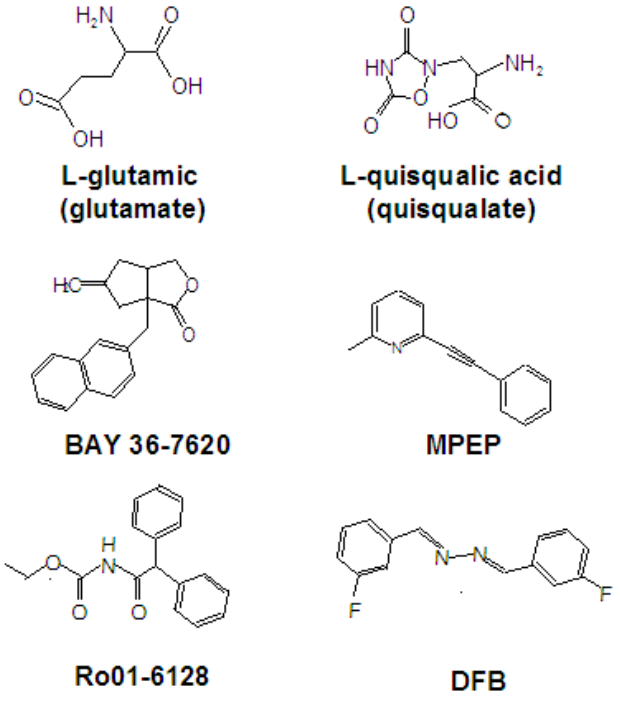


Fig. 22: Chemical structures of representative agonists and antagonists

Non-competitive ligands or allosteric modulators (AMs) **(Fig. 22)** bind within the heptahelical domain, which is highly variable between distinct mGluR members. These compounds stabilize the HD in an active or inactive state. Because these compounds modulate an agonist function, they are called modulators. In the presence of an orthosteric ligand they shift a receptor affinity to higher or lower concentrations of the ligand and are called negative and positive allosteric modulators, respectively. Because some positive AMs were shown to increase agonist-stimulated [^{35}S]GTPγS binding with no effect on agonist affinity, it is suggested that positive AM increases receptor/G-protein coupling (275).

Some of the non-competitive antagonists exert inverse agonism. The mechanism by which they affect the activity of the receptors is not presently known. It is hypothesized that non-competitive antagonists could restrict proper moving of the heptahelical domains and thus protect the signal going from extracellular to intracellular part of the receptor. On the other hand, positive allosteric modulators could facilitate this process by stabilizing the active conformation of HD. Interesting observation was done by Goudet et al. (205) who showed, that mGluR5 lacking N-terminal domain can be activated by using positive allosteric modulators, whereas negative allosteric modulators fully block its activity. This reminds action of class A ligands.

Negative allosteric modulators

The first non-competitive mGluR ligands to be identified were CPCCOEt (7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester) and (-)-PHCCC (*N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1α-carboxamide) (276). CPCCOEt inhibits mGluR1 activity but without affecting the glutamate binding and was shown to be selective for mGluR1 vs. mGluR5 (277). In comparison to CPCCOEt (IC_{50}=6.5 μM), there are other mGluR1 selective non-competitive antagonists exhibiting higher affinity in range of hundreds or even tens nM and could be represented by e.g. BAY36-7620 (**Fig. 22**) {[(3a*S*,6a*S*)-6a-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental [*c*]furan-1-on} (IC_{50}=0.16 μM), (278). Using receptor chimeras and site-directed mutagenesis, the binding sites for all of them were localised within transmembrane domain and binding experiments showed that all of them use a common binding pocket (279, 280).

The potent non-competitive ligand selective for mGluR5 is MPEP (**Fig. 22**) (281) and act also as an inverse agonist (282). Studies using chimeric receptors and site-directed mutagenesis have localized the binding site of MPEP in the pocket formed by HD and surrounded by TMIII and TMVII helices (282, 283). The most critical residues for MPEP interaction are Pro 665, Ser 658 and Ala 810. Mutation of homologous residues in mGluR1 into appropiate residues lead to creation of the MPEP-sensitive mGlu1 receptor. In this mutant mGlu1 receptor [^{3}H] M-MPEP binding is completely inhibited by CPCCOEt, suggesting their common binding pocket (282). Interestingly, MPEP acts as a positive AM at mGluR4 (284) and also exhibits some activity at noradrenaline transporter (285).

Positive allosteric modulators

The first positive AM selective for mGluR1 were synthetized by Roche and were of two chemical bases. Firstly 2-phenyl-1-benzensulfonyl-pyrolidine derivatives represented by Ro67-7476 and secondly diphenylacetyl- and (9H-xanthene-9-carbonyl)-carbamic acid esters represented by Ro01-6128 (**Fig. 22**) and Ro67-4853 (286, 287). The binding site for all these compounds lies within the heptahelical domain (286). Similarly, mGluR5 positive AMs are of two structural classes, first represented by DFB (**Fig. 22**) and second by CPPHA (*N*-288-2-hydroxybenzamide) (288, 289). All these compounds do not exhibit intrinsic agonist activity but markedly facilitate agonist-induced reponses, increasing potency and maximum efficacy.

Compounds of pyridylmethylsulfonamide nature are positive allosteric modulators of group-II mGluRs, e.g. LY487379 (*N*-(4-(2-methoxyphenoxy)phenyl)-*N*-(2,2,2-trifluoroethylsulfonyl)prid-3-yl-methylamine) (290).

Close structural analog of CPCCOEt, a negative AM for mGluR1, *N*-phenyl-7-(hydroxyimino)cyclopropa [*b*]chromen-1α-carboxamide [(-)-PHCCC], exhibits weak antagonistic activity at mGluR1 (276) but increases agonist potency and efficacy at mGluR4 (291, 292). Another negative modulator, MPEP (**Fig. 22**), also act as positive AM at mGluR4 (284).

In summary, because of the high sequence homology of the N-terminal domain, it is difficult to identify orthosteric ligands selective for individual metabotropic glutamate receptors. In contrast, all allosteric modulators identified to date appear to bind within heptahelical domain in respect to different binding preferencies among transmembrane helices. Both positive and negative AMs are of great interest as new therapeutic targets. Of interest is the fact, that a positive allosteric modulator for a relative of the mGluRs, calcium sensing receptor is already on the market. This drug – Cinacalcet hydrochlorid – is succesfully prescribed for treatment of secondary hyperparathyroidism (293).

II. Aim of the study

GPCRs represent the largest group of membrane receptors. Dimerization of GPCRs is their common feature that influences receptor function and extends physiological response of receptors depending on subunit composition, interacting intracellular partners as well as external environment. It is probably as diverse process as diverse the receptors are. The aim of this study was to disclose the mechanism of the activation of a dimeric receptor in terms of molecular processes involved. In class C GPCRs, presence of both and well separated extracellular orthosteric and allosteric binding sites brings new possibilities for pharmaceutical regulation of these receptors. For this we have to know how are dimers organized and activated.

Heptahelical domain is a bridge between extracellular and intracellular space and therefore has a pivotal role in signal transduction. Presence of two HDs within a dimer brings question of whether both HDs are equivalent in terms of organization, activity and roles in signal transduction. Among class C GPCRs, $GABA_B$ receptor is a constitutive heterodimer, in which one subunit binds a ligand whereas the other activates a G-protein (67). In contrast, mGluRs form homodimers. Does in the homodimeric mGluRs also the activation and signaling work in an asymmetrical way or do both HDs-subunits activate a G-protein simultaneously? Do both subunits or their portions (ECD *vs.* HD) play distinct roles and are turned on independently to each other? If the two subunits work asymmetricaly, then it would bring new possibilities for artificial regulation of such a receptor.

It was proposed that receptor undergoes structural rearrengements under the activation (236). How does the relative position of the TM helices and related intracellular loops change during receptor activation and especially what is the kinetics of this rearrengement is not clear yet. It would be interesting to know mechanism, by which allosteric modulators block or enhance the activity of a HD and how these compounds affect the intersubunit movements that could further help to design novel therapeutic compounds.

Thus the questions and hypothesis that are adressed here are as follows:

- What is the role of each HD within a homodimeric mGlu receptor in G-protein signaling?
- Are both HDs activated within one receptor dimer?
- Is activation of a single subunit sufficient for G-protein activation?

- How is this rearrengement influenced by positive and negative allosteric modulators?
- What is the mechanism of action of allosteric modulators in respect of activation of one subunit?

III. Experimental part

1. Materials and methods

1.1. Materials

Chemicals including L-quisqualic acid (quisqualate) and MPEP (2-methyl-6-(phenylethynyl)pyridine) were obtained from Tocris Cookson Ltd. (Bristol, U.K.), BAY 36-7620 has been synthesized by Bayer. Diphenyacetylcarbamic acid ethyl ester (Ro01-6128) (286) and 9*H*-xanthene-9-carboxylic acid 2-(isopropyl-2*H*-tetrazol-5-yl)-amide (XITA) (Jolidon02294) were synthetised in house. 3,3'-difluorobenzaldazine (DFB) was prepared as described previously (205). Serum, culture media and other solutions used for cell culture were purchased from GIBCO (BRL-Life Technologies, Inc., Cergy Pontoise, France), poly-L-ornithine and poly-D-lysine (MW 70-150,000) was obtained from Sigma (Sigma-Aldrich), [^{3}H]Myo-inositol (23.4 Ci/mol) (1 Ci = 37 GBq) was obtained from Amersham Pharmacia (Perkin-Elmer Life Science (NEN), Paris, France). Glutamate-pyruvate transaminase was purchased from Roche Diagnostics. Ethanedithiol (EDT), dimethylsulfoxid and Flash-EDT$_2$ (bis-EDT adduct of 4′,5′-bis(1,3,-dithioarsolan-2-yl)fluorescein) (**Fig. 23**) was obtained from Merck, Fluka and Invitrogen, respectively.

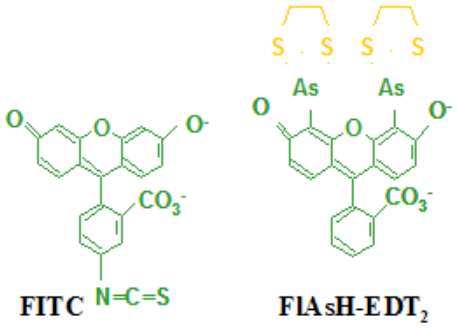


Fig. 23: Chemical structure of FITC and FlAsH

1.2. Methods

1.2.1. Mutagenesis and plasmid construction

To be able to control receptor surface expression, in most constructs an HA (hemagglutinin) or a myc tag was introduced in the N-terminal end after the signal peptide. To that aim, the coding sequence of the mature mGlu1 receptor was introduced after the unique *MluI* restriction site located after the epitope tag of pRKGB1-HA or pRKGB1-myc (67). The resulting constructs consist of the signal peptide of mGlu5, then either the HA or myc epitope, followed by the mGlu1 coding sequence starting at

Ser34. As previously reported for either mGlu5, mGlu2 and mGlu8 receptors (132, 295) or $GABA_B$ receptor subunits (67, 282), the presence of these tags did not modify the functional expression and pharmacological properties of the mGlu1 receptor.

Chimeric mGlu1 and mGlu5 receptors bearing the C-terminal tail of either $GABA_{B1}$ (R1c1) or $GABA_{B2}$ (R1c2), starting at position Met 873 and Gln 761, respectively, were constructed by taking advantage of the restriction site Sph-I ($GABA_{B1}$ or $GABA_{B2}$ tails inserted after His 859 or His 854) in the mGlu1 and mGlu5 sequences, respectively (57).

Mutant mGlu1 receptor sensitive to the mGlu5 selective antagonist MPEP was created by introducing 3 point mutations within the HD. One of them - C671S – in third TMD and other two - T815M and V823A – in the seventh TMD of mGlu1 receptor. The mGlu5 mutant sensitive to Ro01-6128 has been obtained by mutation of three residues in mGlu5, P654S and S657V in the third TMD and L743V in the fifth TMD of the receptor. Another mutation was also introduced in the i3 loop of some constructs (F781P) of R1c2 receptor to prevent coupling of the receptor to G-protein (72, 130, 286). Unless noted otherwise, the mutants were generated using the QuickChange site-directed mutagenesis kit from Stratagene (Chemos, Czech Republic). The entire coding region of all point mutants were sequenced from leading strand using the Big Dye Terminator v. 3,1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

The FlAsH sensitive constructs were prepared by fusion of aligned primers bearing tetra-cystein sequences (**Table 2**) with pRK5-R1c2 digested with *BglII* and *MluI* restriction endonucleases in either i1 or i2 loop of R1 protein. All constructs were sequenced and religated into pRK5-HA-R1c2.

Generation of constructs bearing cDNA of either HA-R1c1-eCFP or HA-R1c2-eCFP was as follows. The eCFP-coding region of pRK5- $GABA_{B1}$-eCFP was religated into pRK5-HA-R1-c1 using *BplI* and *AvrII* restriction endonucleases. For the second construct HA-R1c2-eCFP the coding region of eCFP was amplified using 3'CACTCAGAACAGCATGCACCAGTTCACACAG5' and 5'GCAGATCCTCTCATTCTAGATTA CTTGTACAGC3' primers with *SphI* and *XbaI* restriction sites, respectively. PCR fragment was ligated into pBKS-R1c2 intermediate construct using *SphI* and *XbaI* restriction endonucleases and religated into pRK5-HA-R1c2 using *EcoNI* and *XbaI* restriction sites.

Table 2: FlAsH-binding sequences

FlAsH sequence	3' primer	5' primer
CCPGCC	GATCTGCTGCCCTGGATGTTGCA	CGCGTGCAACATCCAGGGCAGCA
ACCPGCCA	GATCGCCTGCTGCCCTGGATGTTGCGCAA	CGCGTTGCGCAACATCCAGGGCAGCAGGC
AACCPGCCAA	GATCGCCGCATGCTGCCCTGGATGTTGCGCAGCAA	CGCGTTGCTGCGCAACATCCAGGGCAGCATGCGGC
AAACCPGCCAAA	GATCGCCGCAGCATGCTGCCCTGGATGTTGCGCAGCAGCAA	CGCGTTGCTGCTGCGCAACATCCAGGGCAGCATGCTGCGGC
HRWCCPGCCKTF	GATCCATCGTTGGTGCTGCCCTGGATGTTGCAAAACATTCA	CGCGTGAATGTTTTGCAACATCCAGGGCAGCACCAACGATG
AHRWCCPGCCKTFA	GATCGCCCATCGTTGGTGCTGCCCTGGATGTTGCAAAACATTCGCAA	CGCGTTGCGAATGTTTTGCAACATCCAGGGCAGCACCAACGATGGGC
FLNCCPGCCMEP	GATCTTCCTAAACTGCTGCCCTGGATGTTGCATGGAACCAA	CGCGTTGGTTCCATGCAACATCCAGGGCAGCAGTTTAGGAA

1.2.2. Cell culture and transfection of mammalian cells

Human embryonic kidney (HEK293) and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), without sodium pyruvate, supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin 100 U/ml final). Electroporation was performed in a total volume of 300 µl with 2 µg mGluR subunit plasmid DNA and up to 10 µg in total with carrier DNA (pRK6), and 10 million cells in electroporation buffer (K_2HPO_4, 50 mM; CH_3COOK, 20 mM; KOH, 20 mM, pH 7.4). For the functional assays we also added the high-affinity glutamate transporter EAAC1 to prevent the influence of glutamate in the medium. After electroporation (260 V, 1 mF, Bio-Rad Gene Pulser Electroporator), cells were resuspended in DMEM supplemented with 10% FCS and antibiotics, and split in 96 well plates (Costar, Corning Incorporated, NY) (20 million cells per plate) or petri dishes (Techno Plastic Products AG, Switzerland), previously coated with poly-L-ornithine (15 µg/ml; MW, 40,000), (Sigma, Paris, France) to favour adhesion of the cells, unless otherwise indicated.

1.2.3. Control of correct protein expression using immunofluorescence labeling and Western Blotting

To control overall production of the receptor proteins, we used western-blot analysis. HEK293 were transfected and cultured overnight in 6 cm petri dishes. After washing, cells were disrupted at 4°C and protein samples (10 µg per lane) were

separated using SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. The receptors were detected using primary monoclonal anti-HA and anti-myc antibodies from Santa Cruz Biotechnology, Inc. (Bioconsult, Czech republic) and visualized using the substrate SuperSignal® West Pico solution (Pierce, Biotech, Czech Republic) for secondary polyclonal antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc., Bioconsult, Czech republic).

1.2.4. Determination of receptor dimer folding on the cell surface using Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

These experiments were conducted as previously described (57). COS-7 cells were transfected with tagged receptors and cultured overnight in uncoated 96-well black plates. After washing with Hank's balanced salt solution (HBSS, Sigma) buffer with 1% fetal calf serum, cells (200,000 per well) were incubated 6 hours in 4°C in a total volume of 100 µl with the fluorophore labeled anti-HA (12CA5) and/or anti-myc (9E10, ATCC no. CRL-1729) monoclonal antibodies (all provided by CisBio International, Bagnols-sur-Ceze, France) at a concentration of 2 nM and 6 nM, respectively. The donor fluorophore was Pyridine-BiPyridine europium (Eu^{3+}) Cryptate (EuCryptate) whereas the acceptor fluorophore was AlexaFluor® 647 (Alexa647). Anti-HA antibodies carrying either the donor or acceptor fluorophore and anti-myc antibodies labeled with the acceptor fluorophore were used.

After incubation, cells were washed twice with HBSS buffer and fluorescence signals were measured. The bound Alexa647 antibodies were quantified after excitation at 640 nm and emission monitored at 682 nm using an Analyst^{TM} reader (Molecular Devices) equipped with the appropriate filters set (XF47 from Omega Optical). Eu^{3+}-Cryptate fluorescence and TR-FRET signal were measured 50 µsec after excitation at 337 nm, at 620 nm and at 665 nm, respectively, using a RubyStar fluorimeter (BMG Labtechnologies, Champigny-sur-Marne, France). The TR-FRET signal (energy transfer) was measured either as Delta665 (*Delta665=R665pos – R665neg* where *R665pos* is the fluorescence intensity measured at 665 nm in the presence of both fluorophores, and *R665neg* is that measured in the absence of the acceptor molecule), or Delta F (%) ($Delta\ F(\%) = [(R665/620)_{pos} - (R665/620)_{neg}] \times 100/(R665/620)_{neg}$, where $(R665/620)_{pos}$ is the ratio of the 665 signal over that at 620 measured in the presence of

both antibodies, and $(R665/620)_{neg}$ is the same ratio measured in the absence of the acceptor-labeled antibody.

1.2.5. Quantification of cell surface receptors using Enzyme Linked ImmunoAssay (ELISA)

Cell surface expression level of the N-terminal HA-tagged receptors was determined using ELISA assay adapted from (20, 205, 296). HEK 293 cells transfection was performed in 96-well plates (Costar, Corning Incorporated, NY). Twentyfour hours after transfection cells were fixed with 4% paraformaldehyde, permeabilized or not with 0.05% Triton X-100 (5 min) and then blocked with PBS + 1% FBS. Cells were incubated for 1 hour with rat monoclonal anti-HA antibody coupled to horseradish peroxidase (clone 3F10 (Roche) at 0.5 µg/ml). Antibodies were detected and quantified instantaneously by chemiluminescence using SuperSignal® ELISA femto maximum sensitivity substrate (Pierce) and a Wallac Victor^2 luminescence counter (Molecular Devices).

1.2.6. Functional assays

Determination of inositol phosphates (IPs) accumulation

The procedure used for the determination of IP accumulation in transfected cells was adapted for a 96 well plate format as previously described (205). Cells plated into 96 well plates were washed 2 to 3 hours after electroporation and incubated overnight in Glutamax DMEM (BRL-Life Technologies, Inc.,) containing 0.5 µCi/ml [^{3}H]-myo-inositol (0.5 µCi/well). The IP formation determination was performed after a 30-min incubation in the presence of 10 mM LiCl. For basal determination of IP production, GTP (1U/ml) and 2 mM pyruvate were added to the reaction. The antagonist was applied for 10 min followed by 30 min incubation with agonist. The reaction was stopped by replacing the incubation medium with cold formic acid (0.1 M) and plates were kept at 4°C for 30 minutes. Supernatants were recovered and IPs were purified by hydrophilic low protein binding frite-plates (diameter 0.45 µm) (Millipore Corp., Bedford, MA) covered by Dowex resin (AG® 1-X8 Resin, BioRad, Hercules, CA). Total radioactivity remaining in the membrane fraction was counted after treatment with 10% Triton X-100, 0.1 N NaOH for 15 min and used as standard. Radioactivity was

measured using a Wallac 1450 MicroBeta microplate liquid scintillation counter (Molecular Devices). Results are expressed as the ratio between IPs and the total radioactivity present in the membranes and produced by the cells. The dose-response curves were fitted with Graph Pad Prism program (San Diego, CA) and the following equation: $y = [(y_{max} - y_{min}) / 1 + (x / EC_{50})^{nH}] + y_{min}$, where the EC_{50} is the concentration of the compound necessary to obtain 50% of the maximal effect, and nH is the Hill coefficient.

Intracellular Ca^{2+} mobilization assay

Twenty four hours after transfection, HEK293 cells plated in 96 well plates were washed with fresh HBSS buffer and incubated with 1µM Ca^{2+}- sensitive fluorescent dye Fluo-4 AM (Molecular Probes, Leiden, Netherlands), pluronique acid (0.5 µM) (Interchim S.A., Montlucon, France), probenecid (2,5mM p-[dipropylsulfanyl]benzoic acid, Sigma), GPT (10U/ml) and 2 mM pyruvate for 1 hour at 37°C. After washing, cells were incubated with 50 µl of buffer with or without antagonist. Intracellular Ca^{2+} was then monitored measuring fluorescence signal (excitation 485 nm, emission 525 nm) at 1.5 s intervals for a period of 60 s using the microplate reader FlexStation (Molecular Devices). The agonist quisqualate (2x solution, 50 µl/well) was automatically added 20 s after the beginning of the recording at the indicated concentration (**Fig. 24**).

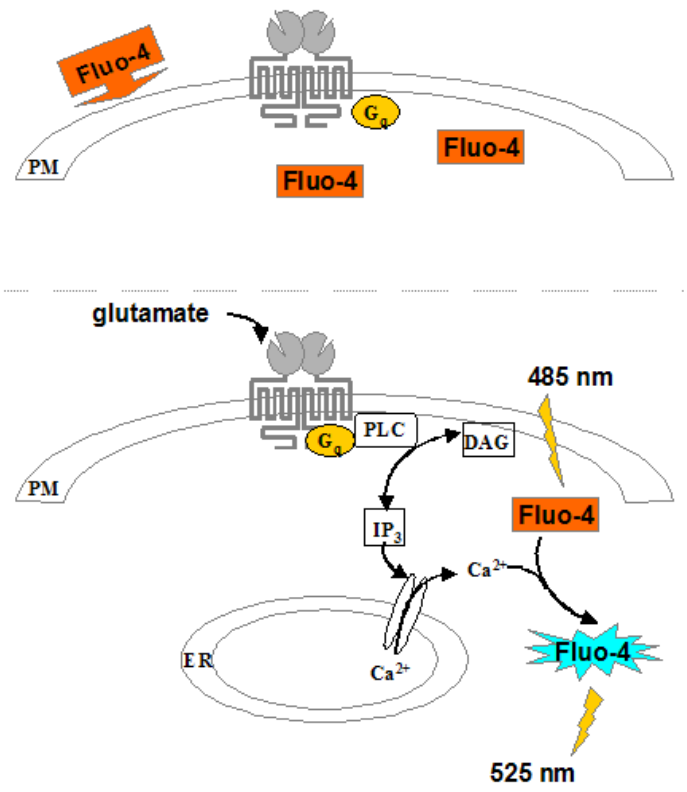


Fig. 24: ***Principle of intracellular Ca2+ mobilization assay***

1.2.7. FlAsH-based experiments

HEK 293 cells were splitted on glass coverslips (Cubreobjetos, P-lab, Czech Republic) coated with poly-D-lysine two days prior assay. After they set in, they were transfected with 2 μg of mGlu receptor DNA/transfection using Effectene reagent (Quiagen) according to protocol. Six hours after transfection, the complete DMEM was exchanged to Glutamax DMEM and left overnight in 37°C, 5% CO_2 incubator. FlAsH-EDT_2 (**Fig. 25**) was used in final concentration of 500 nM in the presence of 12.5 μM EDT. The labeling was performed for 1 hr in 37°C in 1x Hank´s Balanced Salt Solution (HBSS, Gibco-BRL, Invitrogen) supplemented with D-glucose (1g/l). Free and non-specifically bound FlAsH was removed by washing with 250 μM EDT in HBSS + glucose. Cells were maintained in incubator until used.

Fluorescence of FlAsH-labeled living cells was obtained using Leica TCS SP2 system with an Attofluor holder (Molecular Probes) upon excitation at 514 nm of argon laser. Images were taken with a 63x objective using factory settings for YFP fluorescence (530-600 nm).

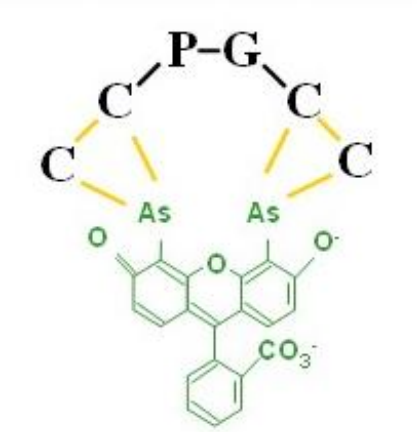


Fig. 25: Chemical structure of FlAsH-EDT bound to the tetracystein motif

2. Results

2.1. Assymetric functioning of dimeric metabotropic glutamate receptors

The main excitatory neurotransmitter, glutamate, acts on either ionotropic or metabotropic receptors. The eight subtypes of metabotropic glutamate receptors belong to the third family of GPCRs. GPCR family is thought to be the most diverse group of membrane receptors (1). Very interesting was the discovery that many GPCRs can form dimers and Dean et al. (297) proposed this property for whole GPCR family. Except newly cloned „orphan" receptors, where the dimerization was not well studied yet, family 3 GPCRs - $GABA_B$ receptor, mGluRs, CaSR and taste receptors - act in dimeric form (19, 21, 39, 43, 45, 59). Among these receptors, $GABA_B$ receptor and taste receptors form heterodimers, whereas mGluRs and CaSR are disulfide-linked homodimers. In the case of taste receptors, heterodimerization of T1R3 with either T1R1 or T1R2 determines specifity of the taste sensing with preference to umami or sweet taste, respectively (45). $GABA_B$ receptor also forms functional heterodimer, where one subunit binds a ligand, whereas the adjacent activates a G-protein (67). On the other hand, mGluRs form homodimers although their oligomerization with other GPCRs is not strictly restricted (46, 226) and is probably utilized as a tool of a bidirectional functional control. But all aspects of proper pairing of two identical subunits still remain unknown. Recently, there is evidence that for full receptor activation two molecules of agonist, each acting on one of the subunits in the homodimer, are required (57, 298). But it is still not known how is the signal transfered into intracellular part of the receptor to be able to activate G-protein.

In our studies we were interested in the role of each heptahelical domain in respect to the G-protein activation. For this purpose, we used mGluR1 and mGluR5 as models of homodimeric receptors. We used the system that allows the functional expression of mGlu dimeric receptors composed of two well-defined subunits, each bearing specific mutations (**Table 3**). We have found out that a single negative AM per dimer does not affect receptor activity. The effect of the non-competitive antagonist is different from that observed when a mutation protecting G-protein coupling is introduced in the i3 loop of a single subunit. In this case, the G-protein coupling efficacy is decreased. On the other hand a single positive AM is sufficient for maximal potentiation of the agonist

effect. Moreover it enhances the receptor activity even when it is bound into the subunit which does not bear the mutation protecting G-protein coupling whereas the adjacent subunit does. These data suggest that a single HD is turned on during receptor signal transduction within a homodimeric receptor and supports an idea of allosteric interaction of two identical subunits similarly to e.g. $GABA_B$ receptor.

Table 3: Nomenclature used for the constructs described in this study

symbol	Description of the modification	Effect of the modification
R1	mGlu1 receptor based construct	
R5	mGlu5 receptor based construct	
c1	C-terminal tail of GB1	Retained in the ER in the absence of a c2 construct
$c1_{ASA}$	c1 with the ER signal mutated into ASA	Not retained in the ER
c2	C-terminal tail of GB2	Allows R1c1:R1c2 to reach the surface
X	1 point mutation (F781P) in the i3 loop	Loss of coupling to G_q-protein
M	3 mutations in HD creating an MPEP site	Inhibited by MPEP
Ro	3 mutations in HD creating an Ro01-6128 site	Potentiated by Ro01-6128
B	2 mutations in VFT (Y236A, D318A)	No activation by agonist

2.1.1. Generation of „heterodimeric“ mGlu receptors

Among receptors of the third family of GPCRs, $GABA_B$ receptor is a well defined heterodimer composed of two different subunits, $GABA_{B1}$ and $GABA_{B2}$. Function of this receptor depends on proper assembling of both subunits already in the ER membranes. $GABA_{B2}$ subunit is required for neutralizing the ER retention signal (RSRR) in the C-terminus of $GABA_{B1}$. Masking of this signal by C-terminal tail of the $GABA_{B2}$ subunit allows the heterodimer to reach the cell surface (**Fig. 26**) (64, 66, 72). In contrast, mGluRs form homodimers whose expression is not controlled by intracellular directed retention. In order to measure response of a single subunit with

engineered mutations in such a homodimeric receptor, we used the quality control system of the heterodimeric $GABA_B$ receptor. This property could be efficiently used for targeted expression of mutated receptors. We preparred chimeric mGlu1 receptors with swapped C-terminal domains with those from either $GABA_{B1}$ (c1-terminus) or $GABA_{B2}$ (c2-terminus), respectively (**Fig. 27**).

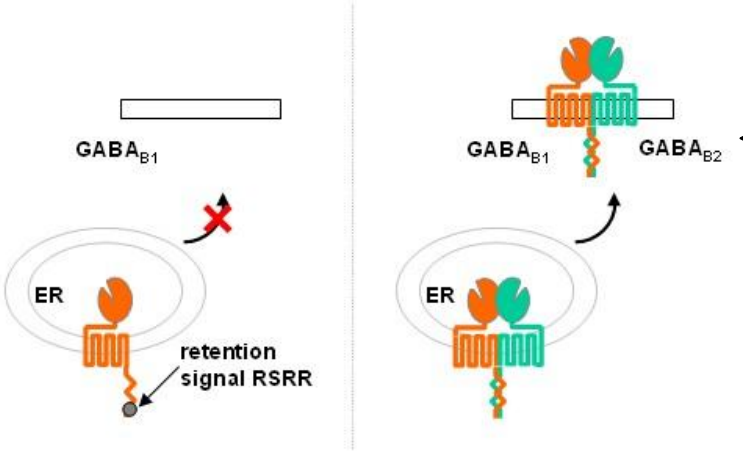
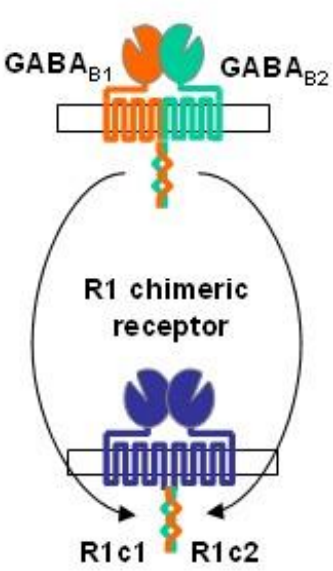


Fig. 26: Association of the two $GABA_B$ receptor subunits is necessary for expression ofthe $GABA_B$ receptor at the cell surface

Fig. 27: Scheme of generation of the mGlu1 (R1) chimeric receptors



As expected, constructs with c1-terminus, does not reach the cell surface alone whereas they do when co-expressed with receptor bearing c2-terminus (**Fig. 28**). Cell surface expression of the receptors was determined by ELISA and TR-FRET analysis (**Fig. 28 and Fig. 30**). TR-FRET experiments were performed with an anti-HA (HA: hemaglutinine) antibody labeled with the donor fluorophore Eu^{3+}Cryptate pyridine bipyridine and an anti-myc antibody labeled with the acceptor fluorophore AlexaFluor647 (**Fig. 29**). As show in **Fig. 30a**, large FRET signal was detected in cells expressing HA-R1c1 and myc-R1c2, as well as HA-$GABA_{B1}$ and myc-$GABA_{B2}$. In contrast, the signal was not observed after mixing cells expressing HA-R1c1 and cells

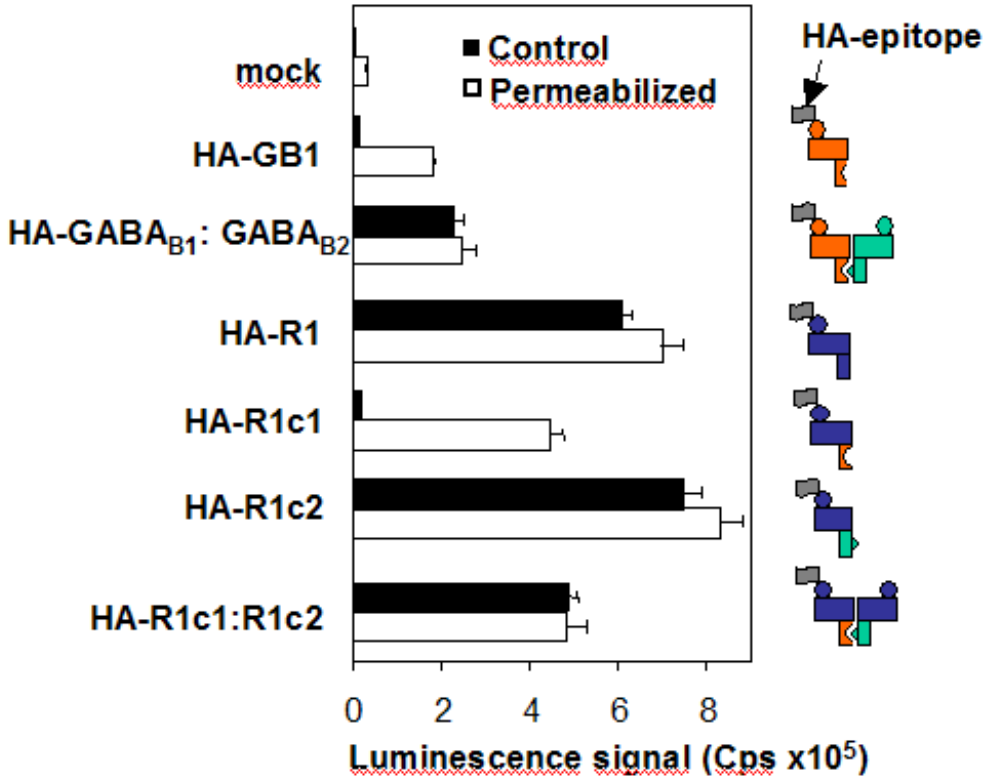


Fig. 28: Cell surface expression of the mGlu1chimeric receptors
Determination of the cell surface expression of GABA$_B$ and WT or chimeric mGlu1 receptors in either non-premeabilized (black) or permeabilized (white) HEK293 cells using anti-HA antibody.

expressing myc-R1c2, and only a small signal was obtained in cells co-expressing HA-R1c2 and the myc-V2 vasopressin receptor, despite a similar expression level of each partner at the cell surface (**Suppl. I, Fig. 2B and C**). Moreover, the FRET signal was directly proportional to the amount of HA-tagged subunit expressed at the cell surface (**Fig. 30b**). These data do not exclude the possibility, that myc-R1c2 allows the homodimer HA-R1c1:HA-R1c1 to reach the cell surface. This is unlikely the case since the FRET signal detected between HA epitopes in cells expressing HA-R1c1 and myc-R1c2 remains low and is comparable to that obtained from cells expressing heterodimeric GABA$_B$ receptor (**Fig. 30a**). Thus it rather comes from over-expression of the receptors (57). In contrast, high FRET-signal measured between HA-tagged receptors, coming from cells co-expressing HA-R1c2 and myc-tagged vasopressin receptor V2, demonstrates that there is high level of HA-R1c2:HA-R1c2 homodimers at the cell surface (**Fig. 30a**) and that this method can be used even for detection of surface-expressed homodimers.

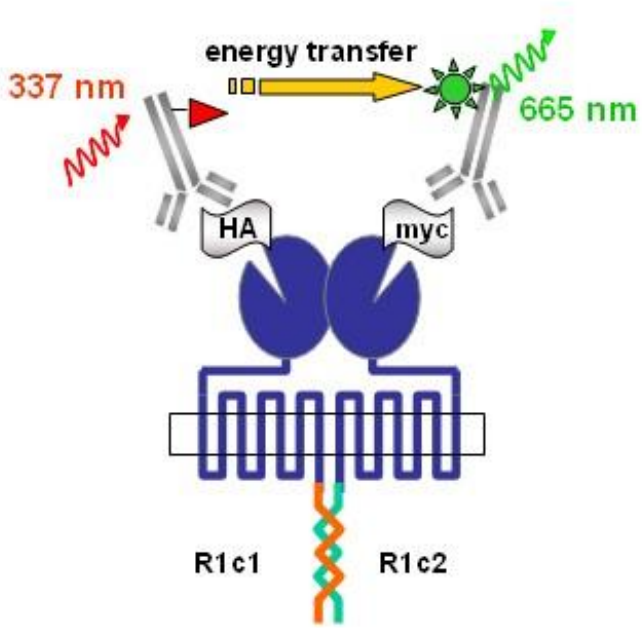


Fig. 29: Scheme of TR-FRET based experiments for determination of cell surface expression of R1c1:R1c2 heterodimers

The ELISA assay was also used to determine percentual proportion of different populations of dimers at the cell surface, homodimers versus heterodimers. We co-transfected HA-tagged R1c1 subunit with either non-tagged or HA-tagged R1c2 subunit (**Fig. 31**). Then anti-HA antibody was used to detect either a single or both subunits. Thus we detected only a heterodimeric or both hetero- and homodimeric populations of receptors. Our data revealed that the amount of the R1c1 subunit is more than one third of the total amount of subunits. According to these observations, we estimated that 72±3% (n=6) of the receptors corresponded to the heterodimer R1c1:R1c2, when an equal amount of plasmid encoding each subunit was used for transfection (**Fig. 31**). This proportion could be further increased using higher amount of plasmid encoding R1c1 subunit. As expected, subunit bearing c1-terminus, R1c1 is not functional because of its intracellular expression, whereas it is when the retention signal RSRR is either mutated into ASAR in R1c1 receptor or masked with R1c2 receptor subunit (**Fig. 32**).

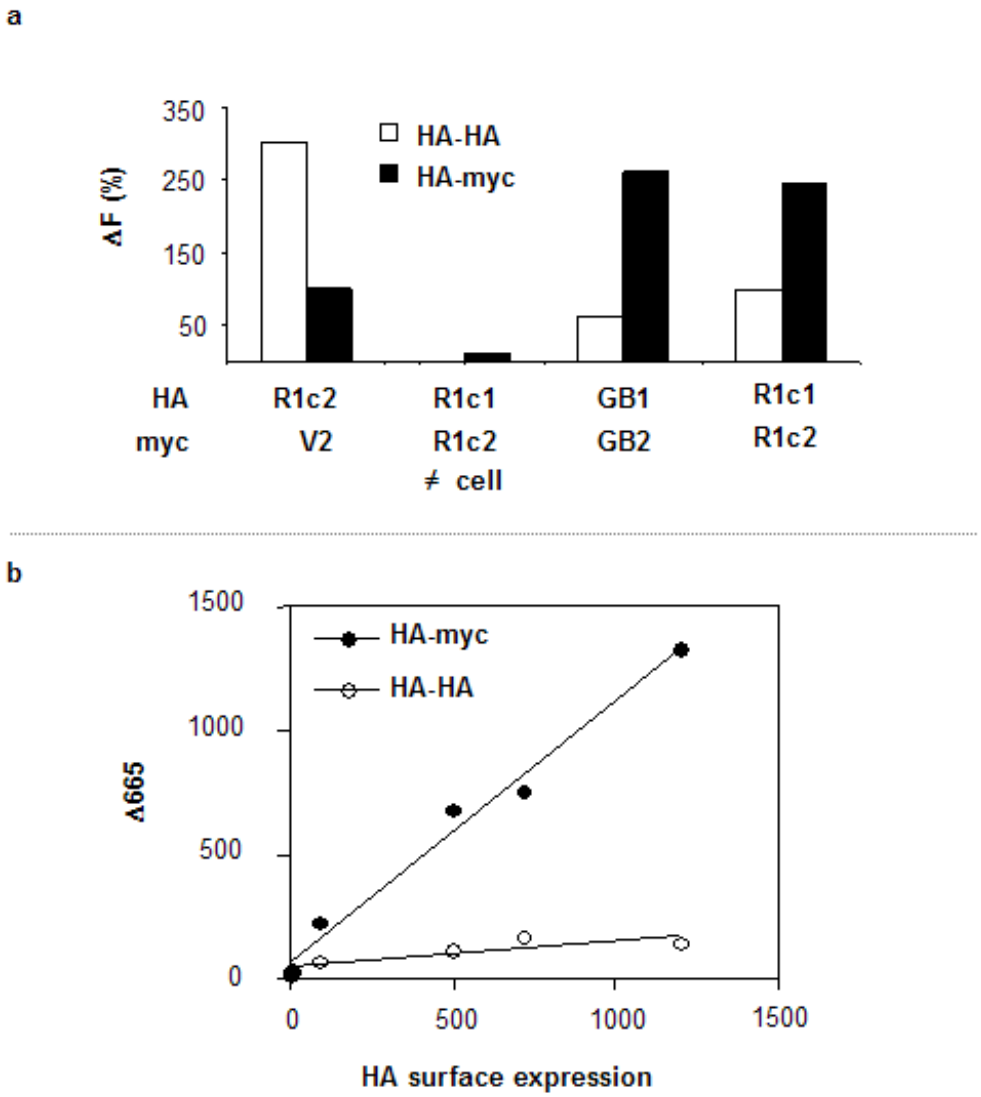


Fig. 30: R1c1:R1c2 heterodimers are expressed on the cell surface

(a) TR-FRET signal measured on intact cells co-expressing indicated subunits. Anti-HA-Eu3+Cryptate and anti-myc-AlexaFluor647 antibodies (black) were used for detection of heterodimers at the cell surface, whereas anti-HA-Eu3+Cryptate and anti-HA-AlexaFluor647 antibodies (white) were used for determination of possible homodimeric interactions. (b) The TR-FRET signal (expressed as D665) was measured in cells transfected with 1µg HA-R1c1 and various amounts of myc-R1c2 (from 0 to 1µg). Anti-HA-Eu3+Cryptate and anti-myc-Alexa647 antibodies were used to estimate HA-R1c1:myc-R1c2 heterodimers (open circles), while anti-HA-Eu3+Cryptate and anti-HA-Alexa647 antibodies were used to estimate the amount of HA-R1c1:HA-R1c1 homodimers (closed circles). The plot shows the TR-FRET signal as a function of the surface expression of the HA-tagged subunits as determined by the specifically bound anti-HA-Eu3+Cryptate antibody. All values are means ± S.E.M. of triplicate determinations from a representative experiment. Similar data were obtained in at least two additional experiments.

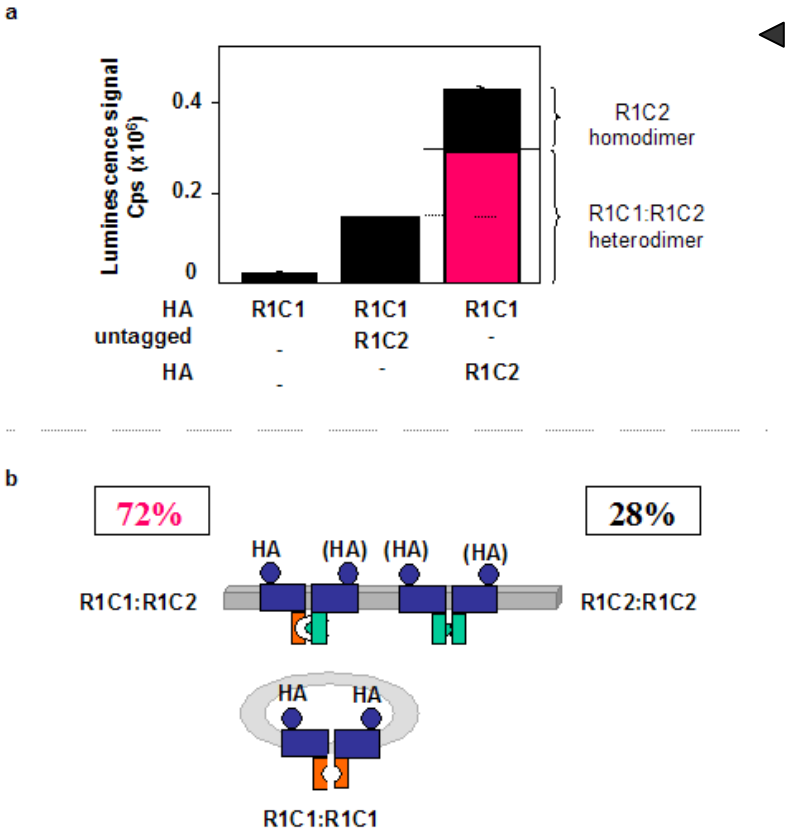
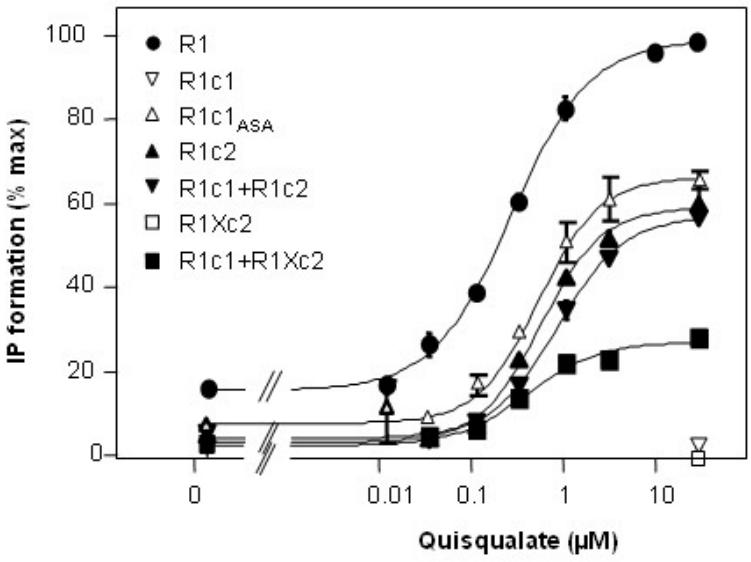


Fig. 31: ***Quantification of the relative expression of R1c1:R1c2 heterodimers and R1c2:R1c2 homodimers***
(a) Determination of the luminiscence signal obtained by ELISA using anti-HA antibody measured in cells expressing the indicated subunits. Values are means ± S.E.M. of triplicate determinations from a typical experiment out of three. (b) Schematic representation of the expected surface expression of the heterodimers vs. homodimers. HA: hemagglutinin tag

Fig. 32: Functional expression of R1c1 and R1c2 chimeras
Effect of increasing doses of quisqualate on chimeric receptors. Increase in IP formation in cells expressing indicated receptor subunits is plotted as a function of quisqualate concentration. Values are normalized to the quisqualate-induced maximal response obtained with wild-type mGluR1 (100%) and are means ± S.E.M. at least three independent experiments performed in triplicate.



2.1.2. Restriction of G-protein coupling

Intracellular parts of GPCRs are involved in recognition and coupling of the G-proteins (131, 132, 299-305). Family 3 GPCRs have short i1 and i3 loop and longer i2 loop (**Fig. 33**). The i2 loop was previously shown to play a critical role in specification of the signaling (130, 306). Among mGluRs, mGlu1 receptor has unique property to activate all, G_q-, G_s- and $G_{i/o}$-proteins (130, 207, 220, 237, 238, 240). Single mutation of several amino acid residues in the i2 loop (Thr 695, Lys 697 and Ser 702) markedly impair PLC activation, whereas mutation of other residue (Pro 698) or deletion of Cys 694/Thr 695 impairs ability to stimulate AC. Furthermore, Lys 690 seems to play role in G_i-mediated signaling (130) (**Fig. 33**). Except determining of the signaling pathway, i2 loop is also responsible for selective recognition of the very end of Gα-subunit (132). Although i2 loop seems to be the main signal-determining part of the mGluRs, other regions, i1 loop, i3 loop as well as proximal part of the C-terminus cooperate with i2 loop to control coupling to the G-proteins (129, 130, 306) (**Fig. 11**).

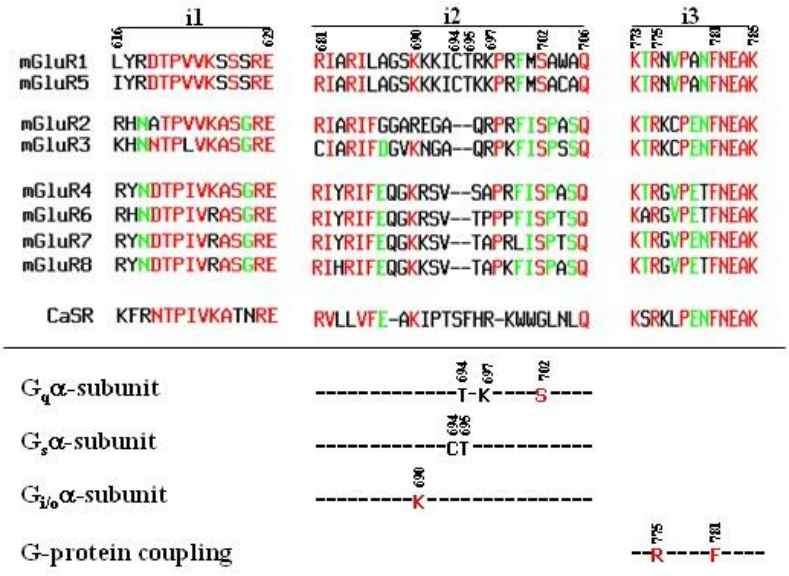


Fig. 33: Sequence alignment of i1, i2 and i3 of CaSR and all eight subtypes of mGluRs
Amino acids highly homological (90%) are highlighted in red, amino acids with low consensus value (50%) are highlighted in green and residues with lower than 50% homology are ilustrated in black colour. The positions of the first and the last amino acids in each intracellular loop and the critical residues are indicated above the aligned sequences and correspond to mGluR1 sequence. The most important amino acids and their selective involvement in distinct signaling pathways are depicted below.

Similarly, several point mutations within i2 and i3 loop of related CaSR decreased or restricted signaling through PLC (307). The importance of i3 loop in coupling of G-proteins was also supported by mutational analysis. Single mutation of either Arg 775 or Phe 781 completely blocks mGluR1-mediated activation of PLC as well as AC pathway (130) (**Fig. 33**). In the related CaSR, equivalent mutation of Phe 707 into Ala

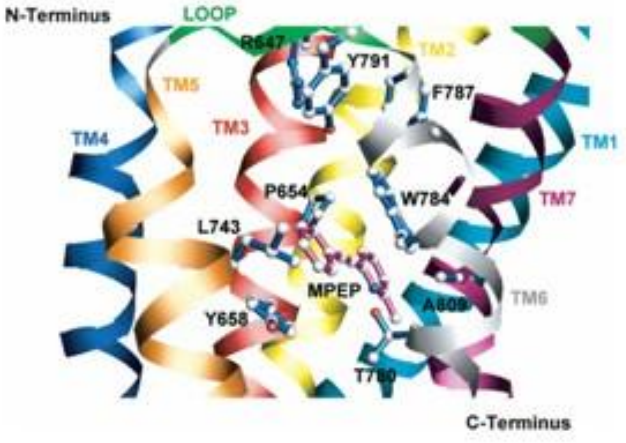
also supresses coupling (307). Loss of the G-protein coupling could be efficiently used for disclosing the role of a single subunit within a dimer.

With this approach we were able to clearly demonstrate, that R1c1:R1c2 receptor is functional. We introduced a point mutation (F781P) into i3 loop of R1c2 subunit (R1Xc2) (**Table 3**). Elimination of homodimeric R1c2 to contribute to overall receptor activation also enabled us to account only response of a single subunit in the „heterodimeric" population. When R1Xc2 was expressed alone, it was not able to activate PLC as measured by inositol phosphate production or Ca^{2+} release (**Fig. 32**), although it was correctly targeted to the plasma membrane. When R1c1 and R1Xc2 were co-expressed, we observed a response, although maximal effect was about one-third of that measured in cells expressing R1c1 and R1c2. Since no response is expected from R1c1 and R1Xc2 homodimers, this demonstrates functional coupling of the R1c1:R1Xc2 heterodimer. We would expect only 30% decrease of the maximal receptor response according to the fact that R1c2 homodimers form only 30% of membraneously expressed receptors. The larger decrease is expected to be due to less efficient activation of PLC by such a mutant receptor. This observation could mean two distinct things in the activation of a homodimeric receptor.

First, each HD is capable to activate a G-protein independently of the other and thus blocking one subunit to couple G-protein decrease the overall activation of receptor approximately to 50%. Second, one or the other subunit, but not both subunits, is turned ON and able to transfer the signal to a G-protein. This suggests that at a time of the receptor activation there are two populations of receptors having activated (turned ON) one or the adjacent subunit and the mutation that prevents G-protein coupling of a single subunit causes decrease in the maximal receptor activity in only one population, hence approximately 50% of receptors (**Fig. 24**).

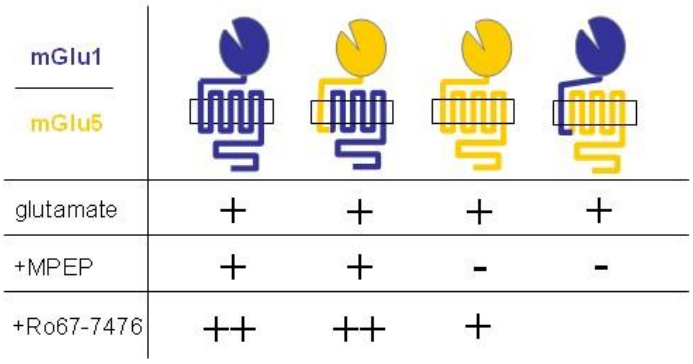
2.1.3. Modulation of a single heptahelical domain by allosteric modulators

With growing number of selective allosteric modulators acting within the heptahelical domain, we decided to use these agents for disclosing the role of a single HD in a homodimeric receptor. It is believed that positive AM could stabilize the active state of HD, whereas negative AM disables HD to reach its active conformation. For both, negative AM of mGluR5, MPEP (281), and positive AM for mGluR1, Ro01-6128 (286), the binding pocket was well described (282, 283, 286) (**Fig. 34**).



◀ ***Fig. 34: Homology model of the mGluR5 TM domain with bound MPEP based on structure of bovine rhodopsin (283)***

Fig. 35: Disclosure of the region where allosteric modulators act
Effect of the MPEP on glutamate-induced changes in $[Ca^{2+}]_{in}$ in COS1 cells transiently expressing indicated constructs (after 282). Enhancement of the glutamate-induced current by Ro67-7476 in GIRK-CHO cells expressing chimeric receptors (after 286); mGluR1 in blue, mGluR5 in yellow. ▼



MPEP inhibits glutamate-stimulated IP production in mGluR5 expressing cells without affecting the EC_{50} value or the Hill coefficient, indicating that MPEP is a non-competitive antagonist acting in the allosteric binding site. Moreover, MPEP acts as an inverse agonist and decreases the constitutive activity of the mGlu5 receptor (282). Chimeric receptors of MPEP-sensitive (mGluR5) and MPEP-nonsensitive (mGluR1) receptors helped to disclose the MPEP binding site and this was localized into transmembrane domain (**Fig. 35**). Binding studies with [^{3}H]-M-MPEP were used to find out the specific region of MPEP binding. It was shown that chimeric receptor lacking TMIII and/or TMVII of mGluR5 do not bind tritiated MPEP, although all of them are functional (282). More detailed study of Malherbe et al. (283) demonstrated that eight residues are crucial for MPEP binding to rat mGluR5. These are: Pro 654, Tyr 658 (in TMIII), Leu 743 (in TMV), Thr 780, Trp 784, Phe 787, Tyr 791 (in TMVI) and Ala 809 (in TMVII) (**Fig. 36**). Although Malherbe et al. (283) showed crucial function of TMVI in the MPEP binding, this helix is identical between mGlu1 and mGlu5 receptors (**Fig. 36**) and thus these four residues probably only contribute to binding of MPEP as shown in **Fig. 34**.

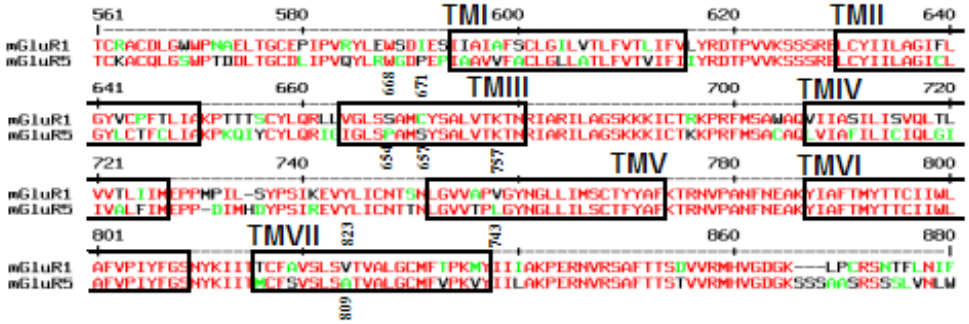


Fig. 36: Amino acid sequence alignment of the TM region of mGluR1 and mGluR5
Each TM helix is boxed. The residues that have been mutated in the studies of Knoflach et al. (286), Malherbe et. al (283) and Pagano et. al (282) are indicated by corresponding number in amino acid sequence of either mGluR1 (above) or mGluR5 (below). Identical amino acids are highlighted in red.

We generated mutant mGlu1 receptor sensitive to MPEP (R1M, **Table 3**) by introducing three point mutations of Ser 863, Cys 671 and Val 823 to corresponding residues in mGluR5, Pro, Ser and Ala, respectively (**Fig. 36**). We show here, that this mutant receptor is sensitive to MPEP but the action of other AM, e.g. BAY 36-7620 (the selective mGlu1 receptor negative AM) is not affected by change of the three amino acids (**Fig. 37**). This indicates, that crucial residues for selective binding of BAY 36-7620 into TMD of mGluR1 were not changed and may lie within other TM helices (see alignment in **Fig. 36**). R1M was fully antagonized by MPEP with IC_{50} of 3.7± 1.3 μM and the combination R1Mc1:R1Mc2 was also inhibited by MPEP with a similar IC_{50} (3.4 ± 1.4 μM) (**Fig. 38**).

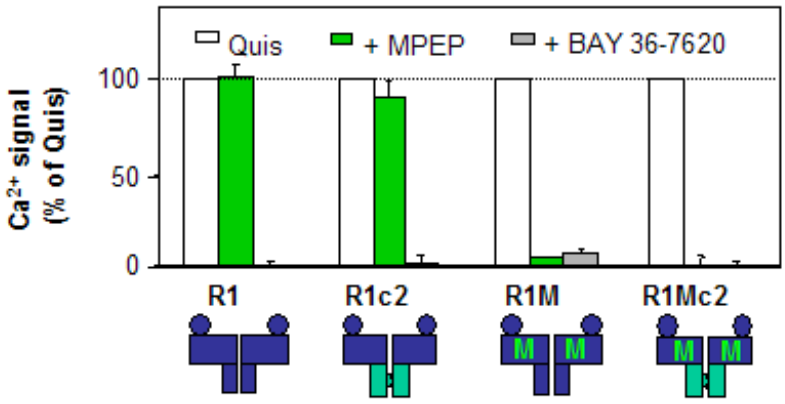


Fig. 37: Generation of MPEP-sensitive mGlu1 receptor
Effect of MPEP and BAY 36-7620 on quisqualate-evoked Ca^{2+} signal on WT, R1c2, R1M and R1Mc2 homodimers the bar graph. Effect of quisqualate (1 μM) alone (open columns), MPEP (100 μM) (black columns) or BAY 36-7620 (10μM) (gray columns) on Ca^{2+} signal was measured in HEK293 cells expressing the indicated subunits (below). Values are expressed as percentage of the maximal quisqualate effect and are means ± S.E.M. of three independent experiments performed in triplicate.

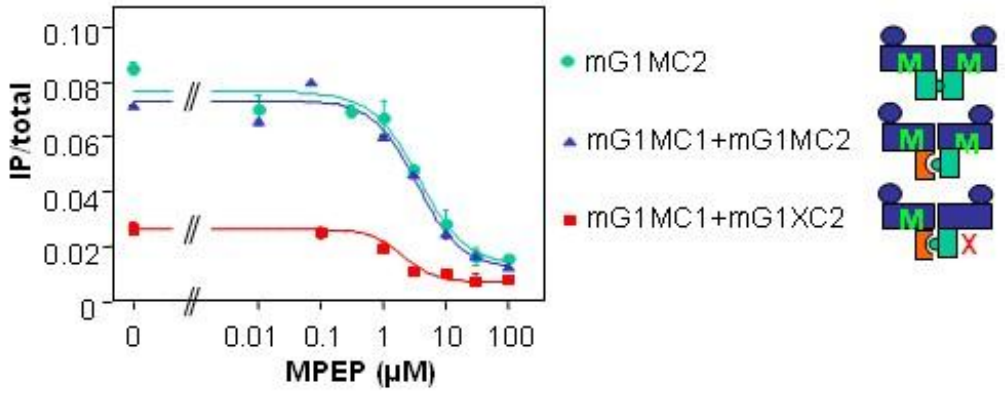


Fig. 38: Dose-dependent effect of MPEP on the quisqualate-stimulated IP production
HEK293 cells expressing R1Mc2 (green circles), R1Mc1:R1Mc2 (blue triangles) or R1Mc1:R1Xc2 (red squares) were monitored for changes in IP formation upon stimulation with quisqualate (1 µM) in the presence of various concentrations of MPEP. Results are expressed as IP rpoduction over total radioactivity remaining in the membranes of the cells. Values are means ± S.E.M. of triplicate determinations from a representative experiment out of three independent experiments.

On the other hand, Ro01-6128 is a mGlu1 receptor enhancer that potentiates glutamate response, although it has no effect when applied alone (286). Using mGlu1/mGlu5 receptor chimeras, the binding site was localized into TM region. The enhancing effect of glutamate response was hardly reduced in V757L and S668P/C671S mGlu1 mutant receptors. Similarly, reciprocal mutation of chosen residues in mGlu5 (P654S/S657C/L743V) caused this receptor to be Ro01-6128-sensitive (286).

Similarly, by introducing these three point mutations into mGluR5, mutant receptor sensitive to Ro01-6128 (R5Ro, **Table 3**) can be generated (Suppl.II Fig. 2). Although Ro01-6128 potentiates quisqualate-stimulated response of mGlu1 receptor, DFB does not affect this response. Similarly, although DFB decreases EC_{50} of quisqualate-induced activity, Ro01-6128 exhibits no effect on mGlu5 wild-type receptor. In contrast, either Ro01-6128 or DFB potentiate quisqualate-stimulated activity of R5Ro mutant receptor, demonstrating that, similarly to MPEP and BAY 36-7620, these two AM do not also share their binding sites.

Two molecules of non-competitive antagonist are required for full receptor inhibition

In order to find out whether one or both HDs must reach its active state for dimeric receptor activation of G-proteins, we examined the effect of MPEP on receptor combinations in which a single subunit was sensitive to MPEP (R1Mc1:R1c2, and R1c1:R1Mc2). As shown in **Fig. 39**, no inhibition by MPEP was observed in cells expressing both R1Mc1 and R1c2 although BAY 36-7620, which can bind both

subunits, was able to fully block the response. When the MPEP site is included in the R1c2 subunit, MPEP inhibits 20% of the agonist-mediated response. This inhibition likely represents the component of the response mediated by the R1Mc2 homodimers, consistent with the heterodimer not being sensitive to MPEP.

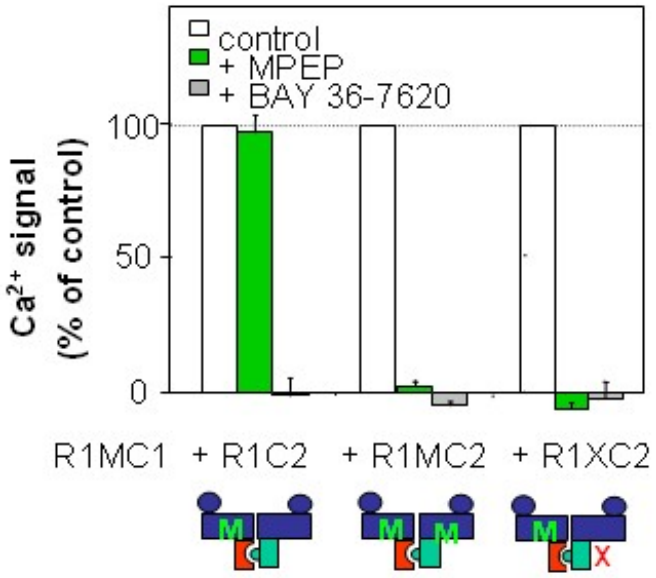


Fig. 39: Two molecules of MPEP are required for inhibition of receptor activity
Two MPEP sites per dimer appear necessary for MPEP inhibition of receptor activity. Effect of quisqualate (1 μM) alone (open columns) or together with MPEP (100 μM) (green columns) or BAY 36-7620 (10 μM) (grey columns) on Ca^{2+} signals was measured in cells expressing the indicated subunits (below). Values are expressed as percentage of the maximal quisqualate effect and are means ± S.E.M. of independent experiments performed in triplicate.

To confirm this suggestion and confirm that MPEP has no antagonist activity on receptor dimers possessing a single MPEP site, we performed additional experiments with dimer combinations made of a R1c2 subunit that does not form a functional receptor alone. To that aim, we introduced two mutations in the agonist-binding site (Y236A and D318A) (**Fig. 40**). It was previously reported that such a mutant receptor cannot be activated when in a homodimeric form, but can still be part of a functional dimer when associated with a subunit possessing a wild-type binding site (57). As shown in **Fig. 39**, whether the MPEP site is introduced in this (c2) or the other (c1) subunit, the effect of quisqualate is not affected by MPEP. Although these data indicate that the presence of a single MPEP site per dimer is not sufficient to allow this inverse agonist to inhibit receptor activity, it is important to know whether or not MPEP can bind in such a site and inhibit activation of this subunit.

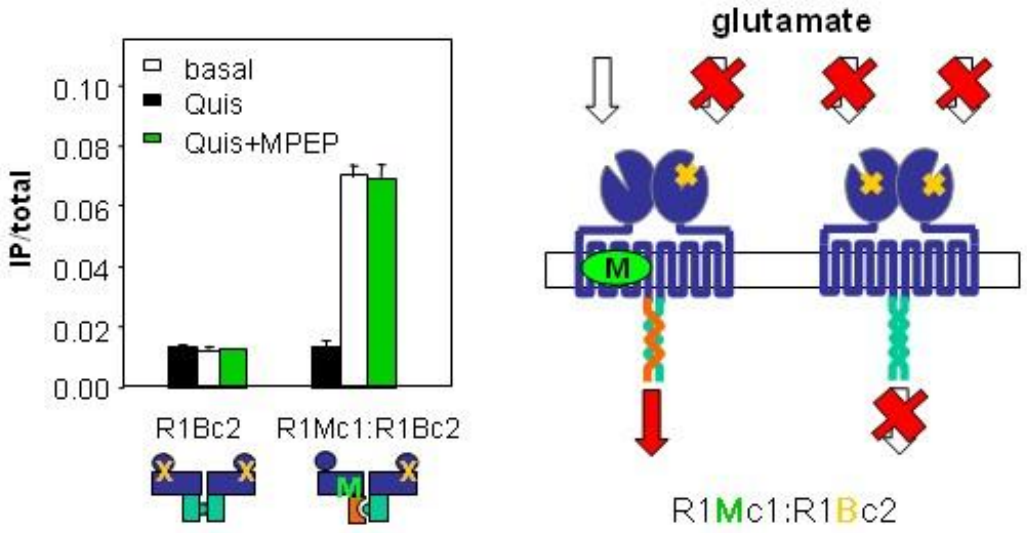


Fig. 40: MPEP does not inhibit activity of receptor dimers containing a single MPEP site
(left) IP production measured under basal condition (black columns), in the presence of quisqualate alone (open columns) or in the presence of quisqualate and MPEP (green columns) in cells expressing indicated subunits. Data are means ± S.E.M. of triplicate determinations from a representative experiment out of five. (right) Schematic representation of the experiment confirming that two MPEP molecules are required for inhibition of the dimer activity.

In summary, these data suggest that one molecule of MPEP is not sufficient to fully block the receptor activation.

One molecule of positive AM is sufficient for maximal potentiation of receptor activity

If two molecules of non-competitive antagonist are required for full inhibition of the receptor activity, we were further interested in whether also two molecules of positive AM are required for maximal potentiation of the receptor activity. In cells co-expressing R5Roc1 and R5c2, both Ro01-6128 and DFB significantly potentiated quisqualate-induced receptor activity (Suppl. II., Fig. 2). As indicated, only the R5Roc1 of the R5Roc1:R5c2 heterodimer bears the Ro-binding site. Thus, the potentiation observed comes only from the action on this single subunit and is similar to potentiation observed with R5Ro homodimer, where two binding sites for Ro01-6128 per dimer exist (Suppl. II., Fig. 2). These data suggest that a single positive AM is sufficient for the full enhancement of agonist action on the dimer.

To further confirm these observations, chimeric R1c1:R5c2 and R5c1:R1c2 heterodimers were generated, in which each subunit can be targeted by a specific positive AM. In this case, according to previous observations indicating that mGlu1 and

mGlu5 receptors do not form heterodimers, it was necessary to examine the correct pairing of indicated receptors. ELISA and TR-FRET experiments were used to achieve that these receptors are targeted to the cell surface and form heterodimers (Suppl. II, Fig. 3). Both modulators efficiently increased the agonist potency, which is expressed by decreased EC_{50}. In cells expressing R1c1:R5c2 heterodimers, potentiation by Ro01-6128 can only result from the effect of this modulator. Similarly, effect of DFB on cells expressing R5c1:R5c2 can only result from the enhancement of the agonist potency by a single molecule of positive AM acting on a single subunit within the heterodimer. Moreover, when both modulators were added simultaneously, no significant further enhancement of the agonist potency was observed (Suppl. II, Fig. 3).

In summary, these data suggest, that a single molecule of positive AM is sufficient to fully enhance the receptor dimer activity.

2.1.4. One heptahelical domain is turned on at a time during the signal transduction through metabotropic glutamate receptors

The effect of intracellular loop mutation (F781P) indicated that both HDs in a receptor dimer can potentially activate G-proteins (**Fig. 32**). The above results suggest that stabilizing a single HD in an inactive state with an inverse agonist (MPEP) has no effect on coupling efficacy of a receptor dimer (**Fig. 39**). Taken together, these data suggest that stabilizing one HD in its inactive state favors coupling by the associated subunit. Furthermore, the fact that potentiation of the receptor activity can be caused by a single molecule of positive AM supports the suggestion that one HD is turned on at a time.

MPEP enhances agonist activity when interacting with the subunit unable to activate G-proteins

To directly test the possibility of the allosteric interaction of both subunits, we examined the effect of MPEP on a receptor dimer in which one HD is wild-type, and the other possesses the MPEP site and is impaired in its ability to activate G-proteins. If the above proposal is correct, then MPEP binding in one subunit should favor receptor activity mediated exclusively by the second subunit. We therefore co-expressed R1c1 that contains a wild-type HD with R1MXc2 that has both an MPEP site and a mutation in the i3 loop (**Fig. 41**). In this case, only the R1c1:R1MXc2 combination is functional,

since the other receptor combination reaching the cell surface, R1MXc2 homodimer is not functional. As expected according to the above proposal, MPEP was found to enhance the effect of quisqualate in a dose dependent manner (**Fig. 41**). This further suggests that preventing the HD of the R1MXc2 subunit to reach its active state facilitates G-protein activation by the associated subunit.

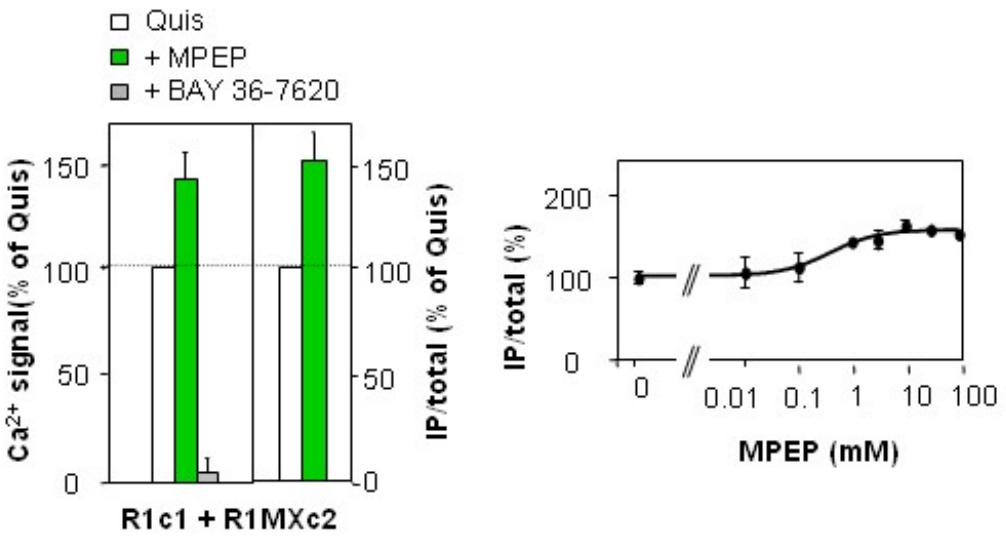


Fig. 41: MPEP potentiates quisqualate-induced response when bound into non-functional subunit
(left) The Ca^{2+}-signal (left panel) and IP production (right panel) induced by quisqualate (10 μM) alone (open columns) or together with MPEP (100 μM) (green columns) or BAY 36-7620 (10 μM) (grey columns) were measured in cells expressing R1c1 and R1MXc2 subunits. Values are expressed as a percentage of the maximal quisqualate effect and are means ± S.E.M. of 3-7 independent experiments performed in triplicate. (right) Effect of increasing concentrations of MPEP on IP production induced by quisqualate in cells expressing the indicated subunits. Values are expressed as a percentage of the quisqualate-induced response in the absence of MPEP and are means ± S.E.M from three independent experiments performed in triplicate.

Ro01-6128 suppresses agonist activity when interacting with the subunit unable to activate G-proteins

Because one positive AM is sufficient to positively modulate the activation of mGlu dimers, similarly to above experimets, the effect of positive AMs on receptor dimers, in which one subunit is impaired in G-protein coupling was examined. Co-expression of one subunit with wild-type HD and second Ro-sensitive subunit unable to activate G-proteins leads to formation of functional heterodimeric R5c1:R5RoXc2 receptor. As shown in Suppl. II, Fig. 4, the mGlu1 positive AM Ro01-6128 acting in the non-functional R5RoXc2 subunit inhibits the maximal receptor activity, whereas the mGlu5 positive AM DFB enhances agonist activity at this receptor, since it can bind into

functional R5c1 subunit. Thus the mGlu1 positive AM bound in the HD unable to activate G-proteins acts as a non-competitive antagonist rather than a positive modulator.

In summary, we examined whether one or both subunits in a dimeric mGlu receptor are turned on during receptor activation.

We have demonstrated that either of two subunits in a homodimeric receptor can potentially activate G-proteins but that only one of them is activated at the moment of signal transduction and hence activates coupled G-protein.

2.2. Determination of the movement of transmembrane helices

In last few years, light resonance energy transfer techniques have been commonly applied to detect protein-protein interactions in intact living cells. These methods require fusion of cDNAs between a protein and bioluminescent (luciferase) and/or fluorescent agents. FRET and BRET methods were used successfully for determination of protein-protein interactions of either receptors or intracellular proteins (57, 75, 205, 308-310).

The most commonly used method taking advantage of either fluorescent proteins or fluorescently labeled antibodies (as described above) is FRET. Initial studies using C-terminaly tagged receptors with either YFP or CFP confirmed oligomerization/dimerization of receptor for neuropeptide Y (311), thyrotropin receptors (79), dopamine D2 receptor (312). This approach was also used for detection of G-protein subunit rearrangements under the activation or for determination of rate constants of GPCR activation (313, 314).

Recently FRET was used for determination of ligand-induced rearrangement of the dimeric mGlu 1 receptor (**Fig. 19**) (236). The FRET was measured in receptor that carried CFP in i1 (i2) loop of one subunit and YFP in i2 (i1) loop of the other. All receptors were able to bind [^{3}H]-quisqualate. Upon ligand binding, the inter-subunit FRET efficiency between the second loops increased, whereas that between first loops decreased, suggesting movement of both i2 loops towards each other and on contrary shifting of the i1 loops away from each other. In contrast, intra-subunit FRET did not change clearly. Interestingly, except dimer carrying CFP or YFP at the C-tails, none of

the constructs was able to trigger mGluR1-mediated Ca^{2+} response, simply because the β-barrel fused with one of the intracellular loop responsible for coupling and activation of G-protein prevent G-protein coupling. The receptors of family 3 GPCRs have commonly very short intracellular loops and engineering of mutant receptors is therefore very difficult. Second, the measuring of the intra-subunit FRET efficiency did not bring clear data. It is not surprising if we take account the fact that the receptor dimer carrying four β-barrels of FPs can undergo strong steric hindrance, although the FRET ratio between CFP and YFP was quite high, probably due to spatial proximity of FPs (**Fig. 19**). Moreover, the authors did not show the expression of each mutant subunit and thus the presence of the CFP and YFP "homodimers" on the cell surface and the possible proximity of both "homodimers" cannot be omitted.

The problem of the size or steric hindrance can be solved by using small organic fluorophores such as fluorescein, rhodamine (17, 38) and europium cryptate (57). These can be fused to studied proteins or antibodies. Recently Tsien's group developed the biarsenical compounds FlAsh and ReAsh (4′,5′-bis(1,3,-dithioarsolan-2-yl)resorufin) (**Fig. 25**), that bind with high affinity to tetracystein motives (CCXXCC). Thus proteins that include this motif can eradiate green or red light depending on used compound (315). Combination of both compounds FlAsh and ReAsh have been used to determine mechanism by which connexin 43 subunit of gap junction channels is added and removed from gap junction plaques or for studing of AMPA receptors trafficking and activity-dependent regulation of their dendritic synthesis (316, 317). Finally, recent study of Hoffman et al. (96) using FRET between FlAsh covalently bound to i3 loop of adenosine A_2 receptor (class A GPCRs) and CFP fused with its C-terminus upon agonist stimulation determined also kinetic constant of tens of miliseconds required for the receptor activation. Different receptors will probably excert different rate constats depending on mechanism of activation of the receptor, concentration of an agonist and its properties and presence of coactivators. It was previously proposed, that α_{2A}-AR from class A GPCRs is activated faster (tens of miliseconds) than receptor for parathyroid hormone (within seconds) from class B GPCRs (314). How fast is the activation of class C receptors is not recently known.

According to complications coming from fusion of two green fluorescent protein (GFP)-like proteins into intracellular parts of a receptor, we proposed to measure FRET between FlAsH bound to tetracystein tag ingeneered in either of the two first

intracellular loops and fluorescent protein (CFP) fused with C-terminus. Thus we could avoid strong steric hindrance and probably could work with a functional protein because as was reported previously, fusion of fluorescent proteins with a C-terminus of $GABA_{B1}$ or $GABA_{B2}$ did not impair receptor activation and following receptor downregulation (318).

Moreover, our data brought a clear evidence for a single subunit being active at a time. Thus, we can engineer fusion mutants of specific fluorophore with one of the intracellular loops and coexpress it with another subunit bearing different fluorescent tag or mutations that specifically alter receptor activation, using quality control system of $GABA_B$ receptor as previously used and enables us to measure FRET between two well defined subunits. The FRET was proposed to be measured between donor (CFP) fused with C-terminus of either subunit (with or without FlAsH-binding sequence) and acceptor (FlAsH) fluorophore as shown in **Fig. 42**. Then the one functional subunit would be sufficient to couple and activate G-proteins and we could determine whether both HDs undergo structural rearrengement upon receptor activation or if only the active HD changes its conformation. Alternatively, of interest is answer the question of how does the rearrengement of active *vs.* inactive HD change under distinct pharmacological influence, e.g. application of negative or positive allosteric modulator. We believe that using this approach we will be able to determine a single subunit movement under allosteric modulation and to more precisely specify molecular aspects of *cis* and *trans* activation. This additional project also deals with activation of mGluRs and therefore it becomes a part of this thesis. Following text present some preliminary data.

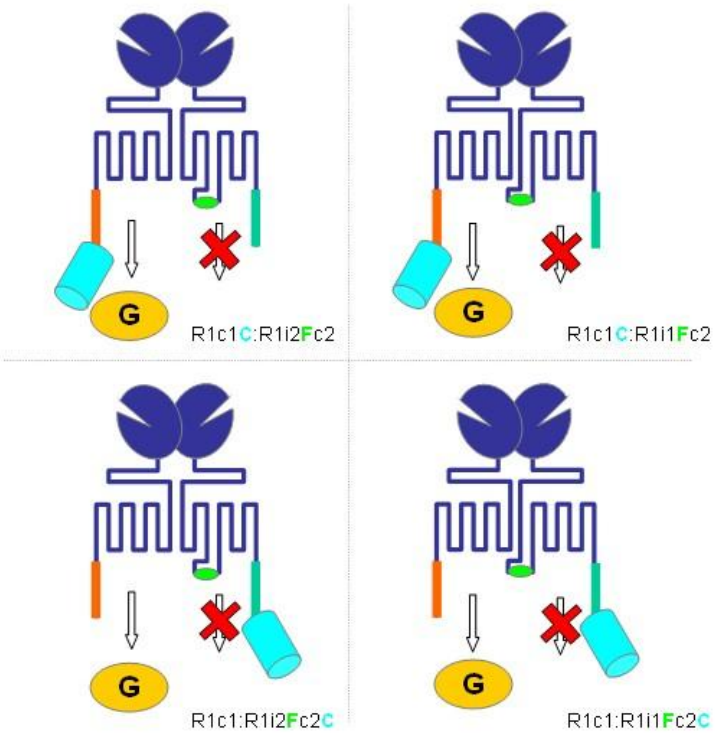


Fig. 42: Proposal of the experiments for disclosure of the movement of transmembrane helices in the homodimeric mGlu1 receptor (R1)
FRET will be measured in cells co-expressing indicated chimeric dimers (R1c1:R1c2) with introduced fluorophores, either CFP (C) or FlAsH binding site (F) in either basal conditions or upon glutamate stimulation. G: G-protein.

First, we have introduced 6- to 12-amino acid FlAsH binding motifs in either i1 or i2 loop of mGluR1 (**Fig. 43**) bearing also the C-terminus of $GABA_{B2}$ subunit (R1i1Fc2 or R1i2Fc2). Thus these mutants should be expressed on the cell surface on their own and this was also confirmed by ELISA assay on intact HEK 293 cells transfected with HA-R1c1 and R1i2Fc2 (R1i1Fc2) receptors using anti-HA-HRP antibody (data not shown). The co-expression of both receptors enables heterodimer to reach the cell surface. None of the R1i2Fc2 or R1i1Fc2 constructs was functional when expressed alone, otherwise co-expressed with R1c1 subunit (**Fig. 44**). Finally, all R1i2Fc2 homodimers were efficiently labeled with FlAsH-EDT_2 (**Fig. 45**) as determined by confocal microscopy.

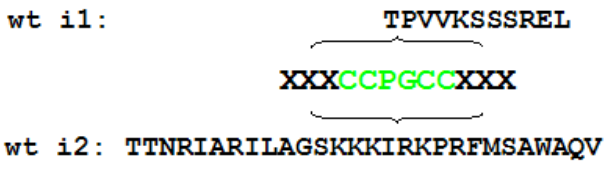
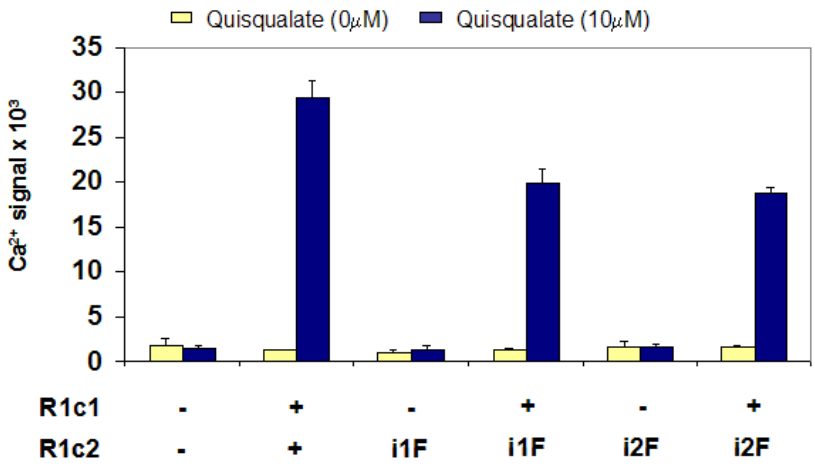


Fig. 43: Scheme of introduced tetracystein tag into either i1 or i2 loop of wild-type (wt) mGlu1 receptor generating R1i1Fc2 and R1i2Fc2, respectively

Fig. 44: A single functional subunit rescues activity of a receptor dimer

Functional analysis of mutated mGlu1 receptors as determined by calcium mobilization assay. Values are means of triplicate of one representative experiment out of three. ▶



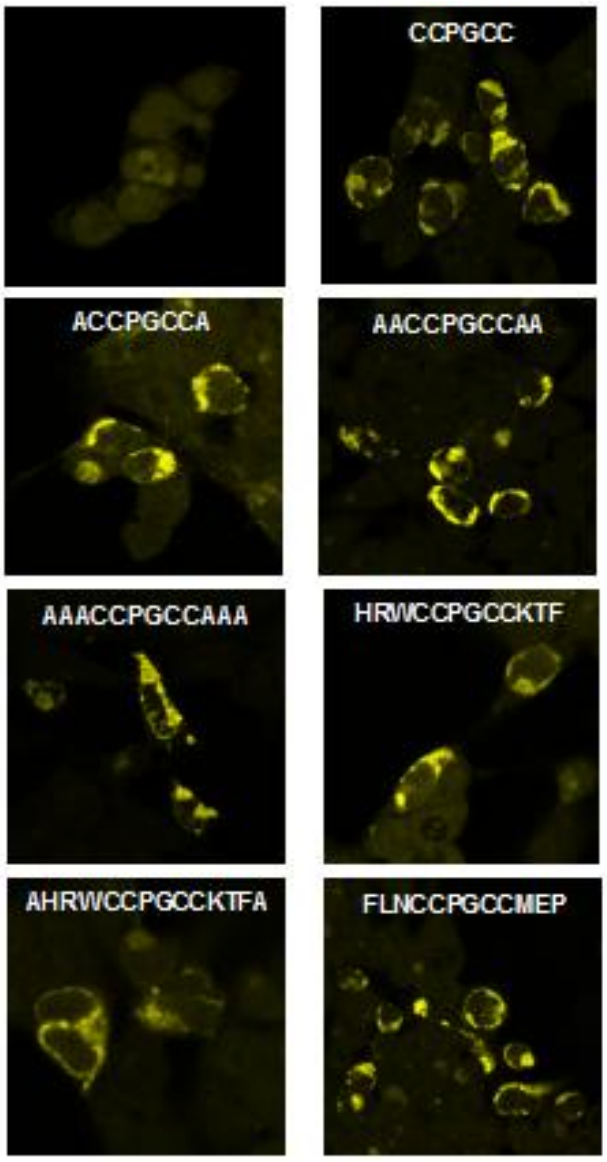


Fig. 45: Confocal microscopy images of wild-type R1c2 receptor and seven R1i2Fc2 receptor constructs with different tetra-cystein tags (as indicated) transiently expressed in HEK 293 cells and labeled with FlAsH

In summary, preliminary data confirm that tetra-cystein tag introduced to either i1 or i2 loop of mGluR1 prevent G-protein activation but not the receptor expression, nor activation when co-expressed with wild-type subunit.

IV. Discussion and perspectives

For many years, it was thought that GPCRs exist in monomeric form, one receptor molecule being activated by a single ligand and activating one heterotrimeric G-protein. Nowadays, a growing number of studies have revealed that GPCRs can form dimers and oligomers, however why and how they function in a dimeric form is not clear (3). Some authors propose that dimerization is important for receptor trafficking, downregulation and internalization (27, 61), others report that subunit composition can influence pharmacological properties of the receptor as well as coupling to intracellular pathways (25, 30, 62). Both possibilities, the agonist-induced dimerization and the formation of constitutive dimers inside of the cells were reported (19, 21, 30, 71). The most probable explanation for existence of constitutive dimers is that two subunits are essential for proper receptor activation like in case of $GABA_B$ receptor.

Among the class C of GPCRs, in contrast to $GABA_B$ receptor, CaSR and mGluRs form constitutive homodimers. Why two identical subunits are needed in these receptors? Do both ECDs bind a ligand in a time? Do both HDs activate G-protein simultaneously or does each of them stimulate one G-protein? As shown previously, ECD of mGluRs closes under glutamate binding and within a dimer, this receptor can adopt Roo, Aco or Acc conformation (103). Both subunits in a homodimer are identical and thus capable of ligand binding. Interestingly, by measuring of tryptophan fluorescence, negative cooperativity in glutamate binding was observed in mGluRs (233). The same has been found for homodimeric glycoprotein hormone receptors (319). Recently, it has been shown that two agonists per dimer are required for full activation of homodimeric mGluRs (57, 298). Hence, it is needed for mGluRs to adopt the Acc conformation to become fully active (57).

How does the finding of requirement of two agonist-molecules per dimer fit with the negative cooperativity observed in mGluRs? The negative cooperativity might serve for extending of concentration range over which can protein work and could simply broaden sensitivity of the receptor. Thus mGluRs can function only in presence of very high concentration of glutamate after e.g. continuous release of neurotransmitter from presynaptic terminal or in presence of co-activator molecule. Negative cooperativity can also serve as a control loop for fast receptor internalization in presence of low concentration of glutamate, preventing loss of cellular energy used for proteosynthesis and protein trafficking. Taken together, allosteric interaction between the two ECDs in mGlu receptors markedly influence the receptor activity. Whether the alosterism can be transferred onto a dimer of HDs in a homodimeric receptor is not recently known.

In our study we studied whether one or both HDs in a dimeric mGlu1 receptor are turned on during receptor activation. To that aim we used a quality control system of the $GABA_B$ receptor to control the formation of dimeric mGlu1 receptors composed of wild-type or differentially mutated subunits. Then we examined the effect of the non-competitive mGlu5 antagonist MPEP on mGlu1 receptor combinations in which a single subunit is made sensitive to MPEP. Alternatively, the effect of mGluR1 non-competitive potentiator Ro01-6128 on mGlu5 receptor chimeras sensitive to this compound was examined.

Either HDs in homodimeric mGlu receptors can activate G-proteins

In $GABA_B$ receptor, a single HD, that of $GABA_{B2}$, is capable of activating G-proteins. Mutation of a single amino acid within the i3 loop of $GABA_{B2}$ generates dimer that cannot activate G-proteins. In contrast, mutation in corresponding sequence within $GABA_{B1}$ subunit does not impair G-protein activation (320). We show that mGlu1 receptor dimers in which one HD is impaired in its ability to activate G-proteins are still able to activate PLC. This illustrates, that a single functional HD of a homodimeric receptor is sufficient for formation of functional complex. Similarly, dimer composed of TSH (thyrotropin release stimulating hormone) receptor unable to bind TSH and of TSH receptor impaired in G-protein activation is still able to activate G-proteins (319). The same was found when LH (luteinizing hormone) receptors with impaired ligand binding and cAMP formation respectively, were co-expressed (68).

We have found out that although mGluR dimers (R1c1:R1Xc2) with one subunit mutated in i3 loop are functional, the maximal response observed with such a receptor has decreased about 60%. We have quantified the proportion of the homodimeric and heterodimeric receptors at the cell surface and loss of the overall activity caused by incapability of homodimer that is unable to activate G-proteins should be less than 30%. We suggest that R1c1:R1Xc2 heterodimer is less efficient in activating G-proteins than the control heterodimer R1c1:R1c2. The same reduction in the receptor activity was observed for chimeric $GABA_B$ receptor ($GABA_{B1/2}$ and $GABA_{B2}$x) in which HD of $GABA_{B1}$ was replaced by HD of $GABA_{B2}$ and $GABA_{B2}$ subunit was unable to activate G-proteins (131). The similar behaviour has been observed for the receptor in which one subunit has not been able to bind glutamate (R5B) in R5c1:R5Bc2 receptor (57). This indicates that either HD is capable to activate G-protein in mGlu dimers. Whether

both HD are activated at a time or only a single subunit is responsible for the activation of G-proteins has to be further determined.

Blocking one HD in its inactive state with an inverse agonist does not impair receptor coupling

As reported previously, the simultaneous introduction of 3 point mutations (one in TMIII, and two in TMVII) in mGlu1 is sufficient to make it sensitive to the mGlu5 selective inverse agonist MPEP. Such mutations did not impair the sensitivity of the receptor to the mGlu1 selective inverse agonist BAY36-7620. Of interest, if a single subunit within the dimer possesses such a site, no effect of MPEP was observed. However, the receptor was fully antagonized by BAY36-7620 that can bind in both subunits of the dimer. The absence of effect of MPEP is unlikely due to the inability of MPEP to act in a dimeric receptor possessing a single MPEP site. Indeed, MPEP fully blocks a receptor combination in which one subunit is sensitive to MPEP and the other is impaired in its ability to couple to G-protein (R1Mc1:R1Xc2 combination). Taken together, these data show that binding of an inverse agonist in one HD within a dimer does not impaired G-protein coupling efficacy of the dimer.

Either one HD in a dimeric mGlu1 receptor is activated at a time.

The effect of the mutation in the i3 loop preventing G-protein coupling and the MPEP action seems to be different. If we accept that mutation in i3 loop impairs only G-protein activation but not the ability of this HD to reach its active state, then our data shed new light on how dimeric receptors are activated (**Fig. 46A-G**). According to the model of a single HD being turned on at a time, upon activation of the receptor, 50% of dimers are active due to the active conformation of one HD (white) and the second half of dimers due to the active conformation of the other HD (black) (**Fig. 46A**). If the HD is impaired in its ability to couple to G-proteins, then only 50% of the dimers are active at a time, because the other 50% have the active subunit that cannot activate G-proteins (**Fig. 46B**). In contrast, when one of the subunits (black) is blocked in its inactive state by negative allosteric modulator (M), then activation of the dimeric ECDs have no other possibility than to activate the other HD (white), leading to 100% active dimers at the cell surface (**Fig. 46C**). According to this model, one molecule of MPEP does not affect the receptor activity. Then the only way how to inhibit receptor activation is to

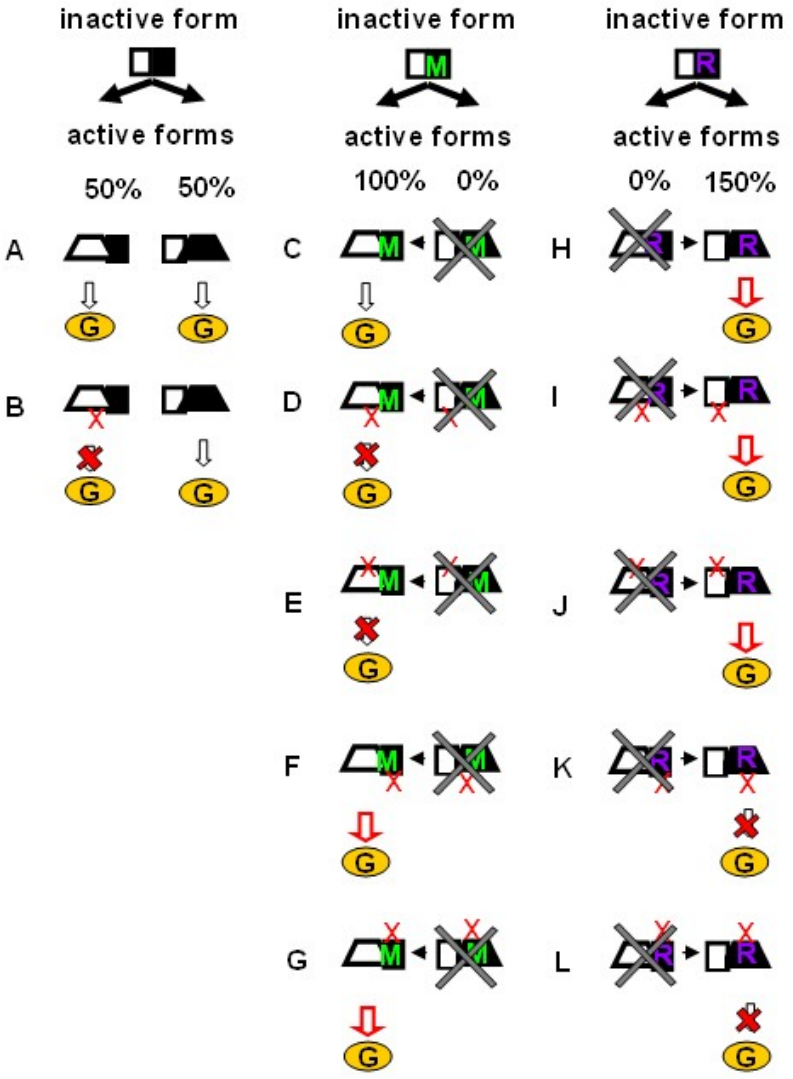
introduce mutation preventing either ligand binding or G-protein coupling which is also in agreement with our data (**Fig. 46D and E**).

Finally, our proposal also explains why MPEP enhances agonist-induced activity in R1c1:R1MXc2 dimer. In such a receptor, only one HD has the ability to reach the active state and activate a G-protein. Addition of MPEP preventing the impaired subunit to reach the active state causes enhancement of the R1c1 action allowing more R1c1 (white) subunits to reach their active state (**Fig. 46F**). The same was truth in case of R1c1:R1BMc2 receptor (Suppl. I, Fig. 9, **Fig. 46G**).

These conclusions were further confirmed by another study using positive AMs..Herein, positive AM for mGluR1, Ro01-6128, on mGlu5 receptor chimeras in which either only a single subunit or both subunits were sensitive to Ro01-6128. In contrast to requirement of two molecules of negative AM for full inhibition of the receptor activity, we have shown, that one molecule of positive AM has been sufficient for full enhancement. It has been further confirmed by the application of positive AMs for both mGluR1 (Ro01-6128) and mGluR5 (DFB) on cells expressing R1:R5 heterodimers. In the presence of both modulators the potentiation did not further increase compared to the one obtained with the most efficient modulator applied alone. These data nicely fit with what was proposed above. Because, if positive AMs stabilize the active conformation of HD (205, 321), then the positive modulator pushes more HDs to reach their active states (**Fig. 46H-L**) and thus a single molecule per dimer is sufficient for full enhancement effect. In the R1:R5 dimers, we expect maximal enhancement after application of either Ro01-6128 or DFB that stabilizes active conformation of R1 or R5 subunit, respectively.

What would happen, if the positive AM binds into HD which is impaired in its ability to activate G-proteins or to bind a ligand? The model of a single HD being turned on at a time proposes non-competitive action of positive allosteric modulator suggesting stabilization of Ro-sensitive HDs in all receptors, although they cannot activate G-proteins or bind a ligand (**Fig. 46K and L**). Similarly, the same principle can be applied on negative AM acting as an enhancer (discussed above).

Fig. 46: Proposed model : one HD being turned on at a time



Effect of Quis	Effect of MPEP	Effect of Ro01-6128
A Full activity	C Full activity	H Enhancement
B Partial activity	D Full inhibition	I Enhancement
	E Full inhibition	J Enhancement
	F Enhancement	K Full inhibition
	G Enhancement	L Full inhibition

*The receptor is represented as a dimer of HDs, one in white, the other in black. The inactive conformation of the HD is represented by a rectangle, whereas the active form is represented by a trapezoid. The presence of the mutations that prevent G-protein activation or agonist binding are indicated by **X**. The presence of an MPEP site is indicated by **M** (C-F). The presence of an Ro01-6128 site is indicated by **R** (G-J). The expected effect of negative and positive modulators, respectively, according to our model proposing that only one HD can reach the active state at a time, are indicated in Table below the illustrations. 100% activity is represented by a black open arrow, enhancement by a red open arrow and inhibition by a red cross. A: control condition, with either HD being activated. B: one HD is mutated in its i3 loop such that only 50% of the dimers activate the G-protein. C: MPEP is supposed to prevent the black HD from reaching the active state, such that all receptors will turn on their white HD, thereby leading to no effect of MPEP. D: MPEP is able to fully block receptor activation when bound to the only active subunit. E: Same like in D. F: By preventing the black HD from reaching its active state, MPEP increases the probability that the white HD will reach its active state, thus leading to a enhancement of the agonist effect. G: same like in F. H: Ro01-6128 stabilizes active conformation of the black subunits thus leading to enhancement of the receptor activity. I, J: same like in G. K: Ro01-6128 stabilizes active conformation of the black subunits which is unable to activate G-proteins result in inhibition of the receptor activity. L: same like in K.n.e. – no effect, G – G-protein.*

Taken together, our data strongly support the asymmetric functioning of the dimer of HDs in homodimeric mGlu receptors (**Fig. 47**). This is quite surprising especially if we take into account that full activity of the receptor depends on closing of both ECDs (57), thus working in a symmetric way (**Fig. 47**).

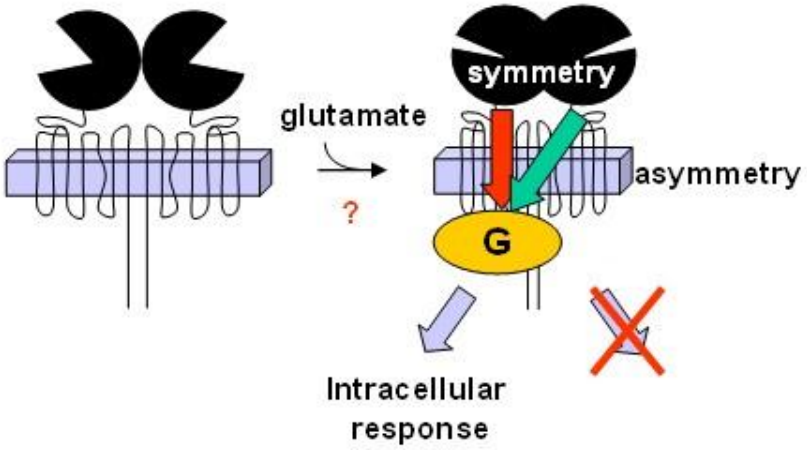


Fig. 47: Mechanism of activation of the mGlu receptor
Upon activation, binding of the two molecules of glutamate into intralobal binding site, both subunits get closer to each other and change in conformation proceeds within whole dimer. In contrast to symmetrical action of ECDs, only a single subunit, turned on at a time, activates a G-protein (G) and triggers signaling cascade leading to appropriate intracellular response. Which HD will be turned on depends probably on the position of a G-protein.

Why is the activated dimer of HDs not symmetric?

In the $GABA_B$ receptor allosteric interactions between two distinct subunits are crucial for the proper function of the receptor. The different role of each subunit in terms of the receptor activation and G-protein coupling arises from the natural heterodimeric composition of the $GABA_B$ receptor. Although ECD of $GABA_{B2}$ subunit binds neither GABA nor any other known natural ligand, its presence improves binding of agonists within $GABA_{B1}$ subunit. On the other hand, although $GABA_{B1}$ is not able to activate G-proteins, it is necessary for activation of G-protein by the $GABA_{B2}$ subunit (67). But which mechanism is responsible for asymmetric functioning of a homodimeric receptor?

It was reported that a single heterotrimeric G-protein interacts with a GPCR dimer (90). Because the surface interface of the receptor dimer and a G-protein is similar in its size, it is likely that one HD will interact with α-subunit, whereas the second HD will interact with $\beta\gamma$ complex (23, 60). It may well be, that such an asymmetric activation of HDs in a dimer is directed by asymmetry of heterotrimeric G-

proteins (**Fig. 48**) on one side and by negative cooperativity of the ligand binding on the other.

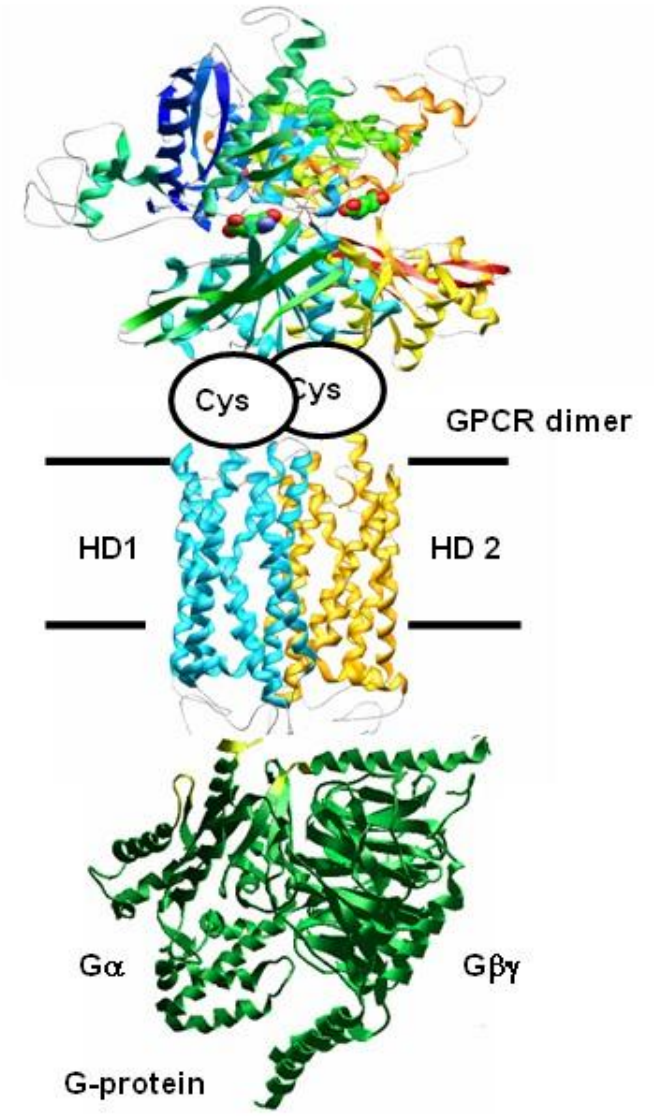


Fig. 48: Schematic representation of association of GPCR dimer and heterotrimeric metabotropic glutamate receptor

Some receptors display certain promiscuity in G-protein activation and it is unlikely that they activate various G-proteins simoultaneously. The activation of distinct intracellular cascades could be influenced by concentration of agonist, presence of co-activators and specific interaction with either intracellular or membrane proteins. Protein-protein interactions could be also part of effector functions of receptors. For example mGluR1 that signals mainly through IP_3-Ca^{2+} intracellular cascade is involved in modulation of calcium influx into cerebellar Purkinje cells through direct interaction with P/Q-type Ca^{2+}-channels. Depending on timing of the activation of mGluR1, P/Q-type Ca^{2+} channels can be either upregulated or inhibited (18).

Why a receptor dimer for G-protein activation?

In rhodopsin-like family, rhodopsin was firstly identified as a monomer but it has been shown recently that this receptor also forms dimers and higher-order oligomers (22, 23, 322). G_t-protein (transducin) can be activated by a single subunit although

dimer activates transducin more efficiently (24). The fact that a single rhodopsin is able to activate transducin is not in contrast with our conclusions.

Heterodimerization of rhodopsin-like receptors, e.g. opioid receptors, appears to be important for many functions, such as potentiation of signal transduction (26, 62), enhancing of the binding and signaling activity of the adjacent subunit (323) and induction or prevention of the receptor desensitization (28, 61). Our proposal of a single HD activated in a time could be applied for all cases described in these studies. The positive synergistic effect of μ- and δ-opioid ligands applied on cells expressing μ:δ opioid receptor heterodimer can result from activation of both receptor subunits (26). But it can also come from a crosstalk between the signaling pathways triggered with μ- or δ-receptor homodimers, since there is not clearly determined proportion of heterodimers *vs.* homodimers and monomers.

In family 3 GPCRs, several receptors form heterodimers are composed of two distinct subunits. In $GABA_B$ receptor, sweet and umami taste receptors, only one HD appears to play an important role in activation of G-proteins (44, 67, 131, 320). Similarly, our data indicate, that only one HD in a homodimer activates a G-protein. Recent data indicating, that the two subunits of the yeast pheromone alpha-factor receptor are activated independently by an agonist but function in cooperation with one another to activate G-proteins (324) are in agreement with our model. Recent studies with receptors from family 1 GPCRs also support the theory of a single HD being capable of G-protein activation (68, 69, 319).

If one subunit is able to activate a G-protein, what is the physiological consequence for existence of dimers? It was proved many times, that dimerization is required for G-protein activation. The relative change in conformation of both ECDs (103, 199) leads to a change in relative position of HDs (236) and enables their activation. Moreover, two agonists per mGlu receptor dimer are required for reaching the full active state of the receptor (57), suggesting the necessity of the two subunits being present in the dimer. Similarly, the negative cooperativity that could be an important controlling mechanism requires two subunits.

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Supplement I

Supplement II

Other supplements