Dimers of serotonin receptors: impact on ligand affinity and signaling

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Abstract

Membrane receptors often form complexes with other membrane proteins that directly interact with different effectors of the signal transduction machinery. G-protein-coupled receptors (GPCRs) were for long time considered as single pharmacological entities. However, evidence for oligomerization appeared for various classes and subtypes of GPCRs. This review focuses on metabotropic serotonin (5-hydroxytryptamine, 5-HT) receptors, which belong to the rhodopsin-like class A of GPCRs, and will summarize the convergent evidence that homo- and hetero-dimers containing 5-HT receptors exist in transfected cells and *in-vivo*. We will show that complexes involving 5-HT receptors may acquire new signal transduction pathways and new physiological roles. In some cases, these complexes participate in disease-specific deregulations, that can be differentially affected by various drugs. Hence, selecting receptor complex-specific responses of these heterodimers may constitute an emerging strategy likely to improve beneficial therapeutic effects.

Keywords: serotonin; receptor; homodimer; heterodimer; signal transduction.

1. Introduction

With more than 900 members encoded in the human genome, the seven-transmembrane domain (TMD) G protein-coupled receptors (GPCRs) represent the largest family of membrane proteins and are the targets of many therapeutic drugs. In recent years, several lines of evidence have revealed the formation of physiologically active oligomers of GPCRs [1, 2]. The initial discovery of the obligatory heterodimeric structure of the γ-aminobutyric acid B, $GABA_{B1}$-$GABA_{B2}$, receptor was triggered through various observations, including (i) the co-localization of mRNA for both receptor subtypes in any cell with a metabotropic $GABA_B$ receptor response and co-immunoprecipitation studies in brain tissue, and (ii) the requirement for co-transfection of cells with $GABA_{B1}$- and $GABA_{B2}$-encoding DNA constructs to produce a functional $GABA_B$ receptor expressed on the plasma membrane and able to recognize its ligand [3]. This $GABA_B$ receptor ($GABA_{B1}$-$GABA_{B2}$ heterodimer), the sweet taste receptor (T1R2-T1R3 heterodimer), and the umami taste receptor (T1R1-T1R3 heterodimer), are well described examples of GPCR obligatory heterodimers. They are all class C GPCRs characterized by large extracellular ligand binding (Venus flytrap) domains, wherein disulfide bonds covalently link together the receptor protomers (i.e. forming units of oligomers). In family C GPCRs, the metabotropic glutamate (mGlu) receptors have also been shown to exist as covalently-linked obligatory dimers [1].

Although well established in class C GPCRs, there is still a substantial debate about the class A GPCR dimers, which are characterized by a binding pocket for small ligands in the seven-helices transmembrane region. Several arguments have been provided for or against the *in-vivo* existence of class A GPCR dimers, see for example [4, 5]. Although several studies have asserted the existence of class A GPCR dimers, others have reported that most of them are mainly monomeric [6]. Nevertheless, new techniques of single-molecule analysis suggest that GPCRs are present at the cell surface in a dynamic equilibrium, with constant formation and dissociation of new receptor complexes [7, 8]. The possibility of heterodimers formation has been proposed not only within GPCRs responding to one same ligand, but also across different families responding to different ligands. These heterodimers may acquire unique biochemical and functional characteristics, including (i) new pharmacology, orthosteric or allosteric binding sites, (ii) new signaling in response to agonists or antagonists, and (iii) unexpected allosteric-like cross-talk between the protomers, as decribed in previous reviews [9-11].

This review will concentrate on metabotropic serotonin (5-hydroxytryptamine, 5-HT) receptors, which belong to the class A-1 GPCR family, display a widespread expression, and are involved in an important array of physiological and pathological processes. They are targeted by a rich pharmacology including many therapeutic drugs. Therefore, a better understanding of their regulation is critical to design new therapeutic compounds. Evidence for the formation of homodimers has been reported for various 5-HT receptor subtypes including 5-HT_{1B} [12], 5-HT_{1A} [13, 14], 5-HT_{2A} [15], 5-HT_{2C} [16], 5-HT_4 [17], and 5-HT_7 [18] receptors in heterologous expression systems. Other evidence pointing for the possibility of different 5-HT receptor subtypes to heterodimerize was later provided for 5-HT_{1B} with 5-HT_{1D} [12, 19], 5-HT_{1A} with 5-HT_7 [20], 5-HT_{1A} with 5-HT_{2A} [21], 5-HT_{2A}, with 5-HT_{2B} or 5-HT_{2C}, and 5-HT_{2B}, with 5-HT_{2C} [22] receptors. There are also observations supporting that some 5-HT receptors form heterodimeric complexes with GPCRs responding to other natural ligands than 5-HT. For example, the formation of heterodimers has been reported for 5-HT_{1A} with μ-opioid [23] and dopamine D2 receptors [24], for 5-HT_{2B} with angiotensin AT1 receptors [25], for 5-HT_{2A} with metabotropic glutamate mGlu2 receptors [26], D2 receptors [27], and cannabinoid CB1 receptors [28], and for 5-HT_{2C} with melatonin MT2 receptors [29].

In this review, we will briefly describe the techniques that allowed the identification of 5-HT receptor oligomers and their limitations. We will summarize the described interactions within 5-HT receptor family and between 5-HT receptors and members of other GPCR families.

In this context, we will show evidence for modified pharmacology and coupling that can originate from various mechanisms.

2. Summary of the main techniques used to identify GPCR dimers.

The evidence that class A GPCRs can form homodimers comes mainly from transfected overexpressing cells and a few from intact living cells or animal models. Various techniques that will only be briefly summarized here, have been discussed with more details in other reviews, for example [2, 8, 30]. Historically, there has been recurrent observations, by Western-blotting against GPCR of immunoreactive bands that could correspond to dimeric or higher order complexes. Importantly, in many cases, the receptor oligomers observed by Western-blotting were relatively resistant to sodium dodecyl sulphate (SDS) denaturation, raising the possibility that they were solubilization artifacts. However, covalent cross-linking before solubilization was also found to increase the proportion of oligomers observed upon Western-blotting. In addition to these observations in whole cell or tissue extracts, co-immunoprecipitation approaches using epitope-tagged receptors provided direct biochemical evidence to support the existence of receptor complexes and are still commonly used. Moreover, using fusion constructs between a GPCR and bioluminescent (luciferase) and/or fluorescent (green fluorescent) proteins, bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) have now been widely used to show very close vicinity (~10 nm) interpretable as dimerization of receptors mainly in living transfected cells [2], when saturation RET is provided. It has to be mentioned that single-point RET assays cannot be reliably interpreted. An additional technique to detect close vicinity of proteins, which has been successfully used to support the presence of GPCR oligomers, is the Proximity Ligation Assay (PLA) with a resolution of ~40 nm. This technique can be used in transfected overexpressing cells but also *in situ* in tissue sections, with the drawback of the poor antibody selectivity against GPCRs [31].

GPCRs are subject to clustering within the membrane [32], which increases their effective local concentration and hence increases the probability of interactions. Clusters of receptors and adaptor proteins can regulate localization, trafficking, signaling and ligand bias of 5-HT receptors [33]. The existence of clusters raises the question of the possible dynamics of these receptor complexes (i.e. monomer/dimer or dimer/tetramer equilibrium) and the functional outcome of receptor stimulation [11]. Such subcellular organization may be problematic for the interpretation of BRET/FRET, PLA, or co-immunoprecipitation studies. PLA has an effective resolution within the range of reported cluster scales, and thus cannot distinguish clustered from dimerized receptors, which can be exacerbated as the high effective concentration makes interactions more likely [34]. Such clustering has been reported for 5-HT_{1A} receptors in human brain slices [35]. Finally, recent techniques of single-molecule tracking and high-resolution imaging microscopy have initiated the view that GPCRs dimers may only be transient, and can be stabilized by agonists [36, 37]. These transient dimers are present at the cell surface in a dynamic equilibrium, with constant formation and dissociation of new receptor complexes [7, 8]. Using single-molecule imaging, dopamine D2 receptor was shown to form transient dimers with a lifetime of 68 ms, which was prolonged upon agonist addition by a factor of ~1.5, suggesting the possibility that dimers are only transiently formed [36]. Single particle tracking revealed that in extrasynaptic membrane, the 5-HT_{1B} receptor movement closely resembles free diffusion, whereas synaptic regions reduce diffusion and lead to confinement [38]. In this review, we will summarize the evidence of 5-HT receptor dimers, mentioning the limitation of the techniques used, although not distinguishing between permanent or transient interactions.

3. Homodimers of 5-HT receptors

The existence of homodimers of 5-HT receptors was first supported by a seminal study using SF9 cells infected by baculovirus to express 5-HT_{1B} or 5-HT_{1D} receptor subtypes. This study reported the observation, on denaturing Western blots, of species with a molecular mass corresponding to multiples of the monomeric receptor [12]. Evidence for homodimers have been subsequently reported for other 5-HT receptors including 5-HT_{1A} transiently overexpressed in N1E-115 neuroblastoma cells [13] or stably expressed in CHO cells [14], 5-HT_{2C} transiently overexpressed in CHO cells [16], 5-HT_{2A} and 5-HT_4 stably expressed in CHO cells [15, 17], and 5-HT_7 receptors stably expressed in HEK293 cells [18]. The combination of techniques strengthened the hypothesis of homodimerization of many 5-HT receptor subtypes including co-immunoprecipitation and FRET saturation for 5-HT_{1A} [13], time-resolved fluorescence anisotropy decay [14], co-immunoprecipitation and FRET or BRET saturation for 5-HT_{2A} [15, 22], co-immunoprecipitation, BRET ratio and BRET saturation, FRET efficiency and more recently fluorescence correlation spectroscopy in choroid plexus epithelial cells for 5-HT_{2C} [16, 22, 39-41], co-immunoprecipitation and single BRET value for 5-HT_4 [17], and receptor reactivation and single BRET value for 5-HT_7 [18]. Despite similar structure, the 5-HT_{2B} receptor that belongs to the same family as 5-HT_{2A} and 5-HT_{2C} receptors, has been shown to have a low propensity to form homodimers by co-immunoprecipitation and BRET saturation in COS7 cells [22].

3.1. Homodimers and intracellular trafficking of 5-HT receptors

Experimental evidence for dimerization of receptors before they reach the plasma membrane includes the detection of positive BRET saturation signals in intracellular membrane fractions [42]. Regarding 5-HT receptors, such evidence was indeed provided for homodimers of 5-HT_{2C} receptors by FRET efficiency [43]. In addition, dimers of 5-HT_{2C} receptors were observed to be co-localized with endoplasmic reticulum and subsequently with Golgi markers, and then at the plasma membrane, during receptor biosynthesis. Mutations that inhibited dimer formation also prevented cell surface targeting of the receptors [43]. These results indicate that receptor dimerization occurs within intracellular compartments, at least for some receptors.

3.2. 5-HT receptor stimulation and stabilization of homodimers

For 5-HT_{1A} receptors, studies showed that specific ligands regulate the degree of homodimer formation. Indeed, the agonist 8-OH-DPAT enhanced, while the antagonist methysergide diminished 5-HT_{1A} receptor homodimerization [13, 44]. For 5-HT_{2C}-receptors, cross-linking experiments indicated substantial conformational changes at the interface between protomers (see below) during stimulation by agonists [45]. However, other studies reported that agonist binding did not promote nor impede the stability of dimers of 5-HT_{2C} [46] or 5-HT_4 receptors [17]. Different sensitivity of the techniques used, cross-linking and fluorescence spectroscopy *vs.* BRET, may explain these apparent contradictory findings, although it is clear that formation of dimers may arise independently of any ligand.

3.3. Interface of 5-HT receptor homodimers

The potential interface involved in the formation of 5-HT receptor homodimers has been investigated. For the 5-HT_{2C} receptors, dimers possess an interface between the TMD4 and TMD5 of each protomer, which conformation is differentially modulated by stimulation with agonists and inverse agonists [45]. Thus, even though some studies claim that ligand binding does not change the stability of homodimers [46], this agonist binding is translated into conformational changes at the TMD4/5 interface and may interfere with G protein coupling. Mutagenesis studies have independently confirmed TMD4 as being also critically involved for

the 5-HT_4 receptor dimer formation [47]. It should also be noted that the currently available 5-HT receptor crystal structures have failed to yield dimer complexes [48-50], but a dimer complex was found in crystals formed around TM1-2 for inactive β2-adrenergic [51], κ-opioid receptors [52] and rhodopsin [53, 54], and around TM5 in active CXCR4 [55], and μ-opioid receptors [56].

3.4. Stoichiometry and asymmetry of G-protein association in 5-HT receptor homodimers

Cross-linking of functional 5-HT_{2C} dimers in which one protomer is covalently linked to Gq (fusion proteins) was used to determine the impact of various cysteine replacement mutations within the dimerization interface. This study indicated that formation and stabilization of dimers in the activated state differ according to whether Gq is fused to a protomer mutated in TMD4 or in TMD5 [45], supporting that the two promoters have distinct conformations. Thus, the 5-HT_{2C} dimer, at least in its active state, can be seen as a "conformational heterodimer", since there is an asymmetry in the interaction to Gq [45]. In addition, mutagenesis of Ser138 to Arg (S138R) in TMD3 produced a 5-HT_{2C} receptor incapable of agonist binding [39]. Expression of this S138R-inactive 5-HT_{2C} receptor mutant with wildtype 5-HT_{2C} receptors had no effect on ligand binding to 5-HT_{2C} receptors but inhibited basal and 5-HT-stimulated inositol phosphate signaling. Dimerization of mutant S138R with wildtype 5-HT_{2C} receptors was confirmed by FRET. Therefore, inactive 5-HT_{2C} mutant receptors inhibit wildtype 5-HT_{2C} receptor function by forming nonfunctional heterodimers [39]. Fluorescence correlation spectroscopy is a technique with single molecule sensitivity that was used to record fluorescence-tagged 5-HT_{2C} receptors diffusing within the plasma membrane of HEK293 cells and rat hippocampal neurons. Photon counting histogram analysis of the fluorescence correlation spectroscopy data was best described by a one-component dimer model without monomers or tetramers, suggesting that 5-HT_{2C} receptors that freely diffuse within the plasma membrane are dimeric [40]. Expression of combinations of 5-HT_4 receptors, in which both protomers were able to bind to agonists but only one was able to couple to Gαs-proteins revealed that activation of one protomer was sufficient to stimulate Gs-protein, although G-protein coupling efficiency was two times greater when both protomers of the dimer were activated by agonist binding in COS7 cells [57]. In this case, the twofold increase in activation suggests that binding to one protomer gives half-maximal activation, such that each protomer contributes equally toward the signaling process [58]. Altogether, these studies indicate that upon agonist occupancy, each protomer does not independently couple to G-proteins.

3.5. Allosteric cooperativity between 5-HT receptor protomers

Several reports indicate the possibility of allosteric cooperativity between 5-HT receptor protomers. Indeed, intracellular signaling cross-talk can be an explanation for observations that suggest allosteric regulation: as previously reported, cross-talk interaction between 5-HT_{1B} and 5-HT_{2B} receptors has been observed in the absence of close interactions as no co-localization could be observed [59]. This should be considered as a valid alternative when close interactions cannot be demonstrated. Data obtained using either the 5-HT_7 or 5-HT_{2A} receptor are strongly supportive of homodimeric functional structures, with little evidence of functional monomers (for review see [3, 58]). Evidence for an allosteric cooperativity between protomers in 5-HT_7 or 5-HT_{2A} homodimers comes from studies, which used a class of serotonergic compounds that antagonize the 5-HT_7 and 5-HT_{2A} receptor-signaling in a "pseudo-irreversible", i.e. non-covalent and wash-resistant, manner. The Hill coefficients of competition curves for antagonists were significantly different from unity for 5-HT_{2A} receptors stably expressed in CHO cells, supporting some allosteric cooperativity [60]. These compounds occlude the orthosteric binding site and thereby inactivate the receptor, producing an apparent irreversible inhibition

("inactivation") [3]. By using subsaturating concentrations of these "irreversible antagonists" in order to occupy only one of the two protomers, it was shown that 5-HT binding to the second protomer is not sufficient for G-protein activation [18, 60]. In contrast, the binding of a competitive antagonist to the orthosteric site of the second unoccupied protomer can release the "inactivator", allowing the receptor to return to an activable state.

These approaches support the existence of some form of allosteric cross-talk between protomers [3]: ligand binding to one protomer within a dimer influences the ability of the other protomer to signal, by inducing conformational changes that modulate ligand binding and/or asymmetric G-protein coupling.

4. Heterodimers of 5-HT receptors

4.1. Heterodimers of Gq-coupled protomers: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors

The 5-HT_{2A} and 5-HT_{2C} receptors are able to form homodimers [15, 22, 40, 41], whereas 5-HT_{2B} receptors overexpressed in COS7 cells are not [22]. However, when co-expressed in heterologous expression systems, saturating BRET experiments indicated that the formation of heterodimers is favored over homodimerization [22]. Signaling from these heterodimers is exclusively driven by the 5-HT_{2C} protomer. Indeed, in 5-HT_{2C}-containing 5-HT_{2A}-5-HT_{2C} and 5-HT_{2B}-5-HT_{2C} heterodimers, the binding of ligands selective for the 5-HT_{2A} or 5-HT_{2B} protomers was inhibited despite normal surface expression of these receptor subtypes. Concomitantly, 5-HT_{2A} or 5-HT_{2B} selective antagonists were unable to block 5-HT signaling in the presence of the 5-HT_{2C} protomer, whereas antagonists of the 5-HT_{2C} protomer were totally inhibiting 5-HT signaling in 5-HT_{2A}-5-HT_{2C} and 5-HT_{2B}-5-HT_{2C} heterodimers [22]. By contrast, signaling in 5-HT_{2A}-5-HT_{2B} heterodimers could be blocked either by 5-HT_{2A} or 5-HT_{2B} selective antagonists. To further investigate this issue, the 5-HT_{2C} receptor was deleted for its C-terminal tail (5-$HT_{2C\Delta Cter}$), still able to bind 5-HT but unable to activate Gq and to generate inositol phosphate production. Co-expression of 5-$HT_{2C\Delta Cter}$ with 5-HT_{2A} or 5-HT_{2B} protomers abolished 5-HT-dependent inositol phosphate accumulation by 5-HT_{2A}-5-$HT_{2C\Delta Cter}$ and 5-HT_{2B}-5-$HT_{2C\Delta Cter}$ heterodimers, due to their retained dimerization ability [22]. Conversely, co-expression of 5-HT_{2C} with 5-$HT_{2B\Delta Cter}$, a 5-HT_{2B} receptor impaired for Gq activation, had no impact on 5-HT_{2C} signaling since inositol phosphate production in response to 5-HT could still be abolished by a 5-HT_{2C} receptor-selective antagonist. These findings demonstrated that in 5-HT_{2A}-5-HT_{2C} or 5-HT_{2B}-5-HT_{2C} heterodimers, there is an asymmetry in Gq-protein coupling, and signaling from 5-HT_{2A} and 5-HT_{2B} protomers is blunted, as only the 5-HT_{2C} protomer can activate the Gq protein [22, 39]. This coupling seems related to a dominant negative effect of the 5-HT_{2C} protomer on ligand binding and coupling ability of the other partner (**Figure 1**).

The putative inhibitory role of 5-HT_{2C} on 5-HT_{2A}-receptor binding and coupling was validated *in-vivo* [22]. It is well established that 5-HT neurons receive ascending noradrenergic neuron afferents originating from the locus coeruleus (LC). These noradrenergic neurons in the LC are themselves negatively controlled by hypoglossal GABAergic interneurons, which express 5-HT_{2A} receptors. This neuronal network is thought to be primarily responsible for the inhibitory effects of 5-HT_{2A}-receptor agonists on noradrenergic neurons firing, and subsequently on 5-HT neurons activity [61]. AAV-5-$HT_{2C\Delta Cter}$ or AAV-GFP were injected in the LC by bilateral stereotaxic injections to infect GABAergic interneurons. As expected, in control mice infected with AAV-GFP, *in-vivo* electrophysiological recordings showed that injection of the 5-HT_{2A} agonist DOI induced a decrease of the firing rate of 5-HT neurons, as an indirect result of increased inhibition of noradrenergic neurons by 5-HT_{2A}-receptor bearing GABAergic interneurons in the LC. Importantly, this indirect inhibitory effect of DOI was

strongly reduced in mice injected with AAV-5-$HT_{2C\Delta Cter}$, consistent with reduced signaling of 5-HT_{2A} receptors due to dimerization with inactive 5-HT_{2C} in the LC GABAergic interneurons [22]. These results support the hypothesis of a dominant negative effect of the 5-HT_{2C} protomer over the 5-HT_{2A} protomer (and potentially 5-HT_{2B}) *in-vivo* and pinpoint the physiological relevance of a putative switch in the pharmacological profile of 5-HT_{2A} receptor expressing neurons, depending on their 5-HT_{2C} receptor expression levels.

4.2. Heterodimers of Gi/o- and Gs-coupled protomers: 5-HT_{1A}-5-HT_7

The G-protein-gated inwardly rectifying potassium channels (GIRKs or Kir3) constitute important physiological downstream targets of 5-HT_{1A} receptors activated by direct binding of βγ-subunits released from Gi/o proteins. Heterodimers between the Gi/o-coupled 5-HT_{1A} and the Gs-coupled 5-HT_7 receptors have been reported by FRET, TIRF microscopy, signal transduction in transfected N1E-115 neuroblastoma [20]. When 5-HT_7 receptors, which alone have no effect on GIRK activity, were expressed in Xenopus oocytes in addition to 5-HT_{1A} receptors and Kir3, both basal and 5-HT_{1A} receptor agonist-induced currents were significantly reduced according to the amount of injected 5-HT_7 plasmid. This inhibition of GIRK currents likely results from 5-HT_7 interacting with and directly modulating 5-HT_{1A} since it is not blocked by selective 5-HT_7 antagonist or reversed by 5-HT_7-targeting shRNA transfected in hippocampal neurons *in-vivo*. In addition to GIRK regulation, 5-HT_{1A} agonists are known to induce MAP kinases ERK1/2 phosphorylation. Interestingly, this signaling is enhanced if 5-HT_7 receptors are co-expressed, supporting that heterodimerization specifically favors activation of 5-HT_{1A}-receptor-mediated ERK signaling whereas it prevents 5-HT_{1A}-mediated activation of Gi/o-GIRK channel activity. In contrast, heterodimerization does not affect 5-HT_7-receptor-mediated Gs signaling, indicating a unidirectional effect of the 5-HT_7 protomer onto 5-HT_1 protomer coupling [20] (**Figure 2 Left**). Further experiments using 5-HT_7 receptor mutants for Gs signaling should be performed to confirm that they affect the switch to 5-HT_{1A}-ERK signaling. Thus, in 5-HT_{1A}-5-HT_7 heterodimers, dimerization markedly increases the ERK1/2 pathway at the expense of the ability of 5-HT_{1A} receptors to activate Gi/o-protein-dependent GIRKs. Asymmetric activation of ERK1/2 and preservation of Gs activation that takes place in 5-HT_{1A}-5-HT_7 heterodimers could have physiological implications. Indeed, since 5-HT_7 receptor expression is known to decrease in prefrontal cortex neurons during postnatal development while 5-HT_{1A} is maintained, the effect of 5-HT on these neurons may vary depending of age, with for example a stronger increase of GIRK currents and thus a stronger hyperpolarizing effect at adulthood than during development.

4.3. Heterodimers of Gi/o and Gq-coupled protomers: 5-HT_{1A}-5-HT_{2A}

The existence of heterodimers between the Gi/o-coupled 5-HT_{1A} receptor and the Gq-coupled 5-HT_{2A} receptor has been shown by BRET saturation assay in cotransfected cells [62]. *In-vivo*, PLA-positive clusters of 5-HT_{1A}-5-HT_{2A} receptors were identified in pyramidal cells of the CA1-CA3 regions in the rat hippocampus [62]. Upon prior forced swimming test, these clusters were reduced in the mouse cortex [21], whereas they were increased by 5-HT_{2A}-receptor antagonist and by low dose of the antipsychotic clozapine. A quantification of the proportion of the dimeric receptors seen by PLA would be further needed to validate these findings. Like for 5-HT_{2A}-5-HT_{2C}, 5-HT_{2B}-5-HT_{2C}, or among 5-HT_7 and 5-HT_{1A} homodimers that were presented above, transduction from 5-HT_{1A}-5-HT_{2A} dimers is not the sum of each protomer's coupling. Indeed, 5-HT_{1A}-receptor radioligand binding assays showed that the 5-HT_{2A}-receptor agonist TCB2 markedly reduced the binding of the 5-HT_{1A}-receptor agonist ipsapirone in membranes of the frontal lobe of the cortex. This suggests allosteric-like inhibitory interactions in the 5-HT_{1A}-5-HT_{2A} heterodimers, with a dominant effect of 5-HT_{2A} over 5-HT_{1A} protomer since activation of the 5-HT_{2A} protomer reduces the affinity of the 5-HT_{1A} protomer for its usual

ligands [62] (**Figure 2 Right**).

Altogether, these studies demonstrate that there can be an asymmetrical coupling in 5-HT-receptor heterodimers, with one protomer being dominant over the other (e.g. 5-HT_{2C} on 5-HT_{2A} and 5-HT_{2B}, 5-HT_{2A} on 5-HT_{1A} receptors). Moreover, the resulting coupling can differ from the main initial signal transduction of each protomer (e.g. 5-HT_{1A}-5-HT_7 dimers). Therefore, variations in the relative expression levels of different 5-HT receptor subtypes in the same cells can have a strong impact on the signaling and pharmacology of these receptors.

5. Heterodimers between 5-HT receptors and other GPCRs

Serotonin receptors can also form heterodimers with receptors from other families of GPCRs, having a different known endogenous ligand. When information is available, we will discuss whether these heterodimers bind both ligands, mainly one of them, or have new binding capacity.

5.1. Heterodimers among Gi/o-coupled protomers

5.1.1. 5-HT_{1A}-μ-opioid heterodimers: reciprocal activation and inhibition

The 5-HT_{1A} and the μ-opioid receptors are both able to activate the pertussis-sensitive Gi/o subfamily of heterotrimeric G proteins, and to induce ERK1/2 phosphorylation. In transiently co-transfected COS7 or HEK293 cells, μ-opioid-5-HT_{1A} receptors heterodimers have been detected by co-immunoprecipitation and by BRETmax determination [23]. Using fusion proteins of protomers with a "reporter" Gα protein (i.e. a subtype of Gα protein which, if free, is not activated by μ-opioid nor 5-HT_{1A} receptor), it was shown that both receptors can induce transactivation of the Gα protein fused to its partner protomer in stably transfected CHO cells. For ERK1/2 activation in these cells, reciprocal (i.e. from 5-HT_{1A} to μ-opioid receptors and from μ-opioid to 5-HT_{1A} receptors) transactivation was demonstrated, as well as reciprocal transinhibition by antagonists. In addition, the μ-opioid receptor-induced ERK1/2 phosphorylation was selectively desensitized by prolonged stimulation and activation of 5-HT_{1A} receptor [23]. Thus, functional heterodimerization of μ-opioid and 5-HT_{1A} receptors is effective in various cell lines and is characterized by reciprocal positive and negative trans-regulation of G protein coupling and of the ERK1/2 pathway. Considering the involvement of both receptors in pain control, this could have interesting therapeutic implications if their heterodimerization is confirmed *in-vivo*.

5.1.2. 5-HT_{1A}-D2 heterodimers: induction of a new signaling pathway

Dopamine D2 and serotonin 5-HT_{1A} receptors both signal through Gi/o, inhibiting adenylyl cyclase and negatively regulating cAMP production when expressed separately. Whereas classical antipsychotics like haloperidol are considered as acting mainly through blockade of D2 receptors, atypical antipsychotics are a class of neuroleptic drugs whose effects are mediated by their higher affinity for 5-HT_{2A} receptors than for D2 receptors, and possibly by other targets. For example, the prototypical atypical antipsychotic clozapine is a high affinity inverse agonist for 5-HT_{2A}, low affinity antagonist for D2, and a partial agonist for 5-HT_{1A} receptors [63]. Interestingly, D2-5-HT_{1A} heterodimers have been detected *in-vivo* by PLA experiments in the mouse prefrontal cortex [21]. A quantification of the proportion of the dimeric receptors seen by PLA would further validate these findings. If stably co-transfected in HEK293 cells, D2 and 5-HT_{1A} receptors form heterodimers that respond in an unexpected way to atypical antipsychotic drugs. Indeed, when cells co-expressing D2 and 5-HT_{1A} receptors were incubated with a single low dose of clozapine and of the 5-HT_{1A} receptor agonist 8-OH-DPAT, a large inhibition of cAMP production was observed, in comparison with the effect of this combination of drugs on cells expressing only one of these receptors. In cotransfected cells stimulated with

clozapine and 8-OH-DPAT, inositol phosphate production and ERK1/2 activation were detected whereas these drugs had no effect on cells expressing only one of these receptors, indicating new coupling pathways [24]. Thus, different second messenger pathways can be activated depending on whether D2 and 5-HT_{1A} receptors are expressed alone or together. The 8-OH-DPAT activation of 5-HT_{1A} receptors together with the D2 blockade by clozapine may trigger a conformational change in the D2-5-HT_{1A} heterodimer allowing the specific recruitment and activation of a Gq-protein [24]. The D2-5-HT_{1A} complex thus possesses functional properties distinct from homodimers. Moreover, clozapine has been shown to increase the level of D2-5-HT_{1A} heterodimers in the mouse prefrontal cortex, while the typical antipsychotic haloperidol, a high affinity D2 antagonist, decreased it [21]. Altogether, these data indicate that D2-5-HT_{1A} receptor heterodimers activate specific pathways, with inositol phosphate production and ERK1/2 activation, and that the level of D2-5-HT_{1A} heterodimers can be differentially regulated by various antipsychotic treatments.

5.2. Heterodimers among Gq-activating protomers: AT1-5-HT_{2B} heterodimers

In *ex-vivo* primary cultures of cardiac fibroblasts, endogenously expressed AT1 receptors for angiotensin II and 5-HT_{2B} receptors shared common Gq-protein-dependent signaling pathways leading to release of cytokines, which triggers cardiac hypertrophy [25]. Through metalloproteinases activation, responsible for Heparin-binding EGF-like growth factor (HB-EGF) shedding, a subsequent EGF-receptor transactivation is induced by either angiotensin II or 5-HT. These findings support that AT-1 and 5-HT_{2B} receptors share common EGF-receptor-dependent signaling pathways leading to cytokine release. Blockade of one of the two receptors prevents cytokine release induced by stimulation of the other receptor at a dose that is inactive to the other receptor in COS7 transfected cells, supporting transinhibition between 5-HT_{2B} and AT-1 receptors [25]. Confocal microscopy to assess colocalization and a pull-down assay in cotransfected COS7 cells demonstrated the interaction of 5-HT_{2B} and AT-1 receptors and their organization in heterodimeric complexes [25]. Signaling of each protomer is not modified by the heterodimerization but inhibiting one protomer is sufficient to block the Gq activation by the second protomer, supporting the presence of a single active G-protein per heterodimer.
A symmetrical Gq coupling between Angiotensin II and 5-HT signal has thus been found in respect to coupling to hypertrophic cytokine release in adult cardiac fibroblasts, with transinhibition and transactivation properties.

5.3. Heterodimers of Gi/o- and Gq-activating protomers

5.3.1. mGlu2-5-HT_{2A} heterodimers

Unbalanced levels of Gq-coupled serotonin 5-HT_{2A} and of Gi/o-coupled metabotropic glutamate 2 (mGlu2) receptors are suspected to be involved in psychosis. Pioneering work demonstrated using various approaches that 5-HT_{2A} receptor (Class A GPCR) and mGlu2 receptor (Class C GPCR) can associate in heterodimeric complex in HEK293 transfected cell lines and *in-vivo* in cortical neurons [26, 64]. These heterodimers display a remarkable inverse cross-regulation that has been brought to light in injected oocytes: in mGlu2-5-HT_{2A} heterodimers, Gi/o signaling from mGlu2 receptor is potentiated whereas the Gq signaling from 5-HT_{2A} receptors is inhibited. Moreover, agonists of one protomer suppress and, conversely, inverse agonists potentiate, the signaling of the endogenous ligand on the associated protomer [65]. Thus, the receptor complex establishes an optimal Gi/o-Gq balance in response to glutamate and 5-HT, with more Gi/o and less Gq signaling. Hallucinogenic drugs (LY341495, a mGlu2 inverse agonist and DOI, a 5-HT_{2A} receptor agonist) invert this balance (strong Gi/o decrease, and strong Gq increase). Antipsychotics (LY379268, a potent mGlu2 receptor agonist, and clozapine, a 5-HT_{2A} receptor inverse agonist), produce the opposite effect on the Gi/o-Gq balance (increasing Gi/o and inhibiting Gq) and noteworthy their behavioral effect in

mice requires expression of both receptors, underlying the functional relevance of these heterodimers *in-vivo* [65]. Formation of these complex involves TMD4 and 5 of mGlu2 receptors [64], a region known to be required for the homo- or heterodimerization of other GPCRs, as previously mentioned. Moreover, a mutagenesis-based approach identified three residues of the fourth TMD as being critical both for the heterodimerization in transfected cells, or eventually higher order complexes formation [66], and for the functional effect of psychoactive drugs in mice [26]. These signaling cross-talks in the mGlu2-5-HT_{2A} heterodimer likely explain the convergent effects of mGlu2- and 5-HT_{2A}-receptor-targeted antipsychotic compounds (and of mGlu2- and 5-HT_{2A}-receptor-targeted pro-psychotics and hallucinogens), which, in summary, regulate by different means but in the same direction the Gi/o-/Gq-coupling balance of these heterodimers [65].

5.3.2. D2-5-HT_{2A} heterodimers

The same Gq-coupled 5-HT_{2A} receptor has been shown to interact with the Gi/o-coupled D2 receptor using BRET and FRET techniques in cotransfected HEK293 cells [67, 68] and using *in-vivo* PLA in rat striatum [69]. Moreover, the formation of dimers can be regulated by some agonists and antagonists [67]. Regarding signaling, it was shown using a Nuclear Factor of Activated T cells (NFAT)-luciferase reporter gene assay and measures of the rise of intracellular calcium levels as read-outs of Gq activation, that activation of 5-HT_{2A} receptor by 5-HT or TCB2 classical agonists was enhanced by the concomitant activation of the D_2 receptor by its agonist quinpirole in cotransfected cells [68]. However, it was conversely found that stimulation of Gi/o coupling by a D2 agonist could be counteracted or inhibited by costimulation with a 5-HT_{2A}-agonist. This demonstrates the existence of a 5-HT_{2A} receptor-mediated trans-inhibition of D_2 receptors, i.e. imbalanced Gq over Gi/o signaling [68]. This latter kind of allosteric-like cross-talk reminds of the inhibiting effect of 5-HT_{2A} agonists onto mGluR2 signaling described above [65], and are ligand-dependent. Indeed, in contrast to classical 5-HT_{2A} receptor agonists, the hallucinogenic agonists LSD or DOI enhanced the inhibition of adenylate cyclase induced by the D2 agonist quinpirole [69] (**Figure 3 Left**). Theses heterodimers may be involved in the response to treatments for schizophrenia, as both D2 and 5-HT_{2A} receptors have affinity for classical and/or atypical antipsychotics [63]. Interestingly, it was shown that the 5-HT_{2A} mutant H452Y, which is a polymorphism existing in humans associated to resistance to the atypical antipsychotic clozapine, has lower dimerization capacity with D2 receptors than the wild-type receptor in cotransfected cells [70]. Altogether, the D2-5-HT_{2A} heterodimer is an example of asymmetrical and ligand-dependent cross-regulations, with transactivation of 5-HT_{2A} by D2 receptors agonists, and transinhibition of D2 by some, but not all, 5-HT_{2A} receptor agonists.

5.3.3. CB1-5-HT_{2A} heterodimers

The Gi/o-coupled CB1 and Gq-coupled 5-HT_{2A} receptors are both expressed in specific brain regions involved in emotion and memory, and it has been shown that the amnesic and anxiolytic effects of the CB1 receptor agonist delta9-tetrahydrocannabinol (THC) require the presence of Gq-coupled 5-HT_{2A} receptors. These functional interactions seem to be related to the existence of heterodimers that have been detected in an exhaustive study using several combined techniques including BRET saturation, PLA verified in knockout animals, bimolecular complementation assays, both *in-vivo,* in behavioral tests, and in transfected cell lines [28] or in *ex-vivo* primary cultures of human olfactory epithelial cells [71]. Functionally, pharmacological data on CB1-5-HT_{2A} heterodimers support the existence of a switch in 5-HT_{2A}-receptor coupling from Gq to Gi/o proteins (thus agonists for any of the two receptors will inhibit cAMP production) and of an antagonist transinhibition (antagonist binding to one receptor blocks signaling of the other receptor) (**Figure 3 Right**) [28]. In addition, stimulation

of both receptors by agonists reduces their pERK1/2 signaling. The CB1-5-HT_{2A} heterodimer appears thus to behave in an asymmetric manner, as only the properties of the 5-HT_{2A} receptor are switched (from Gq to Gi/o-coupling) by heterodimerization. Indeed, heterodimer formation was inhibited by a blocking peptide with the sequence of either TM5 or TM6 (but not TM7 acting as negative control) of CB1 receptors, fused to HIV-TAT (a HIV peptide that can determine cellular uptake of nonpermeant molecules), as revealed by a loss of fluorescence in bimolecular fluorescence complementation assays and confirmed by PLA assays. The cross-antagonism at the level of cAMP, ERK1/2 phosphorylation, and Akt phosphorylation was not observed in cotransfected cells treated with TM5 or TM6 peptides, but the single activation of individual receptors was preserved in the presence of these peptides [28]. The relation between the amnesic effects of THC and heterodimers is also supported by a human study comparing cannabis users and non-users. Although the acute effect of cannabis on memory was not directly addressed in this study, the authors observed a positive correlation between the amount of heterodimers in primary cell cultures from the subjects and their consumption of cannabis, and a negative correlation with their memory performance [71]. In contrast, some of the effects of CB1 receptors agonists, which are therapeutically interesting like their antinociceptive effects, are preserved in *Htr2a*$^{-/-}$ mice and not inhibited by heterodimerization-blocking peptides in wild-type mice, indicating that they do not require heterodimerization of CB1 with 5-HT_{2A} receptors [28]. This dissociation between some beneficial, heterodimer-independent, effects of THC and its detrimental, heterodimer-dependent, amnesic properties may open new perspectives in CB1-receptor-targeted therapies.

5.3.4. MT2-5-HT_{2C} heterodimers

Close interaction between Gi/o-coupled melatonin MT2 receptors and Gq-coupled 5-HT_{2C} receptors has been demonstrated by co-immunoprecipitation and BRET, both in HEK293 transfected cells and in cells from human cortex and hippocampus, and their existence as functional heterodimers is supported by pharmacological experiments [29]. In cells expressing MT2-5-HT_{2C} heterodimers, melatonin can activate the Gi/o pathway, reducing cAMP levels, but 5-HT is without effect on cAMP levels. In contrast, MT2 receptors can transactivate 5-HT_{2C} receptors as melatonin binding to MT2 receptors enhances the Gq-coupled response to 5-HT. Moreover, when expressed alone, only a minor fraction of 5-HT_{2C} receptors is expressed at the cell surface, which is enhanced by expression of MT2 receptors. Pretreatment of cells with 5-HT_{2C}-receptors antagonists completely blocked the melatonin-induced inositol phosphate production, supporting a transactivation mechanism [29]. The MT2/5-HT_{2C} heterodimer appears thus to behave in an asymmetric manner as the properties of the two protomers are differently affected by heterodimerization.

In conclusion, as for 5-HT receptors heterodimers, protomers can maintain their coupling to the same G proteins or not, and pharmacology and signaling of heterodimers can be drastically different from those of the monomeric protomers.

6. Concluding remarks

Although initially proposed as a general mechanism required for proper trafficking of GPCRs to the plasma membrane, the ability of GPCRs to interact as dimers appears to have a large diversity of other functional impacts. From the evidence for the existence of homo- and heterodimers involving various GPCRs, it is clear that heterodimerization can deeply change the modality of signaling of the receptors involved. In particular, 5-HT receptors can form dimers that enable communication between individual receptor protomers, and a general consensus seems to indicate that the transduction originates from a single protomer per dimer. Functional heterodimerization and transactivation of receptor heterodimers can be detected

without modification of protomer signaling as shown in 5-HT_{2B}-AT1 heterodimers. The heterodimers may also behave in an asymmetric manner as the properties of the two protomers are differently affected by heterodimerization and can lead to a switch in their coupling like in 5-HT_{2A}-D2 dimers. Recent studies on 5-HT receptor complexes revealed the existence of allosteric-like cross-talk between protomers, i.e. the presence of one protomer can influence the ability of the other protomer to signal, either by directly promoting conformational changes across the TMD4/5 dimer interface, which will switch G-protein interactions as in 5-HT_{1A}-5-HT_7 dimers or via asymmetric G-protein coupling as in 5-$HT_{2A/2B}$-5-HT_{2C} dimers. Allosteric-like cross-talk between heterodimers may be influenced by the conformational selectivity of the ligand, agonist, inverse agonist, or antagonist; this phenomenon can be observed at distinct levels: (i) at the level of orthosteric binding site where the activation of one protomer can modify ligand binding to other protomer; (ii) at the level of conformation of protomer TMD, opening new intracellular effector interacting sites; and (iii) at signaling complexes modulating receptor-interacting transduction proteins.

Receptor heterodimers are complex molecular entities that result from combinatorial evolution. The newly uncovered functional properties of 5-HT receptors in dimeric interactions could be used for therapeutic purposes. Further work is needed to define the biophysical and cellular processes that modulate heterodimeric assembly, the appropriate ratios of the two protomers, the ligand occupancy effect of one protomer to the binding of the second protomer. The probabilistic effect of a ligand on a defined cell type must be defined by the respective expression levels of various receptor protomers and effectors expressed by this cell type. Targeting these heterodimers constitutes an emerging strategy to select receptor-specific responses and is likely to be useful in achieving specific beneficial therapeutic effects.

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Contributors
LM, CB, and AR wrote and corrected this manuscript.

Conflict of interest
The authors declare no competing financial interests. All authors have approved the final article.

Figure legends

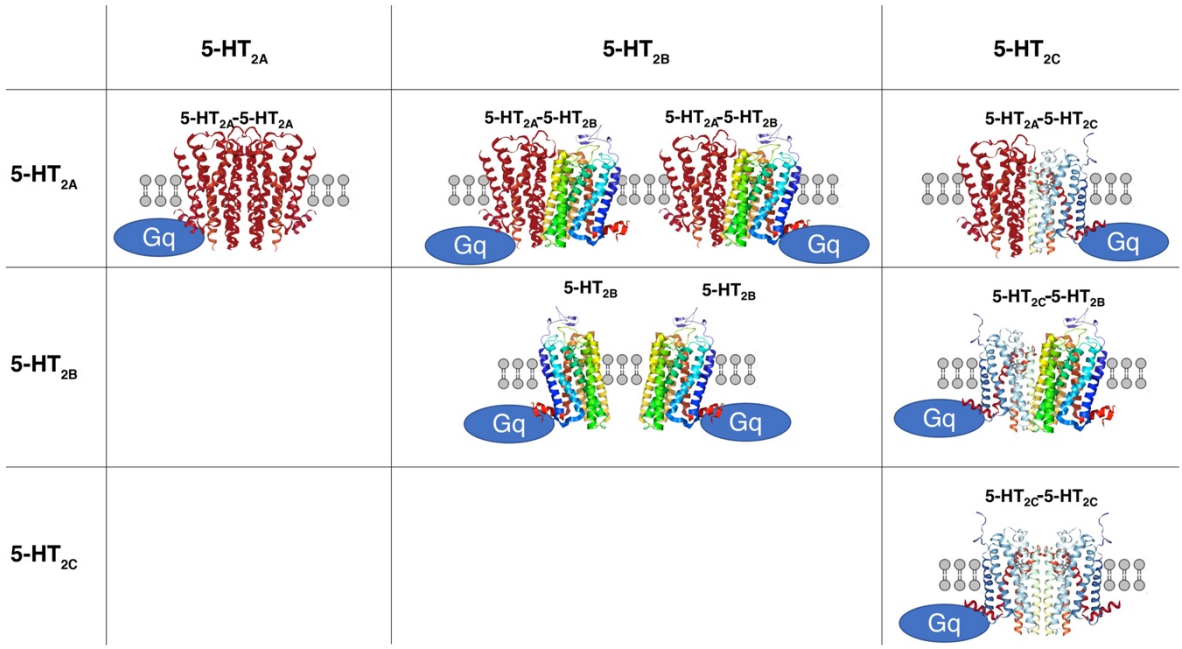

Figure 1: Combinations of 5-HT$_2$ monomers and their coupling to Gq signaling. Whereas in 5-HT$_{2A}$-5-HT$_{2B}$ heterodimers both 5-HT$_{2A}$ or 5-HT$_{2B}$ protomers can activate Gq-signaling, 5-HT$_{2C}$-containing heterodimers signal only from 5-HT$_{2C}$ protomer and show blunted Gq-coupling of 5-HT$_{2A}$ or 5-HT$_{2B}$ protomers. This asymmetry in Gq activation seems related to a dominant negative effect of the 5-HT$_{2C}$ protomer on ligand binding and coupling ability of the other partner as revealed using inactive, C-terminus-deleted receptors. All structures are from RCSB PDB (www.rcsb.org).

Figure 2

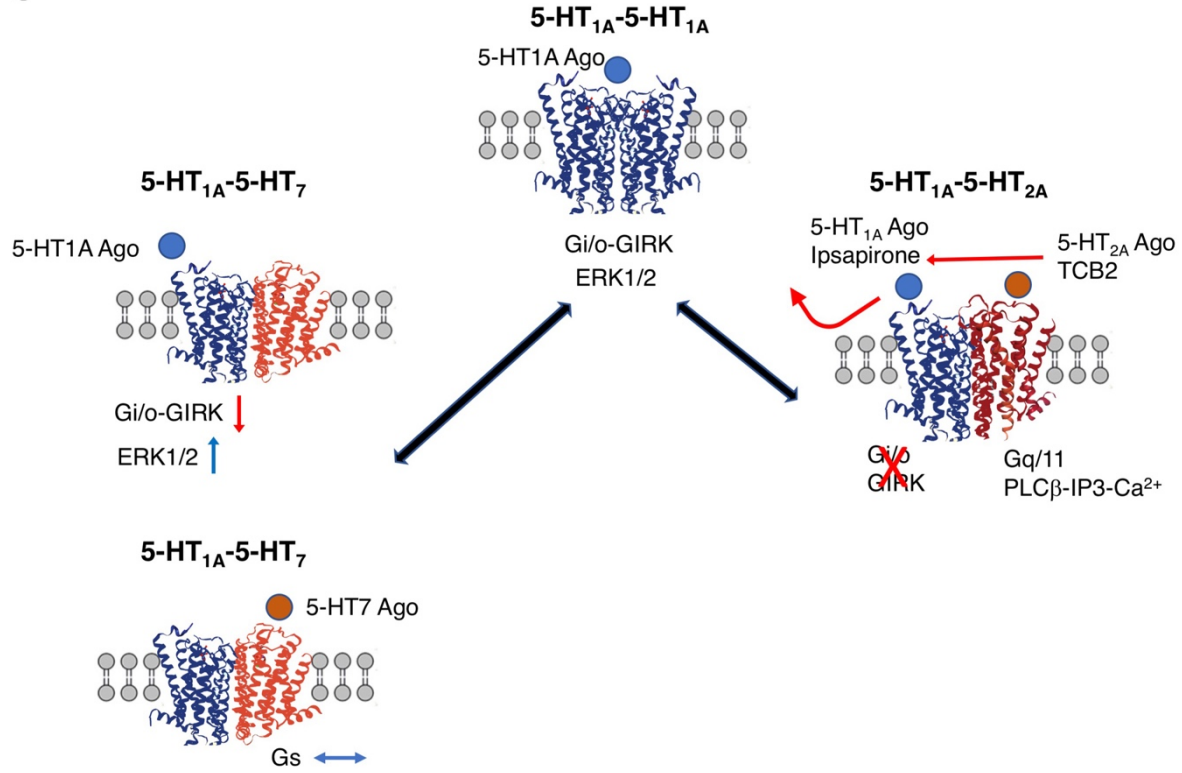

Figure 2: 5-HT_{1A} receptor-containing 5-HT receptor dimers. Top: Homodimers of 5-HT_{1A} receptors are coupled to Gi/o, which stimulates GIRK, and to ERK1/2 activation. **Left:** When 5-HT_{7} and 5-HT_{1A} receptors are coexpressed, the effect of 5-HT_{1A} receptor agonists on Gi/o-GIRK is reduced, but its effect on ERK1/2 phosphorylation is enhanced. This heterodimerization does not, in contrast, affect 5-HT_{7}-receptor-mediated Gs signaling. **Right:** When 5-HT_{1A} receptor is coexpressed with 5-HT_{2A} receptor, the 5-HT_{2A}-receptor-agonist TCB2 reduces the affinity of the 5-HT_{1A}-receptor-agonist ipsapirone for the 5-HT_{1A} receptor sites, leading to a reduction of 5-HT_{1A} receptor signaling and favoring 5-HT_{2A} over 5-HT_{1A} receptor coupling. Ago=agonist. All structures are from RCSB PDB (www.rcsb.org).

Figure 3

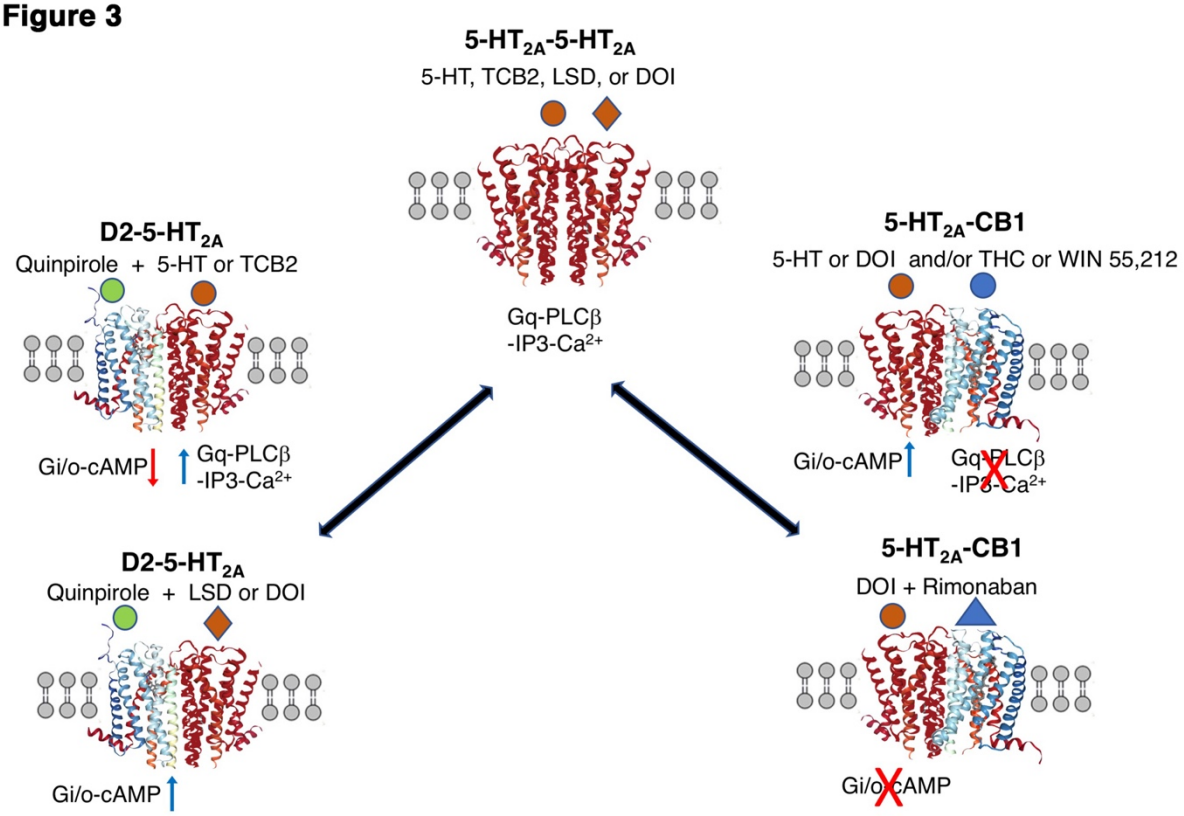

Figure 3: Examples of 5-HT$_{2A}$ receptors and other GPCRs dimers. Top: Homodimers of 5-HT$_{2A}$ receptors are coupled to Gq (and thus to IP$_3$ and Ca^{2+}). **Left:** When D$_2$ receptors, which are coupled to Gi/o and cAMP inhibition, are coexpressed with 5-HT$_{2A}$ receptors, which are coupled to Gq, classical agonists of 5-HT$_{2A}$ receptors 5-HT or TCB2, decrease the cAMP inhibition by the D$_2$ receptor agonist quinpirole, but conversely quinpirole enhances the Gq-dependent intracellular Ca^{2+} rise induced by 5-HT$_{2A}$ receptors agonists. In contrast, the hallucinogenic 5-HT$_{2A}$ receptor agonists LSD and DOI significantly enhance the effect of the D2 receptor agonist on inhibition of cAMP through Gi/o. **Right:** Upon coexpression with the CB1 receptor, which individually is coupled to Gi/o, 5-HT$_{2A}$ appears to switch its coupling from Gq to Gi/o. Thus, upon stimulation either by 5-HT$_{2A}$ receptor agonists, 5-HT or DOI or by CB1 receptor agonists, THC or WIN 55,212, only Gi/o-dependent pathways are activated. In addition, CB1 receptor antagonist Rimonaban blocks signaling of the 5-HT$_{2A}$ receptor, supporting the existence of an antagonist transinhibition. Similar transinhibition was also observed for the 5-HT$_{2A}$ receptor antagonist MDL 100,907 on CB1 receptor-agonist (not illustrated). All structures are from RCSB PDB (www.rcsb.org).

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