Chapter 6
Activation Mechanism and Allosteric Properties of the $GABA_B$ Receptor

Julie Kniazeff, Xavier Rovira, Philippe Rondard, and Jean-Philippe Pin

Abstract The $GABA_B$ receptor is quite original within the large G protein-coupled receptor (GPCR) family. When first identified at the molecular level, it was the only GPCR to require two subunits to form a functional receptor, composed of $GABA_{B1}$ and $GABA_{B2}$. Although part of the mandatory dimeric class C group of GPCRs that also includes the receptors activated by glutamate, calcium, the sweet and umami taste compounds, the $GABA_B$ is unique in that it lacks an essential element, the cysteine-rich domain that interconnects the ligand binding domain to the heptahelical transmembrane domain (7TM) responsible for G protein activation. Here, we will summarize our actual knowledge on the structure, stoichiometry, allosteric properties, and activation mechanism. These reveal some similarities and major differences with the other class C GPCRs and highlight novel possibilities to develop approaches to regulate its activity.

Keywords G protein-coupled receptor • Activation mechanism • Structure • Allostery • Dimer

6.1 Introduction

Since their discovery in the mid 80s as the molecular target of the anti-spasticity drug baclofen, the $GABA_B$ receptors raised much interest with a first goal to elucidate their molecular bases. Based on pharmacological studies, and as already observed for G protein-coupled receptors (GPCRs) activated by other neurotransmitters, several $GABA_B$ receptor genes were expected (Bonanno et al. 1997; Deisz et al. 1997; Zhang et al. 1997). A first clone was identified in 1997 by the Bettler lab (Kaupmann et al. 1997). Although it was found to display the expected pharmacological and brain localization profiles for a $GABA_B$ receptor, agonist affinity was

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lower than expected, and no functional response could be measured in recombinant assays. However, this first clone already revealed a general organization similar to class C GPCRs, with a large extracellular venus flytrap-like domain (VFT) similar to the binding domain of metabotropic glutamate (mGlu) receptors, and where the GABA binding site was rapidly identified (Galvez et al. 1999). However, in contrast to the other class C GPCRs like the mGlu receptors, the VFT was directly linked to the 7TM, such that the cysteine-rich domain (CRD) found in other class C receptors is missing (Kaupmann et al. 1997). This first gene encodes two variants $GABA_{B1a}$ and $GABA_{B1b}$, thanks to an alternative initiation site, adding two sushi domains (SDs) at the N-terminus of the $GABA_{B1a}$ variants (Kaupmann et al. 1997). However, both subunits displayed the same pharmacological properties. Only about 2 years later, a second subunit $GABA_{B2}$ was independently identified by three groups, which was structurally homologous to $GABA_{B1}$ and was absolutely required for agonist high affinity, and for proper coupling to G proteins (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998). $GABA_{B2}$ was also found to be essential for the proper membrane insertion of the $GABA_B$ receptor. Indeed, when expressed alone, $GABA_{B1}$ remains intracellularly retained between endoplasmic reticulum and Golgi because of an intracellular retention signal in its C-terminal tail (Margeta-Mitrovic et al. 2000; Pagano et al. 2001). Only when interacting with $GABA_{B2}$, the retention signal is masked and the heterodimer reaches the cell surface making this receptor unique among all GPCRs known at that time, being the first mandatory heterodimeric GPCR. In addition, it was early demonstrated that $GABA_{B1}$ was responsible for agonist binding while $GABA_{B2}$ was critical for G protein activation (Galvez et al. 2001; Margeta-Mitrovic et al. 2001a, b). These findings were a major breakthrough not only in the $GABA_B$ receptor field, but also in the large GPCR community, where the notion of GPCR dimerization was still the subject of intense debate. Since then, and despite a number of mammalian genomes sequences, no additional $GABA_B$ receptor subunits were identified, making the $GABA_{B1}$-$GABA_{B2}$ heterodimer the only known $GABA_B$ receptor.

As such, the $GABA_B$ receptor is unique in its general structural organization, showing differences with the other class C receptors, and being the first heterodimeric GPCR. This obviously stimulated much effort to elucidate its activation mechanism and allosteric properties, not only to identify novel possibilities to develop drugs to modulate its activity with potential therapeutic application, but also as a model of GPCR heterodimers, with the hope to better understand the possible roles of the still questioned class A rhodopsin-like GPCR heterodimers.

In this chapter, we will report on our actual knowledge of the structure of the various domains of the $GABA_B$ receptor, and how such a multidomain membrane protein is activated by a small ligand to control G protein activity. We will see that despite some similarities with the other class C GPCRs, a different activation mechanism is observed, and we will show how allosteric transitions between the different domains control receptor activity. Such understanding certainly reveals new ideas on how to develop innovative drugs to control this important brain receptor and sheds light on the possible assembly and allosteric interactions between other GPCRs.

6.2 Structure and Organization of the $GABA_B$ Receptor

Both subunits of the $GABA_B$ receptor, $GABA_{B1}$ and $GABA_{B2}$, belong to class C GPCRs together with the mGlu receptors, the calcium-sensing receptor, the sweet and umami taste receptors, and the basic amino acids GPRC6A receptor (Kniazeff et al. 2011). All these proteins share both sequence and structural homology. They are composed of a transmembrane domain (7TM) made of 7 alpha helices (about 260 residues) and of a large and well-structured extracellular domain referred to as the VFT domain (about 410 residues) (Rondard et al. 2011) (see also Chap. 4 of this book) (Fig. 6.1a). Compared to other class C GPCR, $GABA_B$ receptor subunits have the particularity to lack the CRD connecting the VFT to the 7TM that is replaced by a shorter linker (10–15 amino acids) of unknown structure. The intracellular C-terminal tail of $GABA_{B1}$ and $GABA_{B2}$ is rather long (107 and 200 residues, respectively) and contains a well-structured coiled-coil domain that is important for heterodimerization and to guarantee the correct assembly of the heterodimer before proper targeting to the plasma membrane (Margeta-Mitrovic et al. 2000; Pagano et al. 2001; Kammerer et al. 1999).

The VFT domains in class C GPCRs are known to bear the agonist binding site (Okamoto et al. 1998). They share some structural homology with bacterial periplasmic amino acid binding proteins such as the leucine/isoleucine/valine binding protein (LIVBP) (O'Hara et al. 1993). A general folding of these domains was first proposed based on homology modeling and was later confirmed by the structure resolution of VFTs from different mGlu receptors and more recently from both $GABA_B$ receptor subunits in the presence or not of various ligands, agonists, and antagonists (Geng et al. 2012, 2013; Kunishima et al. 2000; Muto et al. 2007; Tsuchiya et al. 2002). The domain is about 70 Å long and 35 Å wide. Each VFT is composed of two opposite lobes linked by three short loops, with lobe 1 being the N-terminal lobe and lobe 2 the C-terminal one (Fig. 6.1b). Both lobes have an αβ-fold with a central β-sheet being surrounded by α-helices. Overall, in the absence of ligand (apo form), $GABA_{B1}$ and $GABA_{B2}$ VFTs share a good structural homology (rms deviation of 1.48 Å for 356 C_α atoms—pdb code 4MQE) (Geng et al. 2013). In addition, when considering separately each lobe of the VFT, there is also a good superimposition with $mGlu_1$ VFT structure (rms deviation ~1.6 Å).

Besides the general homology between $GABA_{B1}$ and $GABA_{B2}$ VFTs, a major difference exists in the relative orientation of the two lobes of the VFTs. Indeed, while the angle defined by the two lobes remains nearly constant for $GABA_{B2}$ VFT in all available structures, it differs for $GABA_{B1}$ VFT depending on the presence and the identity of the bound-ligand in the crystal (Geng et al. 2013). The angle is larger in the apo form and the antagonist-bound form and smaller in the presence of agonists, defining two conformations for the $GABA_{B1}$ VFT, open and close, respectively (Fig. 6.1b). In comparison, the angle defined by the two lobes in $GABA_{B2}$ VFT is in line with the large angle observed for the apo-form and antagonist-bound conformations of $GABA_{B1}$ VFT. Hence, $GABA_{B2}$ is in an open-like conformation, either alone or when associated with the closed or open $GABA_{B1}$ VFT (Geng et al. 2012,

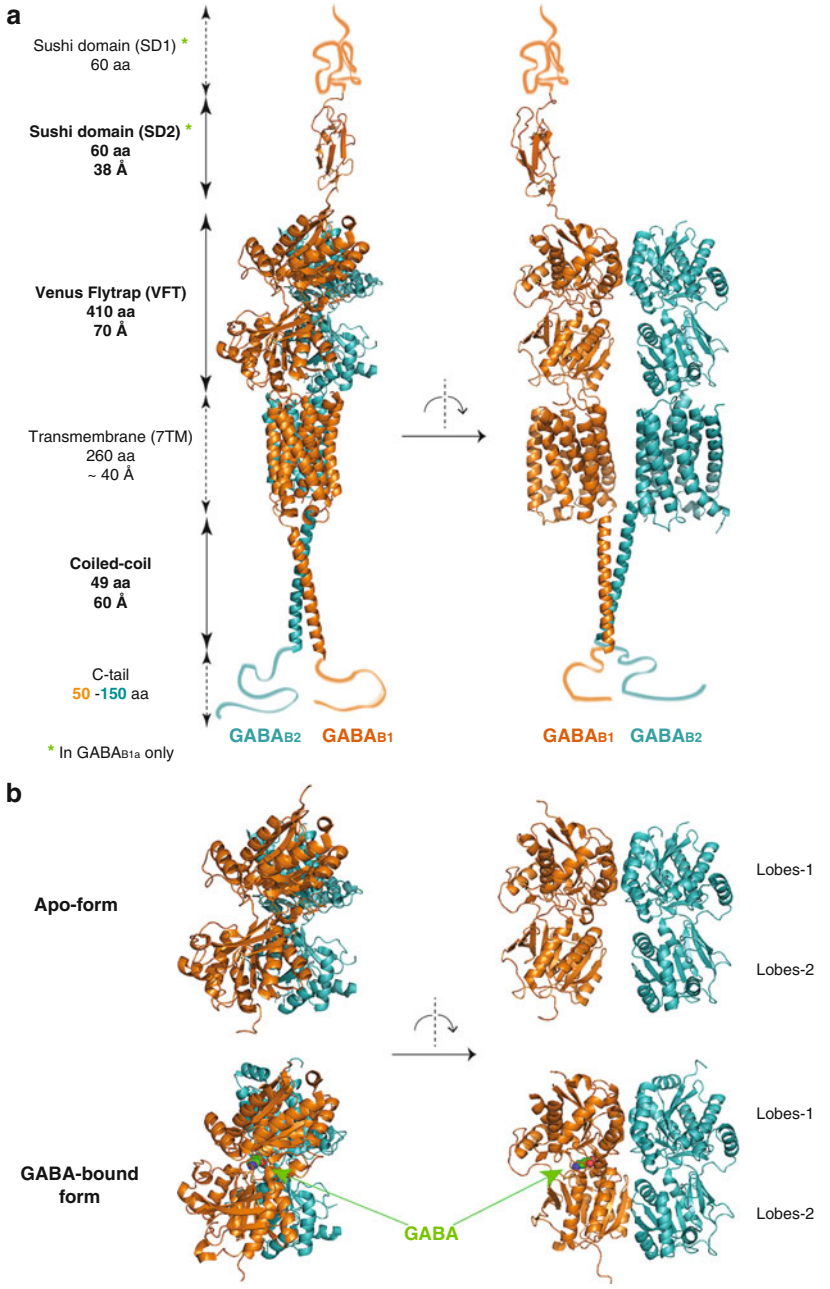

Fig. 6.1 $GABA_B$ receptor structural organization. (**a**) Representation of the structural domains composing the $GABA_B$ receptor. Hypothetical assembly of the receptor heterodimer based on the 3D structures available for the SD2 (pdb code 1SRZ), the VFT dimer (apo form—pdb code 4MQE) and the coiled-coil dimer (pdb code 4PAS), and a model of 7TM dimer. The structures of SD1 and of the C-termini remain unknown and are represented by a cartoon. $GABA_{B1}$ is represented in *orange* and $GABA_{B2}$ in *teal*. SD1 and SD2 are present in the $GABA_{B1a}$ isoform only. The domains for which pdb coordinates are available are noted with *plain-lined arrows* while the others are noted with *dash-lined arrows. Left and right panels* represent the same structure with an approximately

2013). This is in agreement with $GABA_{B1}$ being the only subunit binding agonists in the $GABA_B$ receptor and being responsible for the activation of the entire receptor complex (Kniazeff et al. 2002, 2004).

Thanks to both structural and mutagenesis studies; the binding site of GABA in $GABA_{B1}$ VFT has been described precisely (Galvez et al. 1999, 2000; Geng et al. 2013; Kniazeff et al. 2002). The carboxylate moiety of GABA is at the center of a hydrogen-bound network involving Ser 246 and Ser 269 in lobe 1 and Tyr 366 in lobe 2 (in the whole chapter, indicated residues correspond to $GABA_{B1a}$ numbering). The γ-amino group interacts with His 286 and Glu 465 in lobe 1 and with Trp 394 in lobe 2 through hydrogen-bound and van der Waals contacts (Fig. 6.2). Baclofen, a $GABA_B$ receptor-specific agonist, binds in a similar way than GABA but with a conformational flip of Tyr 366 to accommodate the chlorophenyl moiety of the ligand (Geng et al. 2013; Galvez et al. 2000). Orthosteric antagonists are GABA derivatives that also bind to $GABA_{B1}$ VFT only. Co-crystallization of $GABA_B$ VFTs

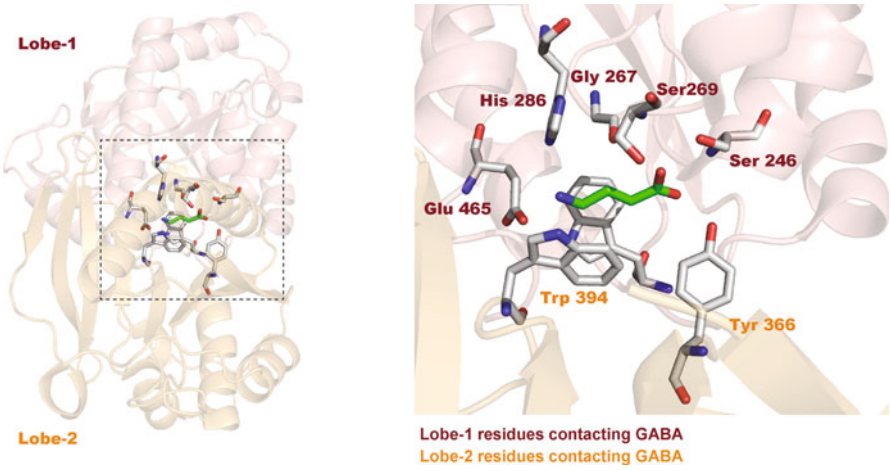

Fig. 6.2 GABA binding site in $GABA_{B1}$ VFT. (*Left panel*) General view of $GABA_{B1}$ VFT in the presence of GABA with lobe-1 colored in *pale red* and lobe-2 colored in *pale orange* (pdb code 4MS3). (*Right panel*) Closer view of the GABA binding site indicated with a *dash-box* in the *left panel*. Residues interacting with GABA (in *green*) are represented by sticks (*gray*). Lobe-1 residues are labeled in *dark red* and lobe-2 residues are labeled in *orange*. For clarity, the residues 171–217 in lobe-2 were removed in the *right panel*. In both panels, the cartoon of secondary structures was set to transparency. Figures were generated using Pymol (Delano Scientific)

Fig. 6.1 (continued) 90° rotation. (**b**) Conformational changes of VFT dimer upon GABA binding. (*Upper line*) Apo-form of $GABA_B$ VFT dimer (pdb code 4MQE); (*Lower line*) GABA-bound form of $GABA_B$ VFT dimer (pdb code 4MS3). The GABA is represented in balls with the carbons in *green*. $GABA_{B1}$ is represented in *orange* and $GABA_{B2}$ in *teal*. *Left and right panels* represent the same structures with an approximately 90° rotation. Figures were generated using Pymol (Delano Scientific)

with the antagonists showed that they bind tightly to the lobe 1 involving similar residues than GABA binding (Ser 246, Ser 269, His 286, Glu 465, and Trp 181). However, compared to agonist-bound conformation, there is only sparse interaction with lobe 2, which is in line with the two lobes being further apart and the stabilization of an "open" conformation (Geng et al. 2013). Of note, the residues involved in GABA binding in $GABA_{B1}$ VFT are not conserved in $GABA_{B2}$ (Kniazeff et al. 2002). In addition, in contrast to the $GABA_{B1}$ VFT cleft where agonists bind, that of $GABA_{B2}$ does no show a specific and high conservation during evolution, strongly suggesting the absence of ligand interaction at this site (Kniazeff et al. 2002).

Crystal structure resolution of the heterodimeric $GABA_B$ VFTs has also shed light on the interaction between $GABA_{B1}$ and $GABA_{B2}$ VFTs (Geng et al. 2013). Lobes 1 of each protomer interact burying a 1400 $Å^2$ surface from solvent accessibility. The interface consists of a central hydrophobic patch surrounded by hydrogen bonds and of a salt-bridge that are well conserved in all available structures. This is in contrast to the equivalent interface in mGlu receptors that is mostly hydrophobic (Kunishima et al. 2000). In the agonist-bound structures, an additional contact between the lobes 2 is present and buries about 1300 $Å^2$ involving mostly polar interactions and showing a lower shape complementarity than lobe 1 interface. Altogether, thanks to structural and mutagenesis studies, we gained a good knowledge of the VFT molecular organization.

Less structural information is available for $GABA_B$ receptor 7TM. Indeed, no crystal structure has been solved so far for this part of the receptor. Only homology models can be obtained based on the crystal structure of $mGlu_1$ and $mGlu_5$ 7TM (Dore et al. 2014; Wu et al. 2014), but not much validation of these models has been obtained so far. The models indicated that $GABA_B$ 7TM is about 40 Å long and 27 Å wide to cross the cell membrane (Fig. 6.1a). Although small molecules identified as positive or negative allosteric modulators were shown to interact in the 7TM domains of the $GABA_B$ receptor (Chen et al. 2014; Malherbe et al. 2008; Urwyler et al. 2001, 2003), their precise binding mode, and the residues involved have not been clearly identified so far. Nonetheless, early models identified an ionic lock stabilizing an interaction between TM3 and TM6 that is important to stabilize the inactive conformation of the $GABA_{B2}$ 7TM (Binet et al. 2007). Such an ionic lock has been confirmed in both $mGlu_1$ and $mGlu_5$ 7TM structures (Dore et al. 2014; Wu et al. 2014). This is consistent with $GABA_{B2}$ 7TM domain undergoing a similar change in conformation leading to G protein activation. Of interest, the ionic lock is absent in $GABA_{B1}$ 7TM (Binet et al. 2007) in agreement with its inability to activate G proteins.

Nothing is known yet on how the 7TM domain of $GABA_{B1}$ interacts with that of $GABA_{B2}$. In mGlu receptors, cysteine cross-linking experiments, associated with functional studies identified TM4 and TM5 as the interface in the inactive form of the dimer, while TM6 appears critical in the active dimer (Xue et al. 2015). However, the heterodimeric nature of the $GABA_B$ receptor, its ability to associate into large complexes in contrast to mGlu receptors (Maurel et al. 2008) and the absence of a large movement between the VFTs (Geng et al. 2013) hence suggesting much smaller movements between $GABA_B$ receptor 7TM indicate a different mode of

subunit interaction in the $GABA_B$ receptor compared to mGlu receptors. More work is then necessary to clarify the general structure, the allosteric interaction, and the modulation of the 7TM domains of each subunit of the $GABA_B$ receptor.

The $GABA_B$ heterodimeric interaction is stabilized by a coiled-coil interaction between the $GABA_{B1}$ and $GABA_{B2}$ C-termini encompassing about 49 residues in each subunit (Ser 772—His 810 in $GABA_{B1}$ and Ser 779 Lys 827 in $GABA_{B2}$) (Kammerer et al. 1999; Burmakina et al. 2014) (Fig. 6.1a). Coiled-coil domains are known structural motives formed of at least two intertwined helices composed of heptad repeats that tightly interact to form a super coil (Mason and Arndt 2004). The structure of $GABA_B$ receptor coiled-coil domain has been solved by X-ray crystallography and highlights the molecular details of the interaction (Burmakina et al. 2014). The two parallel helices form an extended stalk about 60 Å long and 22 Å wide constituted of five complete heptad repeats and additional coiled-coil elements at both ends. The interaction buries a surface of about 2000 $Å^2$. The general packing of the $GABA_B$ coiled-coil domain is in line with the reported interaction of such structural motives. There is a succession of knobs and holes where the knobs of one helix interlock with the holes formed between four residues of the other helix. A particularity of $GABA_B$ coiled-coil interaction is the network of hydrogen-bounds all along the domain, which is favored by the presence of asparagine residues at the center of the coiled-coil interaction. It was proposed to enhance the specificity of the interaction together with the presence of three salt bridges (Burmakina et al. 2014).

An additional structural domain is present on one of the isoforms of the $GABA_B$ receptor. Indeed, two main isoforms, $GABA_{B1a}$ and $GABA_{B1b}$, of the $GABA_{B1}$ subunit are generated through an alternate promoter usage (Steiger et al. 2004). It results in the presence of a repeat of two sushi domains (SD1 and SD2) at the extracellular N-terminus of $GABA_{B1a}$ only (Fig. 6.1a). SDs, which are also named complement control protein (CCP) modules or the short consensus repeats (SCRs), are about 60 residues long and are known to be involved in many recognition processes including that of the complement system (Reid and Day 1989). In the case of the $GABA_B$ receptor, SDs control the specific targeting of the receptor to excitatory terminals most probably through interactions with the extracellular matrix (Vigot et al. 2006). In an attempt to gain a better knowledge on their structural organization, biostructural analyses of the purified $GABA_B$ SDs have been performed (Blein et al. 2004). SD2 is a typical SD with approximately 60 amino acid residues including four cysteines forming two disulphide bridges. Nuclear magnetic resonance (NMR) analysis reveals its 3D structure which is in agreement with previously solved SD structures. It is mostly constituted of five antiparallel β-strands forming part of a barrel-like structure (Fig. 6.1a). An additional two antiparallel β-strands are separated from the other. Compared to other SDs, $GABA_B$ receptor SD2 has a long hypervariable region forming a long loop extending toward its N-terminus. It is suggested that it may interact either with SD1 or with other interacting proteins. In contrast to SD2, SD1 has less sequence homology with typical SDs and is unstable when purified alone or when fused to SD2 (Blein et al. 2004). Mass spectrometry analysis showed, however, the presence of the two typical SD disulphide bridges in SD1 and pull-down

experiments indicated that the purified isolated SD1 maintained its ability to interact with fibulin-2 (an in vitro reported partner for $GABA_{B1a}$). As a consequence of the instability of SD1, the NMR spectra were of poor quality and could not lead to structure determination. Hence, the precise folding of the SD1 remains unknown.

As reported above, precise molecular information is available on the different structural domains composing the $GABA_B$ receptor heterodimers except for the 7TM which is still waiting for structure resolution but which is also rather challenging. A further step would be the resolution of the full-length heterodimeric $GABA_B$ receptor structure that would surely unravel new molecular interactions that remain unknown.

6.3 Activation Mechanism and Allosteric Interaction Between the Various $GABA_B$ Receptor Domains

Having reported the general structure of the $GABA_B$ heterodimeric receptor, we will now describe our current view on how these four main domains can link GABA interaction in the $GABA_{B1}$ VFT, to G protein activation by the 7TM of $GABA_{B2}$ (Galvez et al. 2001).

As well documented for the VFT domains, including the binding domain of the mGlu receptors, GABA interaction in the $GABA_{B1}$ VFT stabilizes its closed state. This is well validated by mutagenesis and modeling studies and confirmed by the crystal structure of the $GABA_{B1}$ VFT (Geng et al. 2013; Galvez et al. 2000). $GABA_{B1}$ VFT closure was indeed found essential and sufficient for $GABA_B$ receptor activation since locking this domain in its closed conformation through an inter-lobes disulfide bridge generates a fully active receptor (Kniazeff et al. 2004). On the opposite, $GABA_{B2}$ VFT could not be observed in a closed conformation, even in the presence of agonist in $GABA_{B1}$ VFT (Geng et al. 2012, 2013). Moreover, any attempt to prevent a putative closure of $GABA_{B2}$ VFT using a glycan wedge approach (insertion of a glycosylation site in the $GABA_{B2}$ VFT cleft) did not affect the properties of the heterodimer, still displaying a high agonist affinity and still being functional with no noticeable differences from the wild-type (Geng et al. 2012). Accordingly, the first effect of agonists on the $GABA_B$ heterodimer is to stabilize the $GABA_{B1}$ VFT in a closed conformation.

In contrast to mGlu VFT dimers, in which domain closure is associated with a major change in the relative orientation of the two VFTs (Kunishima et al. 2000), no such major reorientation is observed in the dimeric $GABA_B$ and instead the relative movement of the VFTs is more subtle (Geng et al. 2013). Indeed, $GABA_{B1}$ VFT closure induces further interactions between the lobes-2 of both VFTs that likely stabilize further the $GABA_{B1}$ closed state promoting an increased agonist affinity (Liu et al. 2004). However, the movement between the lobes-2 was shown to play an important role in the $GABA_B$ receptor activation, since engineering of glycan wedges at this interface prevented G protein activation (Rondard et al. 2008).

At the 7TM level, far less is known, even though a change in conformation in $GABA_{B2}$ 7TM is obviously occurring, as evidenced by the ability of small molecules to activate the isolated 7TM of $GABA_{B2}$ expressed alone (Binet et al. 2004). In addition and as mentioned above, an ionic interaction linking TM3 and TM6 is important to stabilize the inactive conformation of the receptor (Binet et al. 2007). Indeed, removing this lock has been shown to likely stabilize $GABA_{B2}$-7TM in an active state, as indicated by the increased agonist affinity.

But how can the conformational change in the VFT dimer lead to the activation of the $GABA_{B2}$ 7TM? A number of observations revealed an interconnection between all four domains of the $GABA_B$ receptor heterodimer. First, the tighter interaction between the $GABA_{B1}$ and $GABA_{B2}$ VFTs in the presence of agonist stabilizes the closed state of $GABA_{B1}$ increasing agonist potency and revealing a first positive allostery from $GABA_{B2}$ VFT to $GABA_{B1}$ VFT (Geng et al. 2013; Liu et al. 2004) (Fig. 6.3). However, while the interactions between the lobes 2 are strictly required for activation of the wild-type receptor, a $GABA_B$ mutant lacking the $GABA_{B2}$ VFT is still functional although displaying a low agonist potency and a low efficacy (Monnier et al. 2011). These findings highlight a second important allosteric transition between the $GABA_{B1}$ VFT and the 7TM domain of $GABA_{B1}$ leading to an undefined conformational change in this domain that is eventually transmitted to the 7TM of $GABA_{B2}$ through a third allosteric interaction. Of note, this allosteric transition could also be highlighted in full-length receptor since the presence $GABA_{B1}$ 7TM is important to fully activate G proteins (Galvez et al. 2001; Duthey et al. 2002; Havlickova et al. 2002; Robbins et al. 2001). Accordingly, a first activation pathway of the receptor can be defined from the GABA binding site in $GABA_{B1}$ VFT to the G protein coupling site in $GABA_{B2}$ 7TM mediated through $GABA_{B1}$7TM and independent of $GABA_{B2}$VFT (Fig. 6.3). On another end, a second major observation highlighting the conformational transitions of the receptor activation is that a $GABA_B$ receptor mutant lacking the 7TM domain of $GABA_{B1}$ is also functional though displaying a lower coupling efficacy than the wild-type heterodimer (Monnier et al.

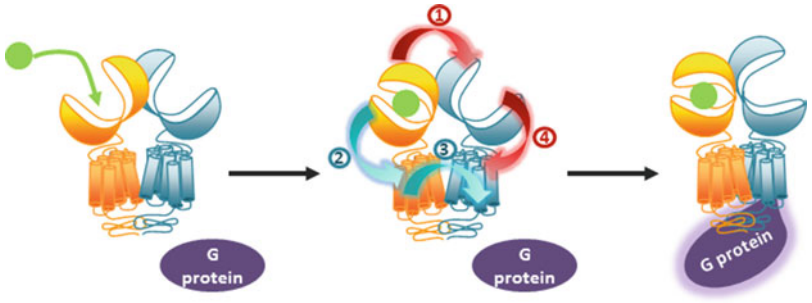

Fig. 6.3 Schematic representation of the allosteric transitions during $GABA_B$ receptor activation. Two independent but concomitant pathways (*one cyan* and *one red*) were defined and are associated with four allosteric transitions (numbered *1–4*) between the four main structural domains of the $GABA_B$ receptor (VFT and 7TM of both $GABA_{B1}$ and $GABA_{B2}$). $GABA_{B1}$ is represented in *orange* and $GABA_{B2}$ in *blue*. The G protein is represented in *purple* and the GABA in *green*

2011). This demonstrates a second activation pathway linking $GABA_{B1}$ VFT to $GABA_{B2}$ VFT and then to the 7TM of $GABA_{B2}$ (fourth allosteric transition), again enabling its coupling to G proteins (Fig. 6.3). This is only when both activation pathways are simultaneously effective that a fully efficient activation is reached.

A model of the possible allosteric coupling between the two 7TM domains of the dimeric mGlu receptors has been proposed. It involves a large relative movement between these domains interacting through TM4-5 in the inactive state and TM6 in the active state (Xue et al. 2015). No such major movement is expected for the $GABA_B$ receptor due to the small conformational changes observed upon activation of the dimer of $GABA_B$ VFTs (Geng et al. 2013). In addition, one has to keep in mind that the $GABA_B$ receptor lacks the rigid CRD linking the VFT to the 7TM in mGlu receptors and that has been shown to also participate in the receptor activation (Huang et al. 2011). This observation indicates further that the precise activation mechanism of $GABA_B$ receptor must differ from that of mGlu receptors. Further studies are required to elucidate the structural bases of the allosteric control of the 7TM of each $GABA_B$ subunit by their respective VFT.

6.4 Higher Order Oligomers of the $GABA_B$ Receptor

As reported above, the $GABA_B$ receptor is an obligatory heterodimer whose heterodimerization plays a critical role in the activation mechanism leading from GABA binding to G protein activation. However, an unexpected property of the $GABA_B$ receptor was reported: the heterodimers assemble to form higher order oligomers. Indeed, two independent studies revealed the oligomerization of the $GABA_B$ receptor. First, a Förster resonance energy transfer (FRET) analysis showed that, at the cell surface, two $GABA_B$ heterodimers are in close enough proximity to promote an inter-heterodimer FRET signal (Maurel et al. 2008). This indicates that the distance between the two heterodimers is below 100 Å, hence that two heterodimers are likely to directly interact. Second, an analysis of fluorescent $GABA_B$ heterodimers diffusion in cell membrane suggested that the $GABA_B$ receptor has a higher propensity to form larger entities than strict heterodimers than any other tested GPCRs starting from tetramers but also even larger complexes (Calebiro et al. 2013).

Several arguments may arise against this occurring in native systems since both studies were performed on transiently transfected mammalian cells. However, most of these could be ruled out. The use of transient transfection is often accounted for leading to expression levels that are way higher than the physiological ones that might favor unspecific interactions. However in both studies, the oligomers were detected already at very low expression levels even though more oligomers or larger ones are present when the expression level was increased as nicely illustrated by the diffusion study (Calebiro et al. 2013). In the FRET study, a comparison of the expression level in the transfected cells relative to the endogenous expression in cortical neurons showed that they were similar in both systems (Maurel et al. 2008;

Comps-Agrar et al. 2011). In addition, one could also exclude that the FRET signal arose from collisional FRET since the site of insertion of the FRET-compatible fluorophores, either on $GABA_{B1}$ or on $GABA_{B2}$, is critical to measure inter-heterodimer FRET: only fluorophore insertion in $GABA_{B1}$ subunit led to a strong and significant FRET signal compared to the low FRET signal obtained when the fluorophores were inserted in $GABA_{B2}$ subunit (Maurel et al. 2008). This furthermore indicates that $GABA_{B1}$ subunit is likely to be at the center of the oligomeric association.

Two additional results obtained with endogenous receptors further support the ability of the $GABA_B$ receptor to form oligomers in the brain. First, the apparent molecular weight of the protein complex pulled down from brain using anti-$GABA_B$ antibodies is compatible with the molecular weight of two $GABA_B$ heterodimers together with their accessory proteins, K^+ channel tetramerization domains (KCTDs) (Schwenk et al. 2010). Second, when performing FRET measurements using fluorescent anti-SD antibodies (i.e., anti $GABA_{B1a}$ antibodies (Tiao et al. 2008)) on membrane prepared from mouse brain, a significant signal was measured indicating that two $GABA_{B1a}$ subunits were in close proximity (Comps-Agrar et al. 2011).

As indicated above, the association of $GABA_B$ receptor heterodimers is mediated by $GABA_{B1}$ subunits. In order to comprehend the molecular determinants of the interaction, the crystal structure of a non-related tetrameric protein that also contains a VFT was taken into account (Sobolevsky et al. 2009). Actually, the N-terminal domain of the ionotropic glutamate receptors subunits has a VFT-fold. In agreement, the crystal structure of the full-length tetrameric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor GluR2 revealed for the first time the interactions that could take place in a VFT tetramer. A first interface very similar to that between $GABA_{B1}$ and $GABA_{B2}$ VFTs is conserved and a second smaller interface is present and may represent a model for the $GABA_{B1}$/$GABA_{B1}$ VFT interface. This interface involves residues at the "lips" of the lobes 2. Using mutagenesis and FRET measurements, it was shown that a similar region in the lobe 2 of $GABA_{B1}$ VFT was indeed important for the proper interaction between $GABA_B$ heterodimers (Comps-Agrar et al. 2011). However, the mutagenesis of this small area did not fully abolish the interaction suggesting that other molecular determinants of the interface, probably at the 7TM level, remain to be identified.

The discovery of the propensity of $GABA_B$ receptor to form oligomers raised some questions starting with the physiological roles of these complexes. A first effort was made in order to determine the differential G protein coupling profiles of the heterodimers and of the oligomers. Since oligomerization is constitutive, a major challenge was to develop strategies to control the oligomerization level of the receptor in cells. By using a competitor of the $GABA_{B1}$/$GABA_{B1}$ interface (a minimal construct consisting of the 7TM part of $GABA_{B1}$ without the VFT and the C-terminal tail), G protein activation upon GABA stimulation showed a better efficacy than in the absence of the competitor (Maurel et al. 2008; Comps-Agrar et al. 2011). The potency of the GABA response was left unchanged. This indicates first that the oligomerization plays a critical role in controlling the G protein coupling efficacy and that oligomers limit G protein coupling compared to heterodimers. To confirm these results, a mutation in $GABA_{B1}$ VFT at the level of the putative $GABA_{B1}$/$GABA_{B1}$

interaction that was shown to decrease the FRET signal of the oligomers was tested for G protein activation. In a similar way than the use of the competitor, introduction of the mutation increased the G protein coupling efficacy without affecting the potency of GABA stimulation (Comps-Agrar et al. 2011). Altogether these data show that the oligomerization surprisingly decreases G protein coupling efficacy, at least when it comes to the canonical Gi/o protein coupling.

One would then wonder what would be the advantage of oligomer formation for the cells if it only limits G protein activation. A first possibility is that oligomers regulate a unique and still undiscovered downstream signaling compared to isolated heterodimers. Alternatively, some physiological conditions could play a regulatory role on the oligomerization and thus control the extent of the $GABA_B$-mediated Gi/o protein activation. Additional studies are required to understand further this phenomenon. In addition, other parameters like trafficking and internalization could also be assessed in the context of the oligomer versus the heterodimer.

An additional intriguing question is to understand the molecular basis of the limitation of G protein coupling in the oligomer. One of the hypotheses is that it may arise from negative allostery in the complex either for ligand binding or for G protein coupling. It could also come from conformational allostery, where activation of one heterodimer hampers the activation of the others. Further studies are required to highlight this mechanism.

Since the propensity of the $GABA_B$ receptor to form oligomers is rather high, one might question the stability of these oligomers. The study of Calibero et al. shows that at any receptor density, several oligomeric species coexist (Calebiro et al. 2013). Also only at very low density strict heterodimers are found and the higher the density, the larger the complexes. Are heterodimers exchanging from one complex to the other leading to transiently existing heterodimers? To assess this, we have developed a methodology in order to measure the stability of the oligomers at the cell surface of HEK293 cells (Comps-Agrar et al. 2012). It consists on following the FRET signal of the oligomers present at the cell surface and targeting new unlabeled (or differently labeled) receptors in a drug-induced manner. We showed that the FRET signal of the preexisting oligomers remained unchanged, which suggests that the $GABA_B$ receptor oligomers are stable at the surface of HEK293 cells. In addition, when targeting new receptors, we could detect the association of these new receptors to receptors that were already present at the cell surface suggesting the constitution of higher order $GABA_B$ receptor oligomers when targeting new receptors to the cell surface, thus when increasing receptor density. This is in agreement with the study of Calibero et al. (2013).

6.5 Conclusions

The discovery that two distinct subunits were requested to form a functional $GABA_B$ receptor was a real breakthrough in the GPCR field. Since this major discovery, major information highlighting the importance of this heterodimeric assembly for

the proper function of the $GABA_B$ receptor was obtained. Not only such an assembly is required for the proper plasma membrane targeting of the receptor, but it is also essential for the allosteric interaction between the various $GABA_B$ receptor domains to allow agonist binding in $GABA_{B1}$ VFT and to activate the 7TM domain of $GABA_{B2}$ leading to G protein activation. Although fewer details are known compared to the dimeric mGlu receptors, the available information already indicates a different activation mechanism for the $GABA_B$ receptor, likely resulting from the lack of a CRD. However, it is clear that the main four domains are tightly linked by allosteric interactions, enabling the information to efficiently reach one domain when the conformation of another is modified. A better understanding of the molecular details undergoing $GABA_B$ receptor G protein coupling will certainly help designing novel $GABA_B$ ligands, and especially allosteric modulators that may have better therapeutic efficacy, with fewer side effects.

Of interest, the $GABA_B$ heterodimeric complex leads to G protein activation through one single subunit only, an observation that is consistent with what is observed with many other dimeric GPCRs. This highlights the interest of studying the $GABA_B$ receptor, not only for the improvement of $GABA_B$ targeting drugs, but also for the more general purpose of elucidating the role and physiological interest of GPCR dimerization.

Today, they are increasing number of papers indicating that GPCRs may assemble into tetramers or larger oligomers (Calebiro et al. 2013; Patowary et al. 2013; Pisterzi et al. 2010). Again, the $GABA_B$ receptor may help unravel the functional consequence of such a receptor assembly since it is clearly one of the best characterized oligomeric GPCRs, being supported not only in recombinant systems, but also in native neurons. Already, allosteric interaction between dimers within such a large receptor complex provides some indication on the possible roles of such complex assembly.

These observations highlight the need for a better understanding of the structural bases of $GABA_B$ receptor assembly and conformational dynamics, as one of the most exciting example of GPCR complex.

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