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Activation mechanism of the heterodimeric $GABA_B$ receptor

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Abstract

The $GABA_B$ receptor was the first heteromeric G-protein coupled receptor (GPCR) identified. Indeed, both $GABA_{B1}$ and $GABA_{B2}$ subunits appear necessary to get a functional $GABA_B$ receptor. Soon after the cloning of both subunits, it was demonstrated that $GABA_{B2}$ was required for $GABA_{B1}$ to reach the cell surface. However, even a mutated $GABA_{B1}$ able to reach the cell surface is not functional alone despite its ability to bind $GABA_B$ ligands. This clearly demonstrated that $GABA_{B2}$ is not only required for the correct trafficking of $GABA_{B1}$ but also for the correct functioning of the receptor. In the present review article, we will summarize our actual knowledge of the specific role of each subunit in ligand recognition, intramolecular transduction, G-protein activation and allosteric modulation. We will show that the $GABA_B$ receptor is an heterodimer (not an hetero-oligomer), that agonists bind in $GABA_{B1}$, whereas $GABA_{B2}$ controls agonist affinity and is responsible for G-protein coupling. Finally, we will show that the recently identified positive allosteric modulator CGP7930 acts as a direct activator of the heptahelical domain of $GABA_{B2}$, being therefore the first $GABA_{B2}$ ligand identified so far.

Keywords: $GABA_B$ receptor; Baclofen; Activation; Dimerization; Allosteric regulator; G-protein coupled receptors

1. Introduction

G-protein coupled receptors have been well recognized as allosteric monomeric membrane proteins capable of stimulating the GDP to GTP exchange in heterotrimeric G-proteins. Accordingly, the general accepted stoichiometry for such receptors was one ligand activating one GPCR that, in turn, activated one set of trimeric G-proteins at a time. Although biochemical data supported this notion, at least for rhodopsin [1], it was found to be quite difficult in the mid-1980s to explain all pharmacological data using this paradigm [2]. Soon, were some GPCR dimers or oligomers were observed using biochemical approaches, and at the end of the 1990s, GPCR oligomerisation in intact cells was observed using resonance energy transfer technologies [2]. However, the functional significance of this phenomenon remains unclear. In particular, it remains a matter of intense debate whether or not such GPCR oligomerisation is required for an efficient activation of heterotrimeric G-proteins.

For one class of GPCRs, the so-called class C, there is a consensus in the field that these receptors function as dimers or higher ordered oligomers, with most of them being constitutive dimers linked by a disulphide bridge [3]. This class includes the GPCRs activated by the two main neurotransmitters, glutamate and GABA, as well as receptors activated by Ca^{2+}, some pheromones and sweet taste compounds [4]. Like other GPCRs, class C receptors have

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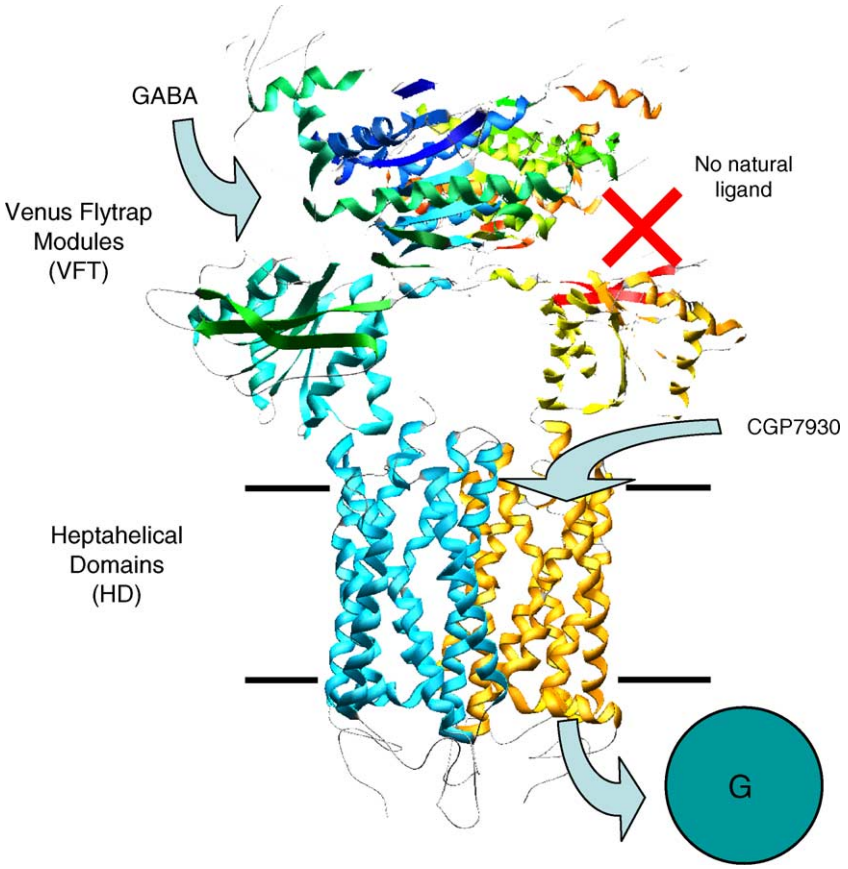

Fig. 1. Proposed structural organization of the heteromeric $GABA_B$ receptor. The $GABA_{B1}$ subunit is in blue, in front, and the $GABA_{B2}$ subunit in yellow, in the back. As shown, each subunit is composed of a Venus Flytrap (VFT) module directly connected to the heptahelical domain (HD). As described in the text, classical $GABA_B$ ligands (both agonists and competitive antagonists) bind in the cleft of the $GABA_{B1}$ VFT, whereas the equivalent domain of $GABA_{B2}$ is unlikely to bind an endogenous ligand. The heptahelical domain of $GABA_{B2}$ plays a critical role in G-protein activation, and has been shown recently to bind the $GABA_B$ positive allosteric modulator CGP7930. Although these subunits are known to interact at the level of a coiled-coil region in their intracellular tail, direct interaction at the level of their VFT has also been reported. Accordingly, VFTs are shown here in a direct interaction at the level of their lobe one, as revealed by the crystal structure of the VFT dimer of the $mGlu_1$ receptor.

a heptahelical domain responsible for activation of heterotrimeric G-proteins, but their ligand-binding site is located in their large extracellular domain (ECD), within a Venus Flytrap (VFT) module [5,6].

Among class C GPCRs, the $GABA_B$ receptor was the first identified as being composed of two distinct subunits, $GABA_{B1}$ and $GABA_{B2}$ [7–10] (Fig. 1). Indeed, although $GABA_{B1}$ bind all known $GABA_B$ ligands, $GABA_{B2}$ is required for the normal trafficking of $GABA_{B1}$ to the cell surface [11–14], as well as for G-protein activation [15–18]. Another role identified for $GABA_{B2}$ is to increase agonist (but not antagonist) affinity in $GABA_{B1}$ [10,19]. As such, the $GABA_B$ receptor represents an excellent model to examine the functional significance of oligomerisation of GPCRs. Indeed, this receptor can be used to examine whether a single agonist per dimer is sufficient for receptor activation, to better understand the allosteric interaction between subunits in a receptor oligomer, and to clarify whether such receptors couple simultaneously to one or two G-proteins. In the present paper, we summarize our recent findings aimed at clarifying these different issues in the case of the $GABA_B$ receptor.

2. The $GABA_B$ receptor is a heterodimer

Expression of $GABA_{B1}$ either in heterologous cells or in neurons [20] revealed that this does not lead to the formation of a functional $GABA_B$ receptor, and that $GABA_{B1}$ does not reach the cell surface. This results from the presence of an endoplasmic reticulum (ER) retention signal (RSRR) located in its intracellular tail [11–14]. In a search for associated proteins possibly required for $GABA_{B1}$ receptor function and trafficking to the cell surface, $GABA_{B2}$ was identified using a yeast two hybrid screen approach [8–10,21]. This subunit share 30% sequence identity with $GABA_{B1}$ (Fig. 1) and is co-localized with $GABA_{B1}$ in the brain. A direct interaction was found to occur between the coiled-coil domains located in the intracellular tails of both $GABA_{B1}$ and $GABA_{B2}$ proteins. This interaction masks the ER retention signal of $GABA_{B1}$, allowing the heteromer to reach the cell surface. This suggests that the heteromer is already formed in the ER. Functional studies also indicated that $GABA_{B2}$ is required for normal G-protein activation [8–10,21] again suggesting that the association of $GABA_{B1}$ and $GABA_{B2}$ is

required for the formation of a functional $GABA_B$ receptor [15,18,22]. Indeed, mutation of the ER retention signal RSRR into ASAR, allows $GABA_{B1}$ to reach the cell surface alone, but even though this subunit binds $GABA_B$ ligands, it is unable to activate G-proteins [12,14]. Only co-expression of $GABA_{B1}$ and $GABA_{B2}$ leads to the formation of a fully functional receptor. But does the $GABA_B$ receptor exists only a heterodimer at the cell surface, or is it an oligomer composed of more than two subunits?

To answer this question, we used a FRET approach that allows for the detection of protein–protein interactions at the surface of intact cells [23]. This technology is based on the transfer of energy between two fluorophores coupled to antibodies that specifically recognize each subunits (Fig. 2). The fluorophore Europium cryptate (EuK) acts as a fluorescent donor molecule, while AlexaFluor®647, or the allophycocyanine XL665, were used as acceptor molecules. Due to the specific long-term emission of EuK, the emission can be measured 50 μm after excitation, therefore allowing a large increase in the signal to noise ratio. This method, known as Time Resolved Fluorescence (TRF), can even be performed in homogeneous assays (HTRF® technology) [24].

To perform this study, $GABA_{B1}$ and $GABA_{B2}$ subunits were tagged at their N-termini, immediately after the signal peptide, with either the HA or myc epitope (Fig. 2). We previously reported that the presence of these tags did not influence $GABA_B$ subunit targeting to the cell surface, ligand binding, or normal function of the $GABA_B$ receptor

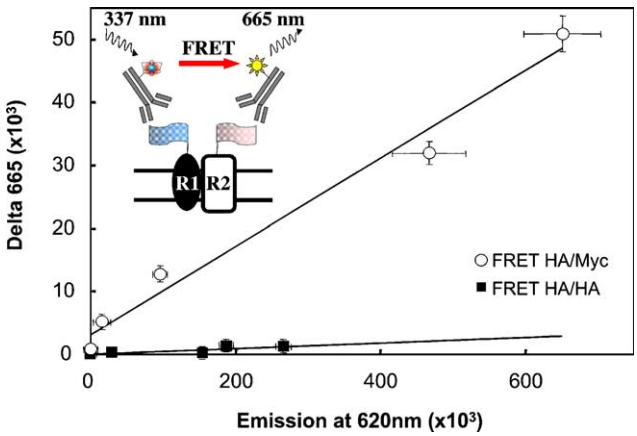

Fig. 2. FRET analysis of $GABA_B$ dimmer formation at the cell surface. The methodology used is schematized in the insert. Proximity between two tagged proteins is measured using antibodies directed against these tags and coupled either to the donor fluorophore EuK, or the acceptor fluorophore AlexaFluor647. The open symbols represent the FRET signals (emission at 665 nm measured in the presence of the acceptor minus that measured in the absence of the acceptor) measured with a combination of anti-HA and anti-myc antibodies with cells expressing various amounts of $GABA_B$ receptors at the cell surface composed of a HA-$GABA_{B1}$ and a myc-$GABA_{B2}$ subunits. The closed symbols represent the FRET signal measured in the same cells but using an equimolar amount of anti-HA antibodies labeled either with EuK or AlexaFluor647. The amount of receptors at the cell surface was estimated by measuring the amount of anti-HA-EuK bound to the receptor.

[12,15]. A strong FRET signal was detected in studies where the HA and myc antibodies coupled to EuK and AlexaFluor647, respectively, were used to label COS cells co-expressing the differentially epitope tagged $GABA_B$ receptor subunits. This signal was directly proportional to the amount of either $GABA_{B1}$ or $GABA_{B2}$ at the cell surface [23] (Fig. 2). No such signal was measured in cells co-expressing HA-$GABA_{B1}$, $GABA_{B2}$ and a myc-tagged version of the V2 vasopressin receptor, indicating the specificity of the signal measured [23]. These data demonstrate the direct interaction of these two proteins at the surface of intact cells.

We then examined whether one or several HA-$GABA_{B1}$ subunits were part of the $GABA_B$ receptor complex. To that aim, cells expressing HA-$GABA_{B1}$ and myc-$GABA_{B2}$ were labeled with an equimolar amount of HA-antibodies coupled either to EuK or AlexaFluor647. Under such conditions, only a small FRET signal was measured (Fig. 2), despite a large signal measured in cells expressing homomeric HA-$mGlu_5$ (data not shown). Accordingly, in cells expressing both $GABA_{B1}$ and $GABA_{B2}$, no association between $GABA_{B1}$ can be detected, in agreement with the $GABA_B$ receptor being a dimer. However, a robust FRET signal was measured using these HA antibodies in cells expressing only HA-$GABA_{B1ASA}$ [23], the mutated version of $GABA_{B1}$ that is able to reach the cell surface by itself. Accordingly, $GABA_{B1}$, in the absence of $GABA_{B2}$, can form dimers, demonstrating that its inability to activate G-protein is not due to its inability to form dimers.

Taken together, these data show that $GABA_{B1}$ and $GABA_{B2}$ mostly form heterodimers at the cell surface, and this is likely required for normal function of $GABA_B$ receptors.

3. The $GABA_B$ heterodimer binds a single GABA molecule in the $GABA_{B1}$ subunit

Since both $GABA_B$ receptor subunits share sequence similarity, and because both subunits possess a VFT module that has been shown to bind glutamate in mGlu receptors, and GABA in $GABA_{B1}$ [25,26], it was assumed that the VFT module of $GABA_{B2}$ also binds $GABA_B$ ligands. It is also possible that $GABA_B$ receptor activation can be obtained with a single agonist per dimer. Indeed, although no binding of radioligand can be detected in $GABA_{B2}$, this may be due to an extremely low affinity of the subunit for these ligands, rather than to a total absence of binding. Using homology modeling and site directed mutagenesis studies, we proposed that Ser246 and Glu465 within the $GABA_{B1}$ VFT module interact with the carboxy and amino group of GABA, respectively [26]. Other residues (Tyr366 and Tyr395) possibly involved in GABA binding were also identified. Surprisingly, none of these residues are conserved in $GABA_{B2}$. Moreover, the residues that line the putative binding pocket within the

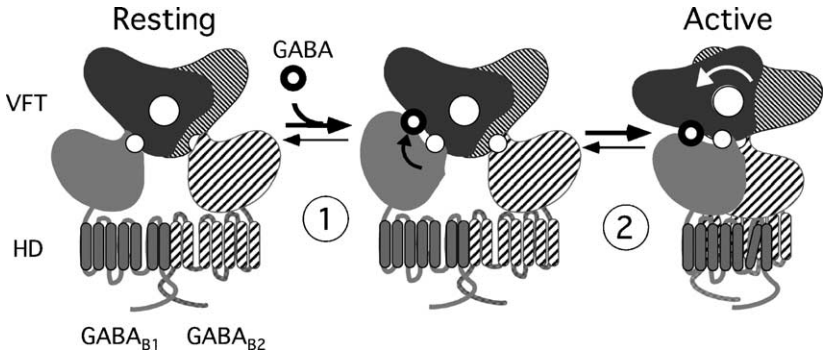

Fig. 3. Proposed activation mechanism of the heterodimeric $GABA_B$ receptor, as based on the known structure of the $mGlu_1$ VFT dimer solved in the absence and presence of bound agonist. In the absence of agonist, both VFTs are in an open form, and in such a relative orientation that their maintain the dimer of HDs in an inactive relative position. Agonist binding in the $GABA_{B1}$ VFT induces or stabilizes its closed state (step 1), and this is proposed to favors a new relative orientation of the VFTs (step 2). In this orientation, the dimer of VFTs in turn favors a new relative position of the HDs, allowing $GABA_{B2}$ HD to reach an active conformation leading to G-protein activation. It is actually not known whether the $GABA_{B2}$ VFT is in an open or closed state in any of these different states of the $GABA_B$ receptor dimer.

$GABA_{B2}$ VFT module were not conserved through evolution. This is in contrast to the high conservation of this binding site within $GABA_{B1}$ receptor sequences identified in various species [26], as well as the high degree of conservation of the glutamate binding site within mGlu receptors [27]. This indicates that there has been no selective pressure on this site in the $GABA_{B2}$ subunit during evolution, suggesting that no conserved natural ligand binds in that site. To further confirm that ligand binding in this putative binding pocket of $GABA_{B2}$ is not required for $GABA_B$ receptor activity, up to five point mutations were simultaneously introduced into this region of $GABA_{B2}$. Nevertheless, normal functioning of the $GABA_B$ receptor was observed [26].

Taken together, these observations demonstrate that a single agonist per heterodimeric $GABA_B$ receptor is sufficient for receptor activation (Fig. 1).

4. Closure of the binding domain of $GABA_{B1}$ is sufficient for receptor activation

Based on the known functioning of VFT modules such as that of $mGlu_1$ [6,28,29], as well as those corresponding to the bacterial periplasmic binding proteins [30], it was proposed that the first action of $GABA_B$ agonists was to stabilize a closed state of the $GABA_{B1}$ VFT module. Indeed, this proposal was consistent with mutations in the $GABA_{B1}$ VFT module that either increase or decrease agonist, but not antagonist, affinity [31]. Although this may well be required, such a $GABA_{B1}$ VFT closure may not be sufficient to fully activate the receptor. To examine this possibility, we introduced cysteine residues on both lobes of the $GABA_{B1}$ VFT ($GABA_{B1}$-CC) that are far apart in an open form model, but that are able to form a disulphide bridge in a closed form model of the receptor [32]. Accordingly, if the disulphide bridge can form, then the $GABA_{B1}$ VFT module will be locked in its closed state.

When such a $GABA_{B1}$-CC mutant is co-expressed with $GABA_{B2}$, a strong constitutive activity can be measured, as revealed by IP formation resulting from the activation of the chimeric G-protein Gqi9 [32]. Indeed, this chimeric G-protein is known to allow an efficient coupling of the $GABA_B$ receptor to activation of PLC. In agreement with the constitutive activity being due to an additional disulphide bridge in the $GABA_B$ receptor, this activity was no longer detectable after reduction with DTT. This was not the result of damage to the receptor since it was still functional, as shown by the normal activation by GABA after DTT treatment.

In agreement with the receptor being locked in an active state, $GABA_B$ receptor antagonists supposed to bind in the open form of $GABA_{B1}$ VFT module were not able to inhibit the constitutive activity of the CC mutant [32]. A more detailed analysis of the $GABA_{B1}$-CC:$GABA_{B2}$ receptor combination revealed that the specific constitutive activity was similar to the agonist-induced activity of the wild-type receptor [32].

Taken together, these data demonstrate that the closure of $GABA_{B1}$ VFT module is sufficient for full receptor activity (Fig. 3), again consistent with a single agonist per dimer being sufficient for full activity.

5. $GABA_{B2}$ ECD controls agonist affinity in $GABA_{B1}$

Expression cloning of the $GABA_{B1}$ subunit revealed that this protein could bind all known $GABA_B$ ligands. However, although $GABA_B$ antagonists display normal affinity, all tested $GABA_B$ agonists have a 10- to 100-fold lower affinity on $GABA_{B1}$ compared to the native receptor in brain membranes [7]. Expression of the mutated $GABA_{B1ASA}$, which is able to reach the cell surface alone, also revealed that this low affinity of $GABA_B$ agonists was not due to the retention of this subunit in the ER and/or to

an incomplete glycosylation of the protein [15]. Indeed, $GABA_B$ agonists still display a low affinity for the $GABA_{B1ASA}$ mutant in intact cells where only binding to surface proteins can be measured. After cloning the $GABA_{B2}$ subunit, it was found that the latter can increase agonist affinity for $GABA_{B1}$ [10]. However, it was not clear how $GABA_{B2}$ allosterically controls agonist affinity for $GABA_{B1}$.

As mentioned above, $GABA_B$ agonists, in contrast to antagonists, likely stabilize a closed state of $GABA_{B1}$ VFT module. In turn, a better stabilization of the closed agonist occupied state is expected to increase agonist affinity [33], likely by slowing the off-rate of the molecule from its binding site. Expression studies, as well as structural studies of the extracellular VFT module of mGlu receptors, revealed that these domains form dimers, as a result of a hydrophobic interaction at the level of their lobe-I [6,28]. Therefore, we speculated that a direct interaction between $GABA_{B1}$ and $GABA_{B2}$ VFTs may be involved in this control of agonist affinity, perhaps because the $GABA_{B2}$ VFT further stabilizes the closed state of the agonist liganded $GABA_{B1}$ VFT.

In order to test this possibility we examined whether the isolated VFT modules of $GABA_{B1}$ and $GABA_{B2}$ can directly interact, and if this interaction is sufficient to increase agonist affinity for the $GABA_{B1}$ VFT module. To that aim, epitope tagged VFT modules were expressed in HEK293 cells and anchored to the surface of the cell either by a single transmembrane domain or by a GPI motif [34]. Using the FRET approach described above, a direct and specific interaction between the two VFT modules was observed, demonstrating the formation of VFT heterodimers [34]. Similarly, a direct interaction of the isolated $GABA_{B2}$ VFT module with the full-length $GABA_{B1}$ was observed. As expected, the $GABA_{B2}$ VFT module was sufficient to increase agonist affinity in the full-length $GABA_{B1}$ more than 10-fold. However, the deletion of the heptahelical domain of $GABA_{B1}$ was already sufficient to increase agonist affinity [19,34]. Finally, the isolated $GABA_{B2}$ VFT increased only threefolds the agonist affinity of the isolated $GABA_{B1}$ VFT.

Taken together, these results demonstrate that, as observed for the mGlu receptors, VFT modules of the $GABA_B$ subunits interact directly. This interaction appears to be important for controlling agonist affinity in $GABA_{B1}$ in two ways: (1) it prevents a negative effect of the heptahelical domain of $GABA_{B1}$ and (2) it directly increases agonist affinity in $GABA_{B1}$ VFT, most likely by stabilizing its closed state.

6. Both $GABA_{B1}$ and $GABA_{B2}$ VFTs are required for agonist-induced activity

Since the $GABA_{B2}$ subunit appears to be necessary for the formation of a functional $GABA_B$ receptor, we wondered which part of the $GABA_{B2}$ subunit is really required. To examine the specific role of $GABA_{B2}$ VFT in receptor activation, chimeric $GABA_B$ receptor subunits were generated in which the VFTs were swapped between the two subunits, leading to the generation of the GB1/2 (a subunit composed of the $GABA_{B2}$ VFT and the HD of $GABA_{B1}$) and the converse GB2/1 chimera. Neither of these subunits expressed alone lead to the formation of a functional receptor, even when the ER retention signal is mutated [15]. However, the co-expression of both chimeras leads to the formation of a functional receptor that exhibits all functional and pharmacological characteristics observed with the wild-type heterodimer. When GB1/2 is co-expressed with $GABA_{B1}$, a heterodimer is formed that binds $GABA_B$ ligands and displays constitutive activity demonstrating its ability to activate G-proteins. However, no GABA-induced activation could be measured [15]. The same constitutive activity was also observed when GB2/1 is co-expressed with $GABA_{B2}$. Similar data were reported thereafter by another group [22].

These data demonstrate that the heterodimer of VFTs is required for two reasons: (1) to maintain the receptor in a low activity state and (2) to allow agonist-induced activation of the receptor (Fig. 3).

7. The $GABA_{B2}$ heptahelical domain is critical for G-protein coupling

To examine whether $GABA_{B1}$ and/or $GABA_{B2}$ heptahelical domains could both couple and activate G-proteins, we generated receptor combinations in which the heteromeric nature of the VFT dimer is maintained, but in which the dimer of HDs is comprised of either $GABA_{B1}$ or $GABA_{B2}$ exclusively. When both HDs are from $GABA_{B1}$, no G-protein activation could be measured despite the fact that mutation of the ER retention signal allowed for correct expression of the receptor combination at the cell surface and that the receptor exhibited native binding properties [15]. In contrast, G-protein activation was observed with the receptor combination made of two $GABA_{B2}$ HDs, although the coupling efficacy is lower than that measured for the wild-type receptor [15]. This clearly demonstrates that the HD of $GABA_{B2}$ possesses sufficient molecular determinants to allow for G-protein activation. Similar data were obtained by others [18]. Further studies demonstrated that the $GABA_{B2}$ HD plays a critical role in G-protein activation within the heteromer. Indeed point mutations in either the i2 [17] or i3 [16] loops of $GABA_{B2}$ suppressed G-protein coupling of the heterodimer, whereas similar mutations in $GABA_{B1}$ had no effect.

Taken together, these data illustrate the critical role of the HD of $GABA_{B2}$ for G-protein activation by the heterodimeric $GABA_B$ receptor (Fig. 1).

8. Both cis- and trans-activation can occur in class C GPCRs

In the wild-type receptor, GABA binding in $GABA_{B1}$ VFT leads to G-protein activation by the $GABA_{B2}$ HD, a mechanism that can be described as trans-activation. However, is this an obligatory mechanism, or is it the consequence of a specific specialization of each subunit? Our finding that the GB1/2 + GB2/1 combination behaves like the wild-type combination suggests that GABA binding in the $GABA_{B1}$ VFT can activate either the HD linked to this domain (cis-activation) or that of the associated subunit (trans-activation). To further examine this point, we assessed the properties of the GB1/2 + $GABA_{B2}$ combination that possesses a homodimer of $GABA_{B2}$ HDs. As mentioned above, such a receptor combination is functional. Of interest, if only one HD is mutated in its i3 loop to prevent its ability to activate a G-protein, a twofold decrease in coupling efficacy is observed, whether the mutation is introduced in the GB1/2 or in the $GABA_{B2}$ subunit [35]. This clearly demonstrates that both cis- and trans-activation can occur in such a receptor, as long as both HDs are able to couple to G-proteins. This is consistent with the proposed activation mechanism of this receptor in which a major change in conformation of the VFT dimer leads to a change in conformation of the dimer of HDs allowing G-protein activation.

This finding also strengthens the conclusion that the $GABA_{B1}$ HD is incapable of G-protein activation. However, it is not clear if the GB1 is also able to reach the active conformation state but cannot couple to G-proteins, or whether it remains always in one rigid inactive conformation.

9. $GABA_B$ receptor allosteric potentiators as agonists of the $GABA_{B2}$ heptahelical domain

Recently, positive allosteric modulators of class C GPCRs have been identified. Such compounds are not able to activate the full-length receptor when applied alone, but facilitate the agonist activation of the receptor. Indeed, such compounds were found to increase the potency, and in some cases the efficacy, of agonists. This has been clearly shown for the $mGlu_1$ [36], $mGlu_5$ [37] and $mGlu_2$ [38,39] receptors as well as for the Ca^{2+}-sensing receptor [40–42]. When identified, the sites of action of these compounds have been found in the heptahelical domain of these homodimeric receptors. Recently, we reported that such compounds act by stabilizing the fully active state of the heptahelical domain, as shown by the full activation of the isolated heptahelical domain of $mGlu_5$ by the positive modulator DFB applied alone [43]. Accordingly, we proposed that such positive modulators can further stabilize the fully active state of the heptahelical domain of the full-length receptor, but this is possible only when the VFT is occupied by an agonist.

A series of positive modulators has also been described for the $GABA_B$ receptor [44,45]. According to our knowledge of the activation mechanism of this receptor, we proposed that such compounds act by stabilizing the active state of the heptahelical domain of the $GABA_{B2}$ subunit, since this domain is crucial for G-protein activation [46]. In agreement with this hypothesis, we recently reported that the positive modulator CGP7930 could directly activate $GABA_{B2}$ expressed alone, as well as a truncated version of this subunit in which the VFT domain is deleted [47]. As such, the isolated heptahelical domain of $GABA_{B2}$ can be directly activated by CGP7930. These data demonstrate that CGP7930 exerts its action by interacting in the $GABA_{B2}$ HD (Fig. 1).

10. Conclusion

The data presented above are consistent with GABA acting by stabilizing the closed state of $GABA_{B1}$ VFT, allowing a specific conformation of the VFT dimer. According to the known structure of the $mGlu_1$ VFT dimer, it is possible that the closure of the $GABA_{B1}$ VFT stabilizes a new relative orientation of the $GABA_{B1}$–$GABA_{B2}$ VFTs, leading to a direct association of lobe-II. This new conformation of the $GABA_{B1}$–$GABA_{B2}$ VFT heterodimer is expected to stabilize the new conformation of the dimer of heptahelical domains, and especially the active conformation of the heptahelical domain of $GABA_{B2}$.

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