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Running title: Dimerization among 5-HT_2 receptor subtypes

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Abstract

The serotonin receptor subtypes 2 comprises 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} that are Gαq-coupled receptors and display distinct pharmacological properties. Although co-expressed in some brain regions and involved in various neurological disorders, their functional interactions have not been studied yet.

We report that 5-HT_2 receptors can form homo and heterodimers when expressed alone or co-expressed in transfected cells. Co-immunoprecipitation and bioluminescence resonance energy transfer studies confirmed that 5-HT_{2C} receptors interact with either 5-HT_{2A} or 5-HT_{2B} receptors. Although heterodimerization with 5-HT_{2C} receptors does not alter 5-HT_{2C} Gαq-dependent inositol-phosphate signaling, 5-HT_{2A}- or 5-HT_{2B}-receptor-mediated signaling was totally blunted. This feature can be explained by a dominance of 5-HT_{2C} on 5-HT_{2A} and 5-HT_{2B} receptor binding: in 5-HT_{2C}-containing heterodimers, ligands bind and activate exclusively the 5-HT_{2C} protomer. This dominant effect on the associated protomer was also observed in neurons, supporting a physiological relevance of 5-HT_2 receptors heterodimerization *in-vivo*. Accordingly, exogenous expression of an inactive form of the 5-HT_{2C} receptor in the *Locus ceruleus* is associated with decreased 5-HT_{2A}-dependent noradrenergic transmission.

These data demonstrate that 5-HT_2 receptors can form functionally asymmetric heterodimers *in-vitro* and *in-vivo* that must be considered when analyzing the physiological or pathophysiological roles of serotonin in tissues where 5-HT_2 receptors are co-expressed.

Many members of the G-protein-coupled receptor (GPCR) family have the capacity to form homo- or hetero-oligomers with biochemical and functional characteristics, including receptor pharmacology, signaling and regulation, which are unique to these oligomeric conformations. These GPCR oligomers have been found not only to occur within a type of GPCR but also across different families and subtypes (1,2).

Metabotropic serotonin (5-hydroxytryptamine, 5-HT) subtype 2 receptors (5-HT_2), which belong to the class A-1 GPCR family, display a widespread expression in the nervous system and are involved in an important array of physiological and pathological processes. The 5-HT_2 subfamily consists in three Gαq/$Gα_{11}$-coupled receptors, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which mediate excitatory neurotransmission (3). Interestingly, 5-HT_2

subtypes coexist in multiple areas of the brain (4,5). For example, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors are co-expressed in GABAergic interneurons and in a subpopulation of pyramidal neurons of the prefrontal cortex (PFC) (6-8), in dopaminergic neurons of the ventral tegmental area (VTA) (9,10) and 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors are expressed in pro-opiomelanocortin (POMC) neurons of the hypothalamic arcuate nucleus (11). Although 5-HT$_2$ receptors are similar in structure, there are differences in their pharmacology and signaling outputs (12). It has been reported that 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors can function as stable homodimers (13-16) whereas the existence of 5-HT$_{2B}$ homodimers has not been documented yet. Dimers have also been reported for other 5-HT receptors, including 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_4$, and 5-HT$_7$ receptor subtypes in heterologous expression systems (13,17-20). In addition, there are observations suggesting that the 5-HT$_2$ receptors subfamily can form heterodimeric complexes with other types of GPCRs. For example, the formation of heterodimers has been reported for the 5-HT$_{2A}$ with mGluR2, D$_2$-dopamine and CB1 receptors (21-23), for 5-HT$_{2C}$ with ghrelin receptors (GHS-R1a) (24) and MT2 receptor (25), for 5-HT$_{1A}$ with μ-opioid (26) and adenosine A$_{2A}$ receptors (27), and for 5-HT$_{2B}$ with angiotensin AT1 receptors (28).

As mentioned above, oligomerization can occur between receptors of different GPCR families (i.e. 5-HT and dopamine for example) but also within the same family. A seminal study reported the identification of the first heterodimer between the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes (18). However, no significant pharmacological differences were reported between homo and heterodimers for these closely related 5-HT receptor subtypes. Recently, a study identified other 5-HT receptor heterodimers with functional implication: heterodimers between 5-HT$_{1A}$ and 5-HT$_7$ receptors have been reported to regulate GIRK channel activity in heterologous systems and in hippocampal neurons (29). Heterodimerization was found to inhibit 5-HT$_{1A}$-mediated activation of Gαi and GIRK channel activity, without affecting 5-HT$_7$-receptor-mediated signaling, indicating a unidirectional dominant effect of the 5-HT$_7$ protomer. Of note, cross talks between 5-HT receptors have been reported without obligatory physical interaction. For instance, co-expression of 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors influences the internalization pathways and kinetics of both receptors without heterodimerization (i.e. lack of FRET signal) (30).

To date, neither the basic pharmacological profiles of putative 5-HT$_2$ heterodimers nor their signaling properties have been characterized. We specifically addressed this issue here, by studying the interactions between the three members of the 5-HT$_2$ subfamily and their functional consequences *in-vitro* and *in-vivo*. Using co-immunoprecipitation and Bioluminescence Resonance Energy Transfer (BRET) approaches, we found that 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ form heterodimers when co-expressed in heterologous expression systems. Although 5-HT$_{2C}$-containing heterodimers did not show alterations in coupling properties, the signaling of associated 5-HT$_{2A}$ or 5-HT$_{2B}$ protomers was blunted, while 5-HT$_{2C}$ protomer maintained its signaling properties. Moreover, no blunting occurred in 5-HT$_{2A/2B}$ heterodimers. We next showed that this asymmetry in Gαq-protein activation was related to a dominant effect of the 5-HT$_{2C}$ protomer on ligand binding to the other partner. Using AAV-mediated exogenous expression of a 5-HT$_{2C}$ receptor truncated C-tail mutant (5-HT$_{2C\Delta Cter}$) in brain regions expressing endogenous 5-HT$_{2A}$ receptor, we also observed a blunting effect of this inactive 5-HT$_{2C}$ receptor leading to a complete binding inhibition of 5-HT$_{2A}$ selective ligand. Accordingly, this lack of ligand binding was associated with impaired 5-HT$_{2A}$-induced excitatory neurotransmission in neurons expressing the 5-HT$_{2C}$ inactive protomer.

Results

Interactions between 5-HT$_2$ receptors

The putative formation of heterodimers between 5-HT$_2$ receptor subtypes was investigated using BRET and co-immunoprecipitation experiments (Fig. 1). The coding region of Renilla luciferase (Rluc, BRET donor) or the yellow variant of the green fluorescent protein (YFP, BRET acceptor), were fused in phase downstream of the coding region of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. Saturation BRET experiments were conducted in HEK293 cells co-transfected with constant amounts of BRET donor plasmids and increasing amounts of BRET acceptor plasmids. In case of a close proximity between the investigated partners, hyperbolic saturation of the BRET signal is expected (see methods). Hyperbolic curves were indeed obtained when 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors were tested for self-association

(5-HT$_{2A/2A}$ and 5-HT$_{2C/2C}$, respectively, Fig. 1a), confirming previous studies showing that these receptors are able to homodimerize in transfected cells (13,14). Noteworthy, the same experiment with 5-HT$_{2B}$ BRET pairs led to a linear plot, consistent with a bystander (non-specific) BRET and thus with the absence of self-association. Hyperbolic curves were also obtained with 5-HT$_{2A}$ and 5-HT$_{2C}$ BRET pairs (5-HT$_{2A/2C}$), 5-HT$_{2B}$ and 5-HT$_{2C}$ BRET pairs (5-HT$_{2B/2C}$) and 5-HT$_{2A}$ and 5-HT$_{2B}$ (5-HT$_{2A/2B}$) BRET pairs (Fig. 1b), suggesting that 5-HT$_2$ receptors can form heterodimers in intact cells. BRET$_{50}$ values (values of YFP/Rluc for half-maximal BRET) reflect the propensity of association between the investigated proteins. Interestingly, BRET$_{50}$ values for heterodimeric association are significantly lower than those measured for homodimeric association of 5-HT$_2$ subtypes, suggesting that in native cells expressing more than one 5-HT$_2$ receptor subtype, heterodimerization is favored over homodimerization (Fig. 1b).

The physical interaction between these receptor subtypes was confirmed by co-immunoprecipitation studies in the same cells, using epitope (FLAG)- or GFP-tagged proteins (Fig. 1c). Consistent with BRET data, FLAG-5-HT$_{2C}$ receptor co-immunoprecipitated with 5-HT$_{2A}$-GFP and 5-HT$_{2C}$-GFP receptors. In complementary experiments FLAG-5-HT$_{2B}$ receptor co-immunoprecipitated with 5-HT$_{2A}$-GFP and 5-HT$_{2C}$-GFP receptors.

Impact of dimerization of 5-HT$_2$ receptors on signaling

We next examined the consequence of 5-HT$_2$ receptors heterodimerization on agonist-induced-Gαq activation. The 5-HT$_2$ receptors consistently activate the PLC-β pathway in native tissues and heterologous cells (3,12). We first determined whether 5-HT$_2$ receptor signaling was altered when expressed in the presence of other 5-HT$_2$ receptors (Fig. 2). Dose-response curves of 5-HT$_2$-mediated production of IP in response to 5-HT or the partial agonist DOI in cells expressing 5-HT$_{2A}$, 5-HT$_{2B}$ or 5-HT$_{2C}$ receptors and combination were analyzed using the operational model (31) to determine the Gαq coupling efficiency of single receptors and heterodimers (Fig. 2). No significant difference in Gαq coupling efficiency as determined by the transduction coefficient (τ/KA) (32) was found among the groups, supporting a lack of major difference in coupling efficiency between individual 5-HT$_2$ receptors to Gαq activation and no modification of this coupling efficiency by heterodimers.

In the absence of highly selective, subtype specific 5-HT$_2$ agonists, we next determined whether agonist-induced 5-HT$_2$ heterodimer signaling was altered in the presence of selective antagonist of the other protomer (Fig. 5). Affinity and selectivity of antagonists were first tested in transfected cell to define the optimal concentration that nearly fully inhibits each individual receptor and avoid cross-reactivity (100 nM-Fig. 3). Dose-response curves of 5-HT$_2$-mediated IP accumulation in response to 5-HT stimulation allowed determining the minimal agonist concentration inducing maximal IP response (1 µM, Fig. 4a). When expressed individually, 5-HT$_2$ receptors displayed significant 5-HT-stimulated IP production (Fig. 5a); in the presence of selective antagonists (MDL100970 for 5-HT$_{2A}$, RS127445 for 5-HT$_{2B}$ and RS102221 for 5-HT$_{2C}$ receptors), the agonist-stimulated IP response was reduced to basal level in cells expressing the cognate 5-HT$_2$ receptor (Fig. 5a).

In the 5-HT$_{2A/2B}$ heterodimers, the 5-HT$_{2A}$ receptor contributed to the activation of the Gαq/PLC-β pathway in response to agonist, as demonstrated by the inhibitory effect of MDL100907 (Fig. 5a); reciprocally, 5-HT$_{2B}$ receptor was also contributing to signaling when co-expressed with 5-HT$_{2A}$ receptors, as demonstrated by the inhibitory effect of RS127445 (Fig. 5a). By contrast in the 5-HT$_{2B/2C}$ heterodimers, only the 5-HT$_{2C}$ receptors continued mediating signaling, because blocking selectively the 5-HT$_{2B}$ receptors with RS127445 did not inhibit the 5-HT-mediated IP accumulation (Fig. 5a), although it can be blocked only by selective 5-HT$_{2C}$ receptor antagonists RS102221 or SB242084 (Fig. 5a, Fig. 4c,d). The 5-HT$_{2A}$-mediated IP accumulation was similarly blunted by expression of 5-HT$_{2C}$ receptor, as MDL100907 had no significant inhibitory effect on IP production, but RS102221 had (Fig. 5a).

The same effect was reproduced for different agonist 5-HT or Nor-(+)-Fenfluramine (NDF) (Fig. 4e,f) and in all tested cell lines, Cos-7 (Fig. 2), HEK293 (Fig. 4-5), CHO (Fig. 6c) or LMTK (Fig. 6a,b) and thus likely independent of agonists or cell-specific effectors. Since it was shown above that under the same expression conditions these receptor isoforms constitute heterodimers, a plausible explanation for the

observed effects is that heterodimerization with 5-HT$_{2C}$ prevents the ability of 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors to signal, whereas in case of heterodimerization of 5-HT$_{2A}$ with 5-HT$_{2B}$ receptors both 5-HT$_{2A}$ and 5-HT$_{2B}$ protomers contribute to signal.

These results are consistent with a model where in 5-HT$_{2A/2C}$ and 5-HT$_{2B/2C}$ heterodimers, only the 5-HT$_{2C}$ protomer couples to the G protein, while preventing coupling of the associated protomer. To investigate this issue, we co-expressed 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor with a 5-HT$_{2C}$ receptor deleted for the C-terminal tail (5-HT$_{2C\Delta Cter}$) (Fig. 5b), a mutant receptor incapable of activating Gαq and stimulating IP production in response to 5-HT stimulation (Fig. 5b). Confirming the hypothesis, in cells expressing 5-HT$_{2A/2C\Delta Cter}$ and 5-HT$_{2B/2C\Delta Cter}$ heterodimers, IP accumulation was dramatically reduced (Fig. 5b). In addition, co-expressing 5-HT$_{2C}$ receptors with 5-HT$_{2B\Delta Cter}$, a 5-HT$_{2B}$ receptor similarly deleted for the C-terminal tail and impaired for IP accumulation, had no impact on 5-HT$_{2C}$ signaling (Fig. 5b) since it was still sensitive to RS102221, supporting that only the 5-HT$_{2C}$ protomer couples to the Gq protein. We confirmed the proper plasma membrane expression of the 5-HT$_{2C\Delta Cter}$ and 5-HT$_{2B\Delta Cter}$ receptor constructs compared to the respective WT form, using a radioligand-binding assay on nonpermeabilized cells. Expression of 5-HT$_{2B}$, 5-HT$_{2B\Delta Cter}$, 5-HT$_{2C}$ or 5-HT$_{2C\Delta Cter}$ receptors in HEK cells, lead to 37, 50, 20 and 53 % of construct surface expression compared to total whole cell membrane expression (permeabilized cells), respectively, suggesting that ΔCter constructs are express properly at the cell membrane. Moreover, BRET assay confirm the ability of the two ΔCter constructs to associate with 5-HT$_{2A}$, 5-HT$_{2B}$, or 5-HT$_{2C}$ protomers (Fig. 7a-d). To confirm these findings, in cell expressing constant 5-HT$_{2B}$ and variable amount of 5-HT$_{2C}$ receptors, the progressive decrease of 5-HT$_{2C}$ expression level allowed the recovery of 5-HT$_{2B}$ receptor-dependent signaling (the fraction of IP accumulation inhibited by the 5-HT$_{2B}$-selective RS127445), likely due to reduced formation of 5-HT$_{2B/2C}$ heterodimers (Fig. 7e).

Impact of dimerization on 5-HT$_2$ receptor binding properties

The results above are thus consistent with a model where in 5-HT$_{2A/2C}$ and 5-HT$_{2B/2C}$ heterodimers, only the 5-HT$_{2C}$ protomer couples to the Gq protein, while preventing ligand binding of the associated protomer. To examine this hypothesis, agonist (^{3}H-5-HT- or ^{3}H-LSD) radioligand binding assays were conducted in the presence or absence of 5-HT$_{2C}$ receptor and of increasing concentrations of selective antagonists (MDL100907 for 5-HT$_{2A}$, RS127445 for 5-HT$_{2B}$, RS102221 for 5-HT$_{2C}$) in cells expressing similar quantities of receptors (Fig. 8). RS127445 could compete for ^{3}H-5-HT (Fig. 8a) or ^{3}H-LSD (Fig. 8c) binding in cells only expressing 5-HT$_{2B}$ receptors, whereas no competition was observed in cells expressing 5-HT$_{2B/2C}$ receptors. In contrast, RS102221 could displace ^{3}H-5-HT (Fig. 8b) or ^{3}H-LSD (Fig. 8d) binding in cells expressing either 5-HT$_{2C}$ receptors alone or 5-HT$_{2B/2C}$ receptors. Similar finding were obtained for 5-HT$_{2A}$ receptors (Fig 8e,f). Noteworthy, ^{3}H-5-HT or ^{3}H-LSD binding was comparable in cells expressing 5-HT$_{2C}$ alone or 5-HT$_{2C}$ receptor and another 5-HT$_2$ isoform, indicating that the presence of 5-HT$_{2C}$ receptors almost completely inhibits ligand binding to co-expressed 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. Strictly similar findings were observed with 5-HT$_{2B\Delta Cter}$ receptor (Fig. 8g,h) or with radiolabeled antagonist (^{3}H-Mesulergine) binding experiments (Fig. 8i-k) supporting that 5-HT$_{2C}$ protomers somehow masked the ligand binding site of the other protomer in 5-HT$_2$ heterodimers, independently of the coupling ability of the complex.

A possibility is that the presence of a 5-HT$_{2C}$ protomer is sufficient to compete with plasma membrane accessibility of either 5-HT$_{2A}$ or 5-HT$_{2B}$ protomers. Surface expression of both receptors was measured by cytometry using a Pacific blue conjugated antibody directed against extracellular 3xFLAG tagged 5-HT$_2$-YFP constructs, YFP being intracellular and reflecting total expression of the receptor (Fig. 9). Ratio of cell surface receptors over total obtained (Mean of pacific-blue signal/Mean of GFP) for each condition and normalized as percentage of 5-HT$_{2A}$ (Fig. 9a,b) or 5-HT$_{2B}$ receptor alone. 5-HT$_{2A}$ (Fig. 9c) or 5-HT$_{2B}$ (Fig. 9d) receptors in cells co-transfected with 5-HT$_{2C}$ receptors was not significantly different compared to control (single expression), although, co-expression of 5-HT$_{2A}$ receptors slightly increased cell surface expression of 5-HT$_{2B}$ receptors. Decreased ligand binding or Gαq-coupling of 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors co-expressed with 5-HT$_{2C}$ receptor cannot be explained by reduced cell surface expression.

Heterodimer properties in-vivo

The inhibitory role of 5-HT$_{2C}$ on 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor binding and coupling, was next analyzed in neurons. Mouse prefrontal cortex (PFC) neurons, which have been shown to express 5-HT$_{2A}$ receptors (33,34), were used as a model system. Accordingly, exogenous 5-HT$_{2C\Delta Cter}$ expression in PFC, upon infection with adeno-associated viruses carrying a 5-HT$_{2C\Delta Cter}$ construct (AAV-5-HT$_{2C\Delta Cter}$) was associated with an increase of [^{3}H]-Mesulergine able to bind to endogenous 5-HT$_{2A}$ and exogenous 5-HT$_{2C\Delta Cter}$ receptors (Fig. 10a). However, a dramatic inhibition of endogenous 5-HT$_{2A}$ receptor-dependent ligand binding compared to AAV mediated GFP expression was also observed (absence of MDL100907-induced [^{3}H]-Mesulergine displacement) (Fig. 10a). We then investigated whether 5-HT$_{2C}$ receptor was also able to suppress 5-HT$_{2A}$ receptor-dependent signaling *in-vivo* via heterodimerization. We thus used the same adenoviral delivery system to express the inactive 5-HT$_{2C\Delta Cter}$ protomer in adrenergic *Locus ceruleus* (LC) neurons. Previous studies reported that the 5-HT$_2$ receptor agonist DOI decreases the firing rate of 5-HT neurons in the dorsal raphe (DR) nucleus (35,36). This inhibitory response was completely blunted in 5-*Htr*$_{2A}^{-/-}$ mice, and was attenuated (30%) by inducing the loss of noradrenergic neurons with the DSP4 neurotoxin (37). Indeed, activation by DOI of 5-HT$_{2A}$ receptors expressed on GABAergic interneurons of the LC (38,39) decreased noradrenergic tone thereby limiting its excitatory influence on DR 5-HT neurons. A corollary of these functional interactions between monoaminergic neurons is a significant decrease of DR 5-HT neuronal activity. We then used this paradigm as a functional read-out for norepinephrine transmission upon expression of the functionally inactive 5-HT$_{2C\Delta Cter}$ protomer (Fig. 10b-d). Bilateral stereotaxic injections of AAV- 5-HT$_{2C\Delta Cter}$ IRES GFP or AAV GFP in the LC were performed one month before *in-vivo* electrophysiological recordings of DR 5-HT neurons (Fig. 10b). Mouse brains samples were examined to verify the distribution of adenovirus-encoded 5-HT$_{2C\Delta Cter}$ and GFP in the LC (Fig. 10c). In mice injected with control AAV (AAV GFP), increasing DOI concentrations induced a progressive decrease of the firing rate of DR 5-HT neurons (Fig. 10d), as observed by previous reports (37). This inhibitory response was strongly reduced (50%) in AAV-5-HT$_{2C\Delta Cter}$ injected mice (Fig. 10d), consistent with an impaired signaling of 5-HT$_{2A}$ receptors. These data indicate that 5-HT$_{2C\Delta Cter}$ receptors inhibit 5-HT$_{2A}$ receptor-mediated signaling in neurons, likely via their heterodimerization with 5-HT$_{2A}$ receptors.

Discussion

Our data demonstrate that 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors heterodimers are favored and able to form *in-vitro* and *in-vivo*. Although, heterodimerization with 5-HT$_{2C}$ receptors does not apparently modify the coupling ability to the Gαq pathway, it appears to blunt binding properties of 5-HT$_{2A}$ and 5-HT$_{2B}$ protomers. These findings can be interpreted as uni-directional dominance of 5-HT$_{2C}$ over the two other protomers in 5-HT$_{2A/2C}$ and 5-HT$_{2B/2C}$ heterodimers. The masking effect exerted by 5-HT$_{2C}$ protomer is specific, since no similar functional consequences could be observed in 5-HT$_{2A/2B}$ heterodimers. This dominance appears to be independent of ligands (agonists or antagonists), of the cell type, or of the plasma membrane accessibility of the different protomers.

Uni-directional activating or inhibiting effects of protomers on ligand binding or coupling properties of the cognate partner were reported for few GPCR heterodimers (40). For example, G protein coupling of angiotensin AT1 receptor is inhibited in AT1/AT2 or AT1/APJ (the receptor for apelin) heterodimers (41,42), but not in AT1/5-HT$_{2B}$ heterodimers (28). In this context, impaired coupling can be caused by different mechanisms. Ligand occupancy of one protomer of the heterodimer dimer can induce conformational changes of the binding pocket of the second protomer resulting in ligand binding inhibition (43). The inhibitory effect of one protomer can also occur in the absence of its ligand. For example, the long carboxyterminal tail of the orphan receptor GPR50 indirectly prevents the binding of melatonin to the MT1 melatonin receptor in GPR50-MT1 heterodimers (44).

Here we show that 5-HT$_{2C\Delta Cter}$, which lacks the capacity of activating Gαq proteins, yet inhibits both Gαq coupling and ligand binding of associated 5-HT$_2$ protomers. These data indicate that asymmetric "sequestration" of Gαq by the carboxy-terminal tail of 5-HT$_{2C}$ receptor or steric hindrance associated does not account for the observed effect. Consistent with previous observations, showing that a 5-HT$_2$ protomer can

promote conformational changes across asymmetrical dimer interface (45,46), 5-HT$_{2C}$-dependent inhibition of 5-HT$_{2A}$ and 5-HT$_{2B}$ protomer binding might occur via a similar mechanism.

Interestingly, when 5-HT$_{2C}$ receptors were co-expressed with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors, binding and functional studies indicated the absence of 5-HT$_{2A}$- or 5-HT$_{2B}$-dependent signaling, consistent with the absence of 5-HT$_{2A}$ or 5-HT$_{2B}$ monomers or homodimers at the cell surface. The analysis of BRET$_{50}$ values in saturation BRET experiments, which reflect the propensity of association between protomers, suggest that in cells expressing simultaneously 5-HT$_{2C}$ and another 5-HT$_2$ receptor isoform, heterodimers are more likely to form than homodimers or monomers and our cytometry analysis revealed that there was no impact of plasma membrane accessibility of 5-HT$_2$ receptor protomers.

Whereas many reports addressing the capacity of GPCRs to form heterodimeric complexes were based on investigations performed in transfected cells, only few studies could document the existence and the functional significance of these complexes *in-vivo* (21,29,47). Here, we show that the expression of exogenous 5-HT$_{2C\Delta Cter}$ receptors in PFC is associated with a dramatic inhibition of 5-HT$_{2A}$ receptor-dependent ligand binding, suggesting that both receptors are able to form heterodimers *in-vivo*. Accordingly, we found that virus-mediated exogenous expression of the inactive protomer 5-HT$_{2C\Delta Cter}$ reduces 5-HT$_{2A}$ receptor-dependent neuronal inhibition of DR 5-HT neurons. Altogether, these results support that 5-HT$_{2C}$ receptors are able to form functional heterodimers with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors when co-express in neurons and pinpoint the physiological relevance of a putative switch in the pharmacological profile of these neurons, depending on 5-HT$_{2C}$-expression levels.

In line with this hypothesis, a recent study showed that high phenotypic motor impulsivity was associated with a diminished PFC 5-HT$_{2A}$/5-HT$_{2C}$ receptors complex (8). Independent findings about 5-HT$_{1A}$/5-HT$_7$ heterodimers (29) demonstrated that heterodimerization markedly decreases the ability of the 5-HT$_{1A}$ receptor to activate G-protein in hippocampal neurons. Interestingly, since 5-HT$_7$ receptor expression decreases during postnatal development, the concentration of heterodimers and their functional significance should change over time. Similarly, it has been demonstrated that the expression of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors varied during prenatal and early postnatal development (48), which represent critical period for synaptogenesis and synaptic refinement (49). The 5-HT$_{2C}$ receptor is transiently express in the cortices from P10 to P28, while the 5-HT$_{2A}$ receptor expression increases progressively from P3 to P21, reaching the adult level. During the early postnatal period, pyramidal cells of layer V of the prefrontal cortex are profoundly depolarized by 5-HT, an effect that is mediated by the activation of 5-HT$_{2A}$ receptor. However, 5-HT$_{2A}$ receptor-induced depolarization declines with increasing age (50,51). In light of our results, 5-HT may progressively bind to the 5-HT$_{2C}$ protomer leading to a switch in signaling pathway activation and downstream membrane depolarization.

Operational model revealed no significant differences in the coupling ability to the Gαq pathway between membranes expressing 5-HT$_2$ receptors alone and membranes expressing 5-HT$_2$ heterodimers. However, heterodimerization may affect 5-HT$_2$ receptors signaling in many other ways: in addition to the activation of Gαq, Gαi/o or Gα$_{12/13}$ proteins, activation of phospholipase A2, phospholipase D (ERK$_{1/2}$ or β-arrestin-dependent pathways) (52,53) can be mediated by 5-HT$_2$ receptors. All these pathways could be differentially affected by heterodimers. Interacting proteins like PSD-95, MUPP-1, RSK2 (54) on 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors might also been affected by the dimerization with the 5-HT$_{2C}$ receptor. For example, the 5-HT$_{2C}$ and 5-HT$_{2B}$ receptors co-expressed in POMC neurons of the arcuate nucleus (11) are involved in feeding behavior but are not necessarily dependent on Gq coupling. Additional studies on other signaling pathways should be performed to ascertain putative changes in 5-HT signaling associated with heterodimerization.

Another consequence of the dominant effect of 5-HT$_{2C}$ receptor is a putative misleading link between pharmacological brain mapping expression and function of 5-HT$_2$ receptors. For example, in the *Globus pallidus*, the expression of 5-HT$_{2A}$ receptors, revealed by immunolabeling, had previously gone unnoticed due to absent or weak ligand binding. The 5-HT$_{2A}$ receptor immunostaining of the amygdala complex and in Purkinje cells of the cerebellum

(55,56) was also inconsistent with negative radioligand binding results previously reported (5,57,58). The reason for this expression discrepancy is unclear but knowing that all these nuclei also express 5-HT_{2C} receptors (4-6,55,59), a plausible explanation would be a pharmacological shielding of 5-HT_{2A} by 5-HT_{2C} receptors, without consequence for the immunocytochemistry detection. More importantly, pharmacological studies targeting 5-HT_{2A} or 5-HT_{2B} receptors in a physiological or a pathological context, like schizophrenia, food intake disorders, or depression, should take into account this dominant effect when brain areas of interest (i.e. frontal cortex, hypothalamus, raphe nucleus respectively) also express 5-HT_{2C} receptors (8,11,47,60,61). This could lead to a misinterpretation of experimental results due to a mismatch between the consistent expression of 5-HT_{2A} or 5-HT_{2B} receptor in particular nucleus, dedicated to a specific physiological function, and a lack of effect of selective pharmacological compounds. The putative formation of these heterodimers must now be taken into account when analyzing physiological and/or pathophysiological role of 5-HT in tissues co-expressing 5-HT_{2C}/5-HT_{2A} or 5-HT_{2C}/5-HT_{2B} receptors.

Experimental Procedures

Animals
Male mice (8–12 weeks old) used in these experiments are in 129S2/SvPAS background (Charles River, France). Animals were housed in groups of 3-5 per cage. The temperature was maintained at 21±1°C, under 12/12h light/dark. Food for laboratory mice (SAFE A03, France; 3200 kcal/kg, moisture 12%, proteins 21%, lipids 5%, carbohydrates 52%, fibers 4%, and mineral ash 6%) and water were available *ad libitum*. Electrophysiological recording and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health's 'Guide for the care and use of Laboratory animals,' and the European Communities Council European Communities Directive 86/609 EEC). All experiments involving mice were approved by the local ethical committee (N° 1170.01).

Plasmid constructs.
Human 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2CINI} receptor cDNAs were subcloned into the p513 vector, a derivative of the pSG5 mammalian expression vector (62), which replicates in SV40 T antigen-expressing cells and drives 5-HT receptors expression under the control of the SV40 early promoter. The C-terminal truncated after amino-acid 370 for the human 5-HT_{2C} and 393 for the human 5-HT_{2B} receptors (63) were generated by PCR deletion mutagenesis. 5-HT receptor coding regions were amplified from their respective cDNAs using appropriate sense and antisense primers. The fragments were then subcloned in frame in either a plasmid encoding C-terminus YFP (Clontech/BD Biosciences, Mountain View, CA), N-terminus FLAG (Clontech/BD Biosciences, Mountain View, CA) or Renilla luciferase (Rluc). The coding regions of all constructs were entirely sequenced.

Cloning of Mouse 5-HT_{2C} Receptor cDNA
From the full-length cDNA sequence of 5-HT_{2CVNV} and $_{VNI}$ receptors from mice that we previously reported (60), we selected the VNV edited isoforms of 5-HT_{2C} receptor for AAV injections, as VNV has been shown to be the most prevalent in C57BL/6J and 129S1/SvImJ mice (64). The C-terminus of the mouse 5-HT_{2CVNV} was truncated after the amino-acid 370.

Cell culture
COS-7, HEK293, LMTK$^-$ and CHO cells were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Biowest) and 1% penicilin/streptomycin (Sigma, St Louis, MO, USA), in 9-cm dishes (*Falcon*). Cells were incubated at 37°C in a 5% CO_2 atmosphere. Cells were 80% confluent when transfected with 10µg of DNA using Nanofectin (PAA), according to the manufacturer's protocol, in an antibiotic-free medium. Four hours later, medium was replaced with fresh medium. Twenty-four hours after transfection, cells were incubated in serum free medium for membrane radioligand binding or trypsinized (Trypsin 1X 0.05% EDTA, Invitrogen) and plated onto 24-well plates for inositol phosphate (IP) accumulation.

AAV-mediated local LC or PFC 5-$HT_{2C}\Delta$Cter expression.
Adeno-associated virus (AAV9)-expressing GFP ($7{,}5x10^{12}$ virus molecules/ml) and 5-$HT_{2C\Delta Cter}$ (IRES) GFP ($8{,}8\ x10^{12}$ virus molecules/ml) under the control of the synapsin promoter (UNC Vector Core, Dr. R. Jude Samulski, Chapel Hill)

were stereotaxically injected into the LC (100 nL) or the PFC (600 nL). Mice were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg) and fixed in a stereotaxic apparatus. A burr hole was drilled above the LC (coordinates: 5.4 mm posterior to bregma, 1 lateral to midline) or the PFC (coordinates: 1.8 mm anterior to bregma, 2 lateral to midline). Stereotaxically guided injections were made through the hole in the dorsal surface of the cranium (3 mm deep for the LC and 1 mm for the PFC). Glass capillary tubes were pulled (HEKA pipette puller PIP5) and tips broken to 40 µm diameters. After 4 weeks recovery and viral expression, the LC and PFC AAV-injected mice were used for electrophysiological or binding experiments, respectively. Proper viral infection was verified by GFP detection on brain fixed sections.

In vivo electrophysiology of DR 5-HT neurons.
Mice were anaesthetized with chloral hydrate (400 m/kg; i.p.) and mounted in a stereotaxic frame. Additional anesthesia (50–100 mg/kg; i.p.) was given as necessary to maintain a full anesthetic state, characterized by the absence of response to a tail pinch. Body temperature was maintained at 37 °C throughout the experiments using a thermistor-controlled heating pad (Astro-Med, Elancourt, France). The extracellular recordings of the 5-HT neurons in the dorsal raphe (DR) were performed using single-barreled glass micropipettes (Stoelting, Dublin, Ireland) pulled on a pipette puller (Narishige, Tokyo, Japan) preloaded with a 2 M NaCl solution. Their impedance typically ranged between 2.5 and 5 MΩ. The single-barreled glass micropipettes were positioned 0.2–0.5 mm posterior to the interaural line on the midline and lowered using a hydraulic micropositioner (Kopf Instruments) into the DR, usually attained at a depth between 2.5 and 3.5 mm from the brain surface. To increase the signal-to-noise ratio, we used a current amplifier (BAK Electronics, Mount Airy, MD, USA) connected to the active filter Humbug (Quest scientific, DIPSI, Châtillon, France). The presumed DR 5-HT neurons were then identified according to the criteria of Aghajanian and Vandermaelen (65), that is, a slow (0.5–2.5 Hz) and regular firing rate and long-duration (2–5 ms) bi- or triphasic extracellular waveform. Neuronal activity was recorded in real time using Spike2 software (Cambridge Electronic Design, Cambridge, UK), which was also used to analyze neurons offline. For all dose–response curves, only one neuron was recorded and tested from each animal.

[^{3}H]Radioligands and drugs
[^{3}H]*Myo*-inositol (51.0 Ci/mmol), [^{3}H]-Mesulergine (99 Ci/mmol), [^{3}H]-5-Hydroxytryptamine (80.0 Ci/mmol) (5-HT), [^{3}H]-lysergic acid diethylamide (50.0 Ci/mmol) were purchased from Perkin Elmer. (+)-norfenfluramine hydrochloride (Sigma-Aldrich, France), RS127445 (Tocris), SB242084 (Sigma-Aldrich), 5-hydroxytryptamine (5-HT), (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI), mesulergine hydrochloride (Tocris), MDL100907 (Tocris), RS102221 (Tocris), SB242084 (Tocris) were dissolved in DMSO as stock solution (1mg/ml).

[^{3}H]Inositol Phosphate (IP) Accumulation Assay
Twenty-four hours before the experiment, cells were incubated in 24-well plates overnight with 20 nM of [^{3}H]*myo*-inositol diluted in an inositol-free medium (BME, Lonza, Basel, Switzerland). Just before receptor stimulation, medium was replaced by Krebs-Ringer-Hepes buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 10 mM Hepes, 10 mM glucose, pH 7.4) supplemented with 20 mM LiCl to prevent IP_1 degradation. Cells were stimulated in duplicate in a final volume of 500 µl for 2 h. The experiment was stopped by replacing the stimulation medium with 10^{-3} M formic acid at room temperature for 20 min, and at 4°C overnight. Thus, IP_1 accumulated from IP_3 and IP_2 hydrolysis, was released from lysed and fixed cells. The accumulated IP_1 was eluted on anion exchange columns (Bio-Rad AG-1X8, BioRad Laboratories, Hercules, CA, USA) with 0.2 M ammonium formiate in 0.1 M formic acid. Scintillation cocktail (Ultima Gold XR, Perkin Elmer) was added to the eluted [^{3}H]IP sample, and radioactivity was counted in a Beckman Coulter scintillation counter. At least three independent experiments were performed in triplicate.

HTRF IP accumulation
COS-7 cells were transfected with 3µg of DNA (1:1 ratio for co-transfection) in 6 wells plate using Genjuice transfectant reagent in complete medium. Twenty-four hours later cells were trypsinized (Trypsin 1X 0.05% EDTA; Invitrogen) and plated in 96 wells plate (30,000 cells/well). The next day, complete medium is replaced by serum free medium. The day of the

experiment, media was replaced by stimulation buffer with LiCl in order to prevent IP_1 degradation (NaCl 146 mM, KCl 4.2mM, $MgCl_2$ 0.5mM, $CaCl_2$ 1mM, Hepes 10 mM, Glucose 5.5 mM, LiCl 50 mM, pH 7.4). Cells were stimulated during two hours at 37°C with different concentration of full and partial agonists, 5-HT and DOI respectively (10^{-11} to 10^{-6} M in stimulation buffer). Stimulation solution was replaced by lysis buffer (IP one HTRF Kit, Cisbio, France) during one hour. Lysates were distributed to 384 well plates and IP was labeled using HTRF reagents. The assay is based on a competitive format involving a specific antibody labeled with Terbium Cryptate (donor) and IP coupled to d2 (acceptor). After one-hour incubation with HTRF reagent the plate was read using Mithras LB940 plate reader according to manufacturer instructions. At least five independent experiments were performed in duplicate.

Operational model

Data obtain in HTRF were transform in fmoles of IP per mg protein per well using standard dose response curve. Operational model first described by Black and Leff in 1983 was used in order to calculate coupling efficiency of each receptor in each condition for full (5-HT) and partial (DOI) agonists. This model allowed to determine if the dimerization could affect the coupling efficiency of the complex to Gαq/phospholipase C (PLC)/IP pathway. The power of a ligand to activate a specific cellular pathway is represented by the τ/K_A or RA for activity ratio. These values are extracted from the description of the model:

Response $A = [A]^n\tau^n Em / [A]^n\tau^n+([A]+K_A)^n$

E_m=maximal response

n = slope

K_A= equilibrium constant dissociation of the agonist-receptor complex or affinity

τ= efficacy

$RA = \tau^n ((2+\tau^n)^{1/n}-1) E_m/K_A (1+t^n)$

Operational model conditions show that for dose-response curves of unit slope (n=1), it can be seen that RA reduces to the term $E_m (\tau/K_A)$. Ratios of these terms for particular agonists cancel the E_m term and are therefore system independent. A theoretically complete term to describe the power of a ligand to active a cellular pathway is τ/K_A, which incorporates both elements of efficacy and affinity. Considering that the most common difference between systems is receptor density, ratios of τ/K_A account for these and are system-independent measures of the relative capacity of ligands to activate a specific pathway.

Radioligand Binding Assays on PFC.

PFC freshly dissected from mouse brain was homogenized with 50 ml of ice-cold buffer per g of wet tissue containing 50 mM Tris and 5 mM $MgCl_2$ (pH 7.4). The homogenate was centrifuged for 20 min at 15,000 *g*. The pellet was resuspended and centrifuged under the same condition three times. Membrane preparations were resuspended in binding buffer to obtain a final concentration of 1 mg of protein/well. Radioligand binding assays were set up in a 96-well plate (1.2 ml/well capacity) using 5 nM [^{3}H]mesulergine (PerkinElmer Life) and increasing concentration of MDL100907 for 60 min at room temperature. [^{3}H]mesulergine ligand choice was based on his selective 5-HT_2 receptors binding properties. This allows the simultaneous measurement of 5-HT_{2A} and 5-HT_{2C} receptors expression, using the specific cold antagonists.

Membrane Radioligand Binding Assay.

Membrane binding assays were performed on transfected cells plated in 10 cm dishes. Cells were first washed with PBS and scraped into 10 ml of PBS on ice, then centrifuged for 5 min at 1,000 *g*. Cell pellets were dissociated and lysed in 2 ml of binding buffer (50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4) and centrifuged for 30 min at 10,000 *g*. Membrane preparations were then resuspended in binding buffer to obtain a final concentration of 0.2-0.4 mg of protein/well. Aliquots of membrane suspension (200 µl/well) were incubated with 25 µl/well of [^{3}H] radioligand at a final concentration near the K_D value, diluted in binding buffer, and 25 µl/well of increasing concentrations of homologous or heterologous compound (from 10^{-11} to 10^{-5} M, diluted in binding buffer) in 96-well plates for 60 min at room temperature. Membranes were harvested by rapid filtration onto Whatman GF/B glass fiber filters (Brandell) pre-soaked with cold saline solution and washed three times with cold saline solution to reduce non-specific binding. Filters were placed in 6-ml scintillation vials and allowed to dry overnight. The next day, 4 ml of scintillation cocktail were added to the samples, which were counted as before. Data in dpm were converted to fmoles and normalized to protein content (ranging from 0.1 to 1 mg/well). At least

three independent experiments were performed in duplicate.

Nonpermeabilized Whole-Cell Radioligand Binding Assay.
Cells expressing 5-HT_{2B}, 5-$HT_{2B\Delta Cter}$, 5-HT_{2C} or 5-$HT_{2C\Delta Cter}$ receptors were plated in 24-well clusters. Twenty-four hours before the experiment, the cells were incubated in serum-free medium overnight. The next day, the medium was replaced by 400 µl/well of Krebs-Ringer-Hepes buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM NaH2PO4, 1.2 mM MgSO4, 10 mM Hepes, 10 mM glucose, pH 7.4). Then, 50 µl of ^{3}H-mesulergine were diluted in Krebs-Ringer-Hepes buffer at a final concentration between half the Kd and the Kd for each 5-HT receptor. The radioligand was competed with 50 µl of increasing concentrations of nonradioactive ligand, also diluted in Krebs-Ringer-Hepes buffer. Cells were then incubated for 30 minutes at room temperature and then washed twice on ice with cold PBS. Washed cells were solubilized by the addition of 500 µl of SDS 1%. The next day, 4 ml of scintillation cocktail were added to the samples, and the radioactivity was counted using a scintillation counter (Beckman Coulter). Data in disintegrations per minute were converted to fmoles and normalized to protein content (0.2–0.4 mg of protein/well). At least three independent experiments were performed in duplicate.

Co-immunoprecipitation.
Flag-tagged 5-HT_{2B} and 5-HT_{2C} receptor cDNA constructs were transfected in Cos-7 cells with various combination of YFP-tagged-5-HT_2 receptors. After forty eight hours, cells were washed in PBS, sonicated and solubilized in lysis buffer [75 mM Tris, 2 mM EDTA, 12 mM $MgCl_2$, 10 mM CHAPS, protease inhibitor cocktail EDTA free, pH 7.4] during 12 hours at 4°C. Lysates were centrifuged at 12,000 *g* during 30 min at 4°C. Immunoprecipitations were performed using EZview Red FLAG M2 Affinity Gel (Sigma) according to manufacturer's recommendations. Immunoprecipitated proteins and 100 µg of total proteins were combined with Laemmli buffer, heated at 70°C for 10 min and run on 10% Bis-Tris gel. Immunoblots were probed with rabbit anti-gfp (Abcam) or anti-flag rabbit (Sigma) antibodies diluted 1/2000 and immunoreactivity was revealed using secondary antibody coupled to 680 nm fluorophores using the Odyssey LI-COR infrared fluorescent scanner.

BRET assays.
Bioluminescence Resonance Energy Transfer (BRET) assays were performed according to published methods (66). Briefly, HEK cells (5 x 10^5 per well of a 6-well plates) were transfected with 30-100 ng plasmid DNA coding for the BRET donor (5-HT_2-Luc) and increasing amounts of BRET acceptor plasmids (5-HT_2-YFP; 100-4000 ng per well). Twenty-four hours after transfection, cells were washed in PBS, detached using 10 mM EDTA in PBS, centrifuged (1,400 *g* for 5 minutes), resuspended in Hank-balanced salt solution and distributed in 96-well plates (PerkinElmer plates; 10^5 cells per well). After addition of the luciferase substrate, coelenterazine-h (5 µM final concentration), luminescence and fluorescence were measured simultaneously (at 485 and 530 nm, respectively) in a Mithras LB940 plate reader. The BRET ratio was calculated as: ([emission at 530 nm/emission at 485 nm] – [background at 530 nm/background at 485 nm]), where background corresponds to signals in cells expressing the Rluc fusion protein alone under the same experimental conditions. For better readability, results were expressed in milli-BRET units (mBRET), 1 mBRET corresponding to the BRET ratio multiplied by 1000. BRET ratios were plotted as a function of ([YFP-YFP0]/YFP0)/(Rluc/Rluc0), where YFP is the fluorescence signal at 530 nm after excitation at 485 nm, and Rluc the signal at 485 nm after addition of coelenterazine- h. YFP0 and Rluc0 correspond to the same values in cells expressing the Rluc fusion protein alone.

Receptor cell-surface export analysis.
To study the putative impact of 5-HT_{2C} receptor co-expression on 5-HT_{2A} or 5-HT_{2B} receptor targeting at the cell surface, Cos-7 cells were transiently cotransfected with 5-HT_{2C}-mCherry or 5-HT_{2A}-mCherry and a construct coding for 5-HT_{2A} or 5-HT_{2B} displaying the Flag epitope at the N-terminus and fused C-terminally to the YFP. Empty vector, p513 was used to maintain identical the total amount of transfected DNA. 48 h after transfection, cells were harvested, washed in PBS and fixed in 4% PFA. The expression of each receptor was assessed by measuring GFP and mCherry fluorescence using a Miltenyi MacsQuant VYB cytometer. These determinations allowed us to quantify the amount of single- (less than 5%) of double-

transfected cells (60%) and shows that these values are identical whatever combination of transfected receptors. To determine cell surface 5-HT_{2A} or 5-HT_{2B} expression, cell aliquots were stained with a primary antibody directed against the extracellular Flag epitope conjugated to Pacific Blue Dye (Cell Signaling) following to manufacturer's protocol. Pacific Blue and YFP signals correspond to surface and total Flag-5-HT_2-YFP receptors. Cells expressing both GFP and mCherry were analyzed for Pacific-Blue signal. Ratio of cell surface receptors over total were obtained (Mean of pacific-blue signal/Mean of GFP) for each conditions and normalize as percentage of 5-HT_{2A} or 5-HT_{2B} receptor alone (single transfection), see Doly et al. (67).

Data analysis.
Binding data were analyzed using the iterative non-linear regression model (GraphPad Prism 6.0). This allowed the calculation of inhibition constants (K_i) and the maximal number of sites (B_{max}). All values represent the average of independent experiments ± SEM (n = number of experiments as indicated in the text).

Statistics.
Comparisons between groups were performed using Student's unpaired *t* test or one- or two-way ANOVA with Bonferroni's posthoc test were used depending on the experiment. Significance was set at $p < 0.05$.receptors.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Authorship Contributions.
Participated in research design: S Doly, L Maroteaux.
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Performed data analysis: S Doly, B Guiard, E Quentin and I Moutkine.
Wrote or contributed to the writing of the manuscript: S Doly and L Maroteaux.

References

1. Ferre, S., Baler, R., Bouvier, M., Caron, M. G., Devi, L. A., Durroux, T., Fuxe, K., George, S. R., Javitch, J. A., Lohse, M. J., Mackie, K., Milligan, G., Pfleger, K. D., Pin, J. P., Volkow, N. D., Waldhoer, M., Woods, A. S., and Franco, R. (2009) Building a new conceptual framework for receptor heteromers. *Nature Chem Biol* **5**, 131-134
2. Bulenger, S., Marullo, S., and Bouvier, M. (2005) Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci* **26**, 131-137
3. Millan, M. J., Marin, P., Bockaert, J., and Mannoury la Cour, C. (2008) Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol Sci* **29**, 454-464
4. Bonhaus, D. W., Bach, C., DeSouza, A., Salazar, F. H., Matsuoka, B. D., Zuppan, P., Chan, H. W., and Eglen, R. M. (1995) The pharmacology and distribution of human 5-hydroxytryptamine2B (5-HT2B) receptor gene products: comparison with 5-HT2A and 5-HT2C receptors. *Br J Pharmacol* **115**, 622-628
5. Pompeiano, M., Palacios, J. M., and Mengod, G. (1994) Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors. *Brain Res Mol Brain Res* **23**, 163-178
6. Carr, D. B., Cooper, D. C., Ulrich, S. L., Spruston, N., and Surmeier, D. J. (2002) Serotonin receptor activation inhibits sodium current and dendritic excitability in prefrontal cortex via a protein kinase C-dependent mechanism. *J Neurosci* **22**, 6846-6855
7. Nocjar, C., Alex, K. D., Sonneborn, A., Abbas, A. I., Roth, B. L., and Pehek, E. A. (2015) Serotonin-2C and -2a receptor co-expression on cells in the rat medial prefrontal cortex. *Neuroscience* **297**, 22-37
8. Anastasio, N. C., Stutz, S. J., Fink, L. H., Swinford-Jackson, S. E., Sears, R. M., DiLeone, R. J., Rice, K. C., Moeller, F. G., and Cunningham, K. A. (2015) Serotonin (5-HT) 5-HT2A Receptor (5-HT2AR):5-HT2CR Imbalance in Medial Prefrontal Cortex Associates with Motor Impulsivity. *ACS Chem Neurosci* **6**, 1248-1258
9. Bubar, M. J., Stutz, S. J., and Cunningham, K. A. (2011) 5-HT(2C) receptors localize to dopamine and GABA neurons in the rat mesoaccumbens pathway. *PLoS One* **6**, e20508
10. Esposito, E. (2006) Serotonin-dopamine interaction as a focus of novel antidepressant drugs. *Curr Drug Targets* **7**, 177-185
11. Yadav, V. K., Oury, F., Suda, N., Liu, Z. W., Gao, X. B., Confavreux, C., Klemenhagen, K. C., Tanaka, K. F., Gingrich, J. A., Guo, X. E., Tecott, L. H., Mann, J. J., Hen, R., Horvath, T. L., and Karsenty, G. (2009) A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell* **138**, 976-989
12. Raymond, J. R., Mukhin, Y. V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T. W., Grewal, J. S., and Garnovskaya, M. N. (2001) Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol Ther* **92**, 179-212
13. Brea, J., Castro, M., Giraldo, J., Lopez-Gimenez, J. F., Padin, J. F., Quintian, F., Cadavid, M. I., Vilaro, M. T., Mengod, G., Berg, K. A., Clarke, W. P., Vilardaga, J. P., Milligan, G., and Loza, M. I. (2009) Evidence for distinct antagonist-revealed functional states of 5-hydroxytryptamine(2A) receptor homodimers. *Mol Pharmacol* **75**, 1380-1391
14. Herrick-Davis, K., Grinde, E., Harrigan, T. J., and Mazurkiewicz, J. E. (2005) Inhibition of serotonin 5-hydroxytryptamine2c receptor function through heterodimerization: receptor dimers bind two molecules of ligand and one G-protein. *J Biol Chem* **280**, 40144-40151
15. Herrick-Davis, K., Grinde, E., Lindsley, T., Teitler, M., Mancia, F., Cowan, A., and Mazurkiewicz, J. E. (2015) Native serotonin 5-HT2C receptors are expressed as homodimers on the apical surface of choroid plexus epithelial cells. *Mol Pharmacol* **87**, 660-673
16. Herrick-Davis, K., Grinde, E., Lindsley, T., Cowan, A., and Mazurkiewicz, J. E. (2012) Oligomer size of the serotonin 5-hydroxytryptamine 2C (5-HT2C) receptor revealed by fluorescence correlation spectroscopy with photon counting histogram analysis: evidence for homodimers without monomers or tetramers. *J Biol Chem* **287**, 23604-23614

17. Berthouze, M., Ayoub, M., Russo, O., Rivail, L., Sicsic, S., Fischmeister, R., Berque-Bestel, I., Jockers, R., and Lezoualc'h, F. (2005) Constitutive dimerization of human serotonin 5-HT4 receptors in living cells. *FEBS Letters* **579**, 2973-2980
18. Xie, Z., Lee, S. P., O'Dowd, B. F., and George, S. R. (1999) Serotonin 5-HT1B and 5-HT1D receptors form homodimers when expressed alone and heterodimers when co-expressed. *FEBS Lett* **456**, 63-67
19. Smith, C., Toohey, N., Knight, J. A., Klein, M. T., and Teitler, M. (2011) Risperidone-induced inactivation and clozapine-induced reactivation of rat cortical astrocyte 5-hydroxytryptamine(7) receptors: evidence for in situ G protein-coupled receptor homodimer protomer cross-talk. *Mol Pharmacol* **79**, 318-325
20. Pellissier, L. P., Barthet, G., Gaven, F., Cassier, E., Trinquet, E., Pin, J. P., Marin, P., Dumuis, A., Bockaert, J., Baneres, J. L., and Claeysen, S. (2011) G protein activation by serotonin type 4 receptor dimers: evidence that turning on two protomers is more efficient. *J Biol Chem* **286**, 9985-9997
21. Albizu, L., Holloway, T., Gonzalez-Maeso, J., and Sealfon, S. C. (2011) Functional crosstalk and heteromerization of serotonin 5-HT2A and dopamine D2 receptors. *Neuropharmacology* **61**, 770-777
22. Moreno, J. L., Muguruza, C., Umali, A., Mortillo, S., Holloway, T., Pilar-Cuellar, F., Mocci, G., Seto, J., Callado, L. F., Neve, R. L., Milligan, G., Sealfon, S. C., Lopez-Gimenez, J. F., Meana, J. J., Benson, D. L., and Gonzalez-Maeso, J. (2012) Identification of three residues essential for 5-hydroxytryptamine 2A-metabotropic glutamate 2 (5-HT2A.mGlu2) receptor heteromerization and its psychoactive behavioral function. *J Biol Chem* **287**, 44301-44319
23. Vinals, X., Moreno, E., Lanfumey, L., Cordomi, A., Pastor, A., de La Torre, R., Gasperini, P., Navarro, G., Howell, L. A., Pardo, L., Lluis, C., Canela, E. I., McCormick, P. J., Maldonado, R., and Robledo, P. (2015) Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors. *PLoS Biol* **13**, e1002194
24. Schellekens, H., van Oeffelen, W. E., Dinan, T. G., and Cryan, J. F. (2013) Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *J Biol Chem* **288**, 181-191
25. Kamal, M., Gbahou, F., Guillaume, J. L., Daulat, A. M., Benleulmi-Chaachoua, A., Luka, M., Chen, P., Kalbasi Anaraki, D., Baroncini, M., Mannoury la Cour, C., Millan, M. J., Prevot, V., Delagrange, P., and Jockers, R. (2015) Convergence of melatonin and serotonin (5-HT) signaling at MT2/5-HT2C receptor heteromers. *J Biol Chem* **290**, 11537-11546
26. Cussac, D., Rauly-Lestienne, I., Heusler, P., Finana, F., Cathala, C., Bernois, S., and De Vries, L. (2012) mu-Opioid and 5-HT1A receptors heterodimerize and show signalling crosstalk via G protein and MAP-kinase pathways. *Cellular signalling* **24**, 1648-1657
27. Lukasiewicz, S., Blasiak, E., Faron-Gorecka, A., Polit, A., Tworzydlo, M., Gorecki, A., Wasylewski, Z., and Dziedzicka-Wasylewska, M. (2007) Fluorescence studies of homooligomerization of adenosine A2A and serotonin 5-HT1A receptors reveal the specificity of receptor interactions in the plasma membrane. *Pharmacological reports : PR* **59**, 379-392
28. Jaffre, F., Bonnin, P., Callebert, J., Debbabi, H., Setola, V., Doly, S., Monassier, L., Mettauer, B., Blaxall, B. C., Launay, J. M., and Maroteaux, L. (2009) Serotonin and angiotensin receptors in cardiac fibroblasts coregulate adrenergic-dependent cardiac hypertrophy. *Circ Res* **104**, 113-123
29. Renner, U., Zeug, A., Woehler, A., Niebert, M., Dityatev, A., Dityateva, G., Gorinski, N., Guseva, D., Abdel-Galil, D., Frohlich, M., Doring, F., Wischmeyer, E., Richter, D. W., Neher, E., and Ponimaskin, E. G. (2012) Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking. *J Cell Science* **125**, 2486-2499
30. Janoshazi, A., Deraet, M., Callebert, J., Setola, V., Guenther, S., Saubamea, B., Manivet, P., Launay, J. M., and Maroteaux, L. (2007) Modified receptor internalization upon coexpression of 5-HT1B receptor and 5-HT2B receptors. *Mol Pharmacol* **71**, 1463-1474
31. Black, J. W., and Leff, P. (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**, 141-162

32. Kenakin, T., Watson, C., Muniz-Medina, V., Christopoulos, A., and Novick, S. (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci* **3**, 193-203
33. Doly, S., Madeira, A., Fischer, J., Brisorgueil, M. J., Daval, G., Bernard, R., Verge, D., and Conrath, M. (2004) The 5-HT2A receptor is widely distributed in the rat spinal cord and mainly localized at the plasma membrane of postsynaptic neurons. *J Comp Neurol* **472**, 496-511
34. Cornea-Hebert, V., Riad, M., Wu, C., Singh, S. K., and Descarries, L. (1999) Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J Comp Neurol* **409**, 187-209
35. Boothman, L. J., and Sharp, T. (2005) A role for midbrain raphe gamma aminobutyric acid neurons in 5-hydroxytryptamine feedback control. *Neuroreport* **16**, 891-896
36. Bortolozzi, A., and Artigas, F. (2003) Control of 5-hydroxytryptamine release in the dorsal raphe nucleus by the noradrenergic system in rat brain. Role of alpha-adrenoceptors. *Neuropsychopharmacology* **28**, 421-434
37. Quesseveur, G., Reperant, C., David, D. J., Gardier, A. M., Sanchez, C., and Guiard, B. P. (2013) 5-HT(2)A receptor inactivation potentiates the acute antidepressant-like activity of escitalopram: involvement of the noradrenergic system. *Exp Br Res* **226**, 285-295
38. Szabo, S. T., and Blier, P. (2001) Functional and pharmacological characterization of the modulatory role of serotonin on the firing activity of locus coeruleus norepinephrine neurons. *Brain research* **922**, 9-20
39. Szabo, S. T., and Blier, P. (2002) Effects of serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibition plus 5-HT(2A) receptor antagonism on the firing activity of norepinephrine neurons. *J Pharm Exp Ther* **302**, 983-991
40. Maurice, P., Kamal, M., and Jockers, R. (2011) Asymmetry of GPCR oligomers supports their functional relevance. *Trends Pharmacol Sci* **32**, 514-520
41. Siddiquee, K., Hampton, J., McAnally, D., May, L., and Smith, L. (2013) The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition. *Br J Pharmacol* **168**, 1104-1117
42. AbdAlla, S., Lother, H., Abdel-tawab, A. M., and Quitterer, U. (2001) The angiotensin II AT2 receptor is an AT1 receptor antagonist. *J Biol Chem* **276**, 39721-39726
43. Jastrzebska, B. (2013) GPCR: G protein complexes--the fundamental signaling assembly. *Amino Acids* **45**, 1303-1314
44. Levoye, A., Dam, J., Ayoub, M. A., Guillaume, J. L., Couturier, C., Delagrange, P., and Jockers, R. (2006) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *The EMBO journal* **25**, 3012-3023
45. Herrick-Davis, K. (2013) Functional significance of serotonin receptor dimerization. *Exp Br Res* **230**, 375-386
46. Mancia, F., Assur, Z., Herman, A. G., Siegel, R., and Hendrickson, W. A. (2008) Ligand sensitivity in dimeric associations of the serotonin 5HT2c receptor. *EMBO Rep* **9**, 363-369
47. Fribourg, M., Moreno, J. L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., Park, G., Adney, S. K., Hatcher, C., Eltit, J. M., Ruta, J. D., Albizu, L., Li, Z., Umali, A., Shim, J., Fabiato, A., MacKerell, A. D., Jr., Brezina, V., Sealfon, S. C., Filizola, M., Gonzalez-Maeso, J., and Logothetis, D. E. (2011) Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. *Cell* **147**, 1011-1023
48. Li, Q. H., Nakadate, K., Tanaka-Nakadate, S., Nakatsuka, D., Cui, Y., and Watanabe, Y. (2004) Unique expression patterns of 5-HT2A and 5-HT2C receptors in the rat brain during postnatal development: Western blot and immunohistochemical analyses. *J Comp Neurol* **469**, 128-140
49. Gaspar, P., Cases, O., and Maroteaux, L. (2003) The developmental role of serotonin: news from mouse molecular genetics. *Nat Rev Neurosci* **4**, 1002-1012
50. Beique, J. C., Campbell, B., Perring, P., Hamblin, M. W., Walker, P., Mladenovic, L., and Andrade, R. (2004) Serotonergic regulation of membrane potential in developing rat prefrontal cortex: coordinated expression of 5-hydroxytryptamine (5-HT)1A, 5-HT2A, and 5-HT7 receptors. *J Neurosci* **24**, 4807-4817

51. Beique, J. C., Chapin-Penick, E. M., Mladenovic, L., and Andrade, R. (2004) Serotonergic facilitation of synaptic activity in the developing rat prefrontal cortex. *J Physiol* **556**, 739-754
52. Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X. P., Vardy, E., McCorvy, J. D., Jiang, Y., Chu, M., Siu, F. Y., Liu, W., Xu, H. E., Cherezov, V., Roth, B. L., and Stevens, R. C. (2013) Structural features for functional selectivity at serotonin receptors. *Science* **340**, 615-619
53. Bohn, L. M., and Schmid, C. L. (2010) Serotonin receptor signaling and regulation via β-arrestins. *Critical reviews in biochemistry and molecular biology* **45**, 555-566
54. Roth, B. L. (2011) Irving Page Lecture: 5-HT(2A) serotonin receptor biology: interacting proteins, kinases and paradoxical regulation. *Neuropharmacology* **61**, 348-354
55. Bombardi, C. (2014) Neuronal localization of the 5-HT2 receptor family in the amygdaloid complex. *Frontiers in pharmacology* **5**, 68
56. Maeshima, T., Shutoh, F., Hamada, S., Senzaki, K., Hamaguchi-Hamada, K., Ito, R., and Okado, N. (1998) Serotonin2A receptor-like immunoreactivity in rat cerebellar Purkinje cells. *Neuroscience letters* **252**, 72-74
57. Pazos, A., Cortes, R., and Palacios, J. M. (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. II. Serotonin-2 receptors. *Brain research* **346**, 231-249
58. Wright, D. E., Seroogy, K. B., Lundgren, K. H., Davis, B. M., and Jennes, L. (1995) Comparative localization of serotonin1A, 1C, and 2 receptor subtype mRNAs in rat brain. *J Comp Neurol* **351**, 357-373
59. Ward, R. P., and Dorsa, D. M. (1996) Colocalization of serotonin receptor subtypes 5-HT2A, 5-HT2C, and 5-HT6 with neuropeptides in rat striatum. *J Comp Neurol* **370**, 405-414
60. Banas, S. M., Doly, S., Boutourlinsky, K., Diaz, S. L., Belmer, A., Callebert, J., Collet, C., Launay, J. M., and Maroteaux, L. (2011) Deconstructing antiobesity compound action: requirement of serotonin 5-HT2B receptors for dexfenfluramine anorectic effects. *Neuropsychopharmacology* **36**, 423-433
61. Diaz, S. L., Doly, S., Narboux-Neme, N., Fernandez, S., Mazot, P., Banas, S. M., Boutourlinsky, K., Moutkine, I., Belmer, A., Roumier, A., and Maroteaux, L. (2012) 5-HT(2B) receptors are required for serotonin-selective antidepressant actions. *Mol Psychiatry* **17**, 154-163
62. Green, S., Issemann, I., and Sheer, E. (1988) A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. *Nucl. Ac. Res.* **16**, 396
63. Deraet, M., Manivet, P., Janoshazi, A., Callebert, J., Guenther, S., Drouet, L., Launay, J. M., and Maroteaux, L. (2005) The natural mutation encoding a C terminus-truncated 5-hydroxytryptamine 2B receptor is a gain of proliferative functions. *Mol Pharmacol* **67**, 983-991
64. Du, Y., Davisson, M. T., Kafadar, K., and Gardiner, K. (2006) A-to-I pre-mRNA editing of the serotonin 2C receptor: comparisons among inbred mouse strains. *Gene* **382**, 39-46
65. Aghajanian, G. K., and Vandermaelen, C. P. (1982) Intracellular recording in vivo from serotonergic neurons in the rat dorsal raphe nucleus: methodological considerations. *J Histochem Cytochem* **30**, 813-814
66. Achour, L., Kamal, M., Jockers, R., and Marullo, S. (2011) Using quantitative BRET to assess G protein-coupled receptor homo- and heterodimerization. *Methods Mol Biol* **756**, 183-200
67. Doly, S., Shirvani, H., Gata, G., Meye, F. J., Emerit, M. B., Enslen, H., Achour, L., Pardo-Lopez, L., Yang, S. K., Armand, V., Gardette, R., Giros, B., Gassmann, M., Bettler, B., Mameli, M., Darmon, M., and Marullo, S. (2015) GABA receptor cell-surface export is controlled by an endoplasmic reticulum gatekeeper. *Mol Psychiatry* **21**, 480-490

Footnotes

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Nonstandard abbreviations used are:
Locus Coeruleus (LC) ;
Seven transmembrane receptors (7TMRs);
Serotonin (5-hydroxytryptamine, 5-HT);
(±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI);
Nor-(+)-Fenfluramine (NDF);
Inositol Phosphate (IP);
Dulbecco's Modified Eagle's Medium (DMEM);
Phosphate buffer saline (PBS);
Bovine serum albumine (BSA);
Wild type (WT);
C-terminal deletion (ΔCter);
Renilla luciferase (Rluc);
N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4).
(2-amino- 4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine hydrochloride) (RS127445)
8-[5-(2,4-dimethoxy-5-(4-trifluoro- methylphenylsulfonamido)phenyl-5-oxopentyl)]-1,3,8-triazaspiro[4.5]de- cane-2,4-dione hydrochloride (RS102221)
([6- chloro-5-methyl-1-(6-(2-methylpiridin-3-yloxy)pyridine-3-yl carbamoyl] inodoline dihydrochloride) (SB242084)
R-(+)-α-(2,3-dimethoxyphenyl)- 1-[2-(4-fluorophenylethyl)]-4-piperidine methanol (MDL100907)
Adeno-Associated Virus (AAV)
Internal ribosome entry site (IRES)
Green Fluorescent protein (GFP)
Dorsal raphe (DR)
Phospholipase C (PLC)

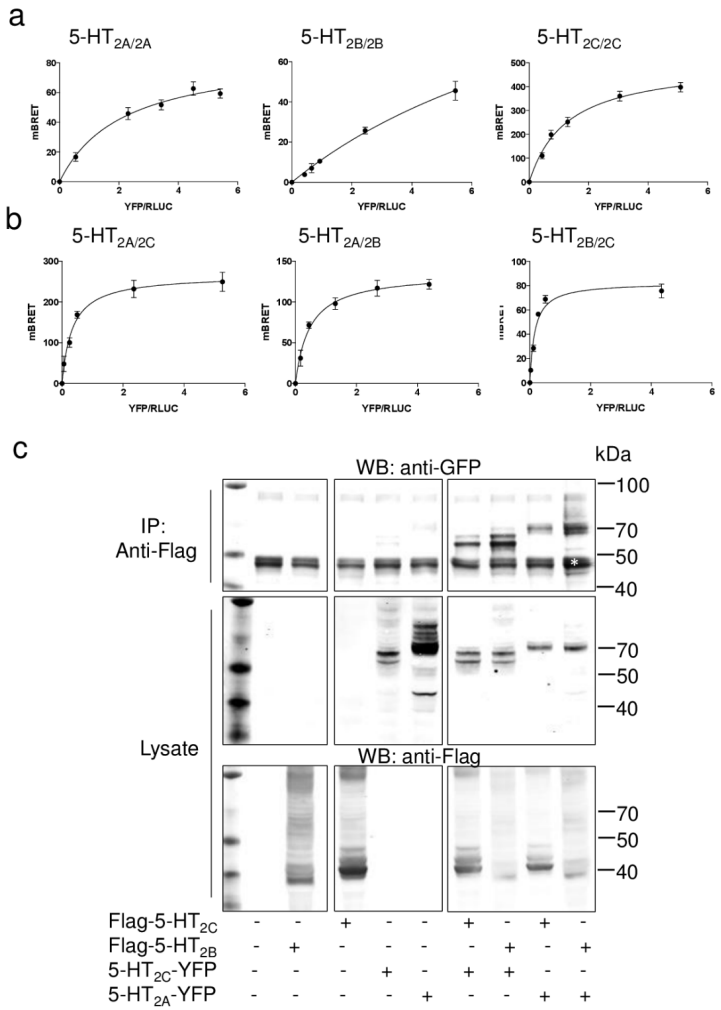
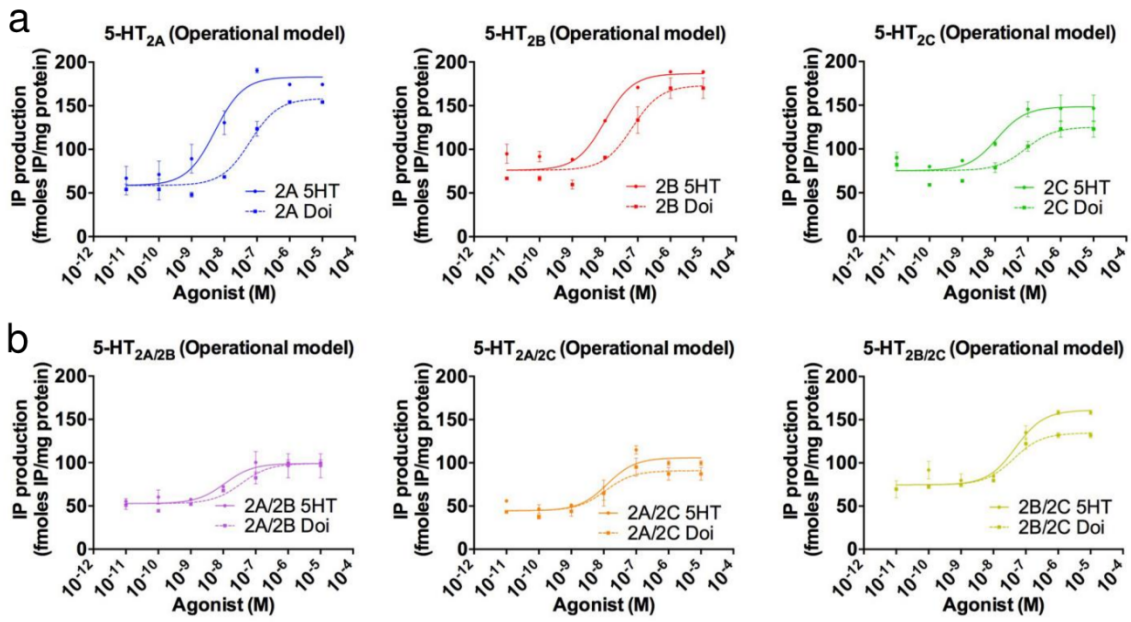


Figure 1: Constitutive heterodimerization of 5-HT$_2$ receptors and co-immunoprecipitation in living HEK293 cells. a) BRET proximity assays between homodimers of 5-HT$_{2A}$, 5-HT$_{2B}$ or 5-HT$_{2C}$ receptors. HEK-293 cells were co-transfected with plasmids coding for a constant amount of RLuc-5-HT$_{2A}$, RLuc-5-HT$_{2B}$ or RLuc-5-HT$_{2C}$ (BRET donors) and increasing concentrations of the corresponding YFP tagged 5-HT$_2$ homodimer (the BRET acceptor). **b)** BRET proximity assays between heterodimers of 5-HT$_{2A}$, 5-HT$_{2B}$ or 5-HT$_{2C}$ receptors. HEK-293 cells were co-transfected with plasmids coding for a constant amount of RLuc-5-HT$_{2A}$, RLuc-5-HT$_{2B}$ or RLuc-5-HT$_{2C}$ (BRET donors) and increasing concentrations of YFP tagged 5-HT$_2$ heterodimer (the BRET acceptor). For each heterodimers, BRET donor and acceptor were swap as a control experiment (not shown). Energy transfer was measured after addition of membrane permeable luciferase substrate coelenterazine-h. The BRET signal was determined by calculating the ratio of light emitted at 530 nm and that emitted at 485 nm, as described in "experimental procedures". Error bars indicate SEM of specific BRET-ratio values obtained from triplicates determination. BRET values (Bmax, BRET$_{50}$) were obtained from 6 independent experiments. Plots were established using Graphpad software. 5-HT$_{2A/2A:}$ BRETmax 76±16, BRET$_{50}$ 2.4±0.5; 5-HT$_{2B/2B}$ Ambiguous; 5-HT$_{2C/2C}$ BRETmax 434±32 BRET$_{50}$ 1.2±0.4; 5-HT$_{2A/2C}$ BRETmax 266±24 BRET$_{50}$ 0.33±0.04; 5-HT$_{2A/2B}$ BRETmax 90±32 BRET$_{50}$ 0.19±0.07; 5-HT$_{2B/2C}$ BRETmax 85±6 BRET$_{50}$ 0.16±0.04. **c)** Interactions of 5-HT$_2$ subtypes in co-immunoprecipitation experiments. Cos-7 cells were transfected with various combinations of plasmids coding for 5-HT$_{2A}$-YFP, 5-HT$_{2C}$-YFP and Flag-epitope-tagged 5-HT$_{2B}$ and 5-HT$_{2C}$, as indicated. Immunoprecipitation with a monoclonal anti-Flag antibody coated beads (EZview Red FLAG M2 Affinity Gel) was performed from 1 mg of protein of cell lysates. The presence of 5-HT$_2$-GFP and 5-HT$_2$-Flag was revealed with anti-GFP and anti-Flag antibodies, respectively. 100 µg of protein of cell lysates were analyzed to determine receptor-GFP receptor-FLAG expression (input). white star: IgG heavy chain.



c

Log (τ/K_A)	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$	5-HT$_{2A/2B}$	5-HT$_{2A/2C}$	5-HT$_{2B/2C}$
	-7.11±0.31	-7.58±0.19	-6.69±0.22	-7.30±0.20	-7.26±0.38	-7.22±0.19

Figure 2: IP production induced by stimulation by a full (5-HT) and a partial agonist (DOI)
a) 5-HT and DOI dose response curves in single receptor transfections. COS-7 cells transiently expressing 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, receptor alone, were stimulated with serotonin (5-HT) or DOI and IP levels were determined. For each conditions stimulation with 5-HT induces full dose response curve and DOI a partial dose response that can be modelized using the operational model. **b**) 5-HT and DOI dose response curves in two receptor transfections. COS-7 cells transiently co-expressing 5-HT$_{2A/2B}$, 5-HT$_{2A/2C}$, 5-HT$_{2B/2C}$ receptors, were stimulated with serotonin (5-HT) or DOI and IP levels were determined. These are representative curves of at least 4 independent experiments performed in duplicates. **c**) Dose response curves allow to calculate the mean Log(τ/K_A), which is not affected by dimerization (one-way ANOVA, n=4-5). Values are given ± SEM.

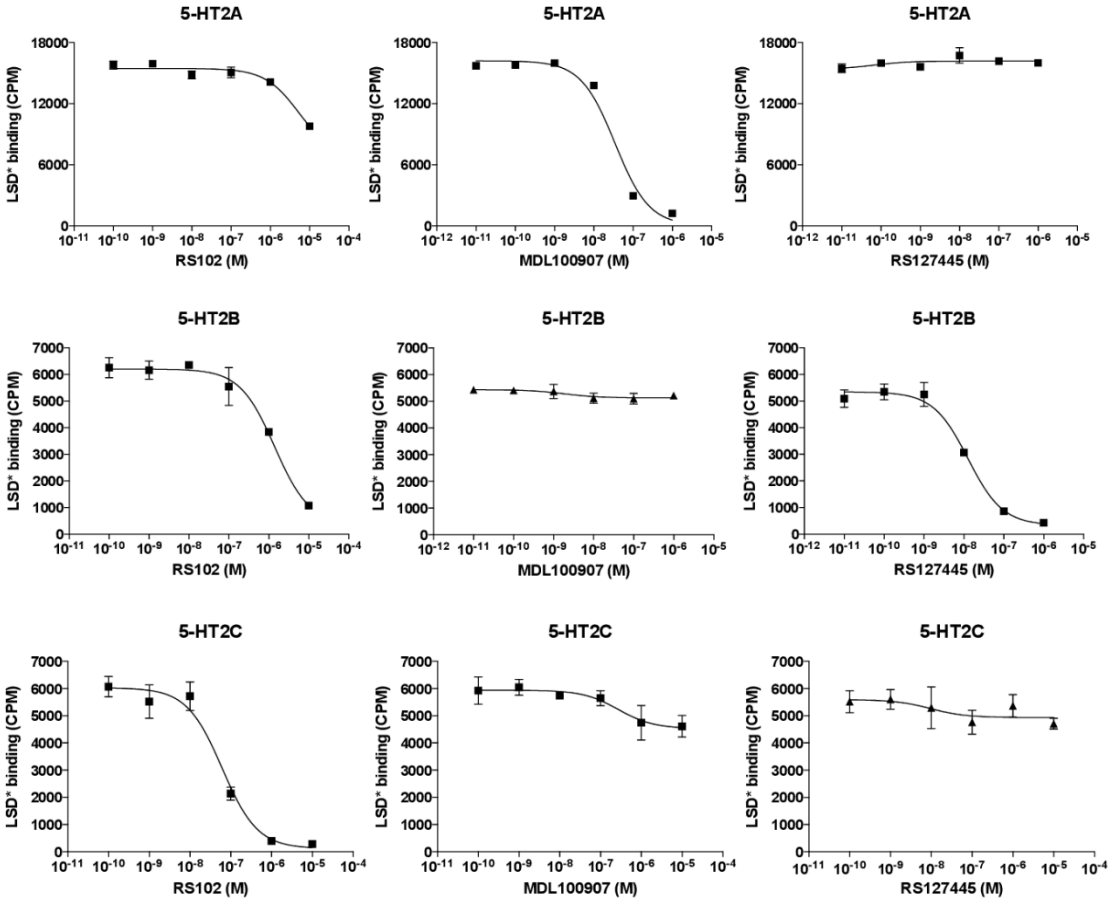


Figure 3: Affinity and selectivity of antagonists used in this study. Affinity and selectivity of antagonists were tested to define the optimal concentration and avoid cross-reactivity. Representative examples of ^{3}H-LSD radioligand binding heterologous competition experiments performed on membrane preparation. HEK293 cells transiently expressing 5-HT$_{2A}$, 5-HT$_{2B}$ or 5-HT$_{2C}$ receptors were incubated with ^{3}H-LSD and increasing concentrations of the 5-HT$_{2A}$ (MDL100907), 5-HT$_{2B}$ (RS127445) and 5-HT$_{2C}$ (RS102221) antagonist. Thus, we used in this study 100 nM concentration for all the antagonists. Graphs are representative of one experiment performed in triplicate. Bars represent ± SEM from triplicates. Binding curves were done using Graphpad software.

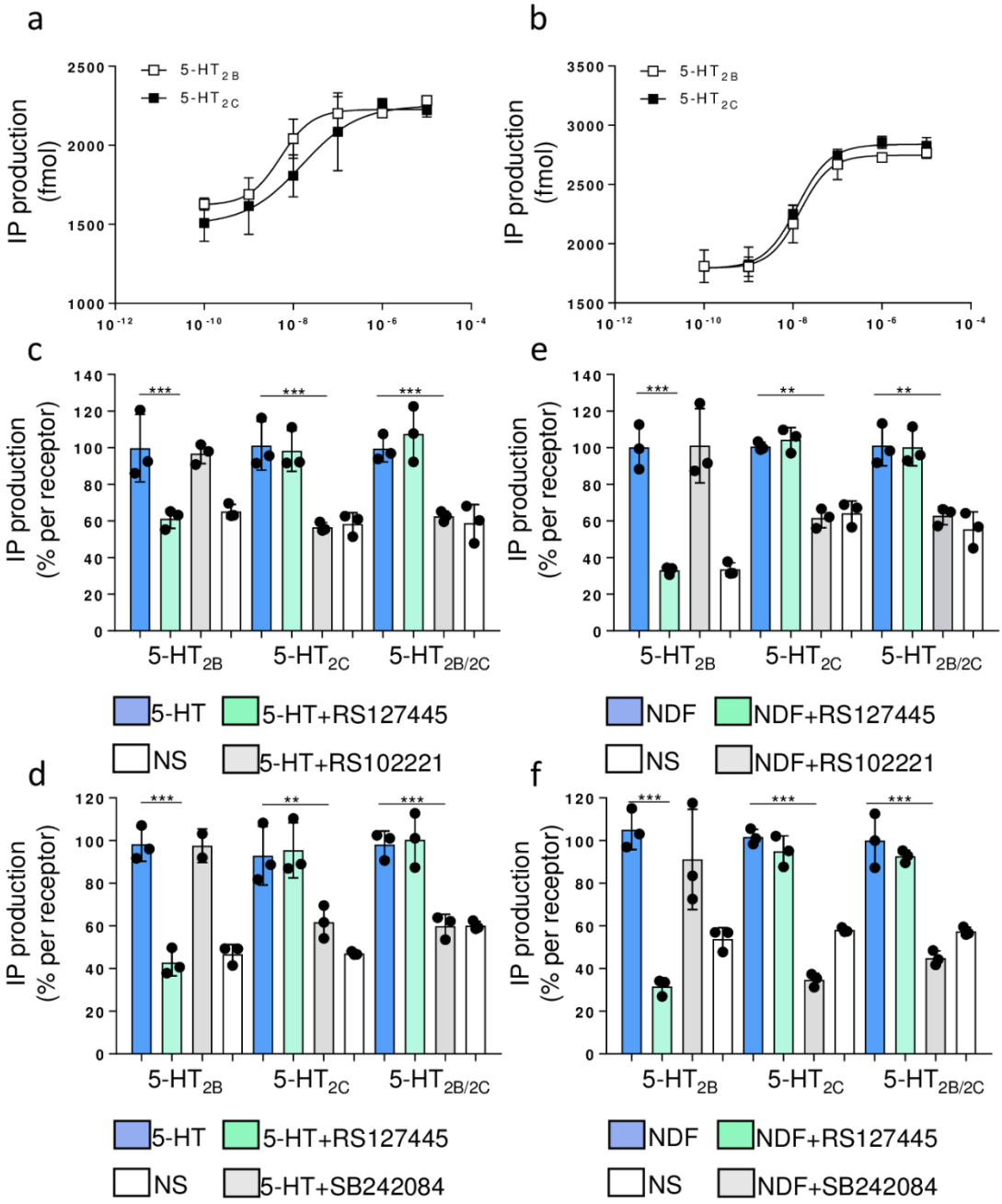


Figure 4: 5-HT$_{2C}$ receptor expression blocks 5-HT$_{2B}$ receptor signaling. a) Agonist concentration-induced IP response. Serotonin (5-HT) and Nor-(+)-Fenfluramine (NDF) dose-response curves for stimulation of IP production in cells expressing the same amount of 5-HT$_{2B}$ (white box) or 5-HT$_{2C}$ (dark box) receptors were used to obtain the minimal agonist concentration-induced maximal IP response (1µM for both agonists). Bars represent ± SEM from triplicates. **b,c)** HEK293 cells transiently expressing 5-HT$_{2C}$ and 5-HT$_{2B}$ receptor alone or in combination (5-HT$_{2B/2C}$), were stimulated with 1 µM of the 5-HT$_2$ agonist serotonin (5-HT) or Nor-(+)-Fenfluramine (NDF) and IP accumulation was determined. The selective 5-HT$_{2B}$ (RS127445-green) or 5-HT$_{2C}$ antagonists (RS102221-grey) or (SB242084-grey) were co-incubated with agonists. Data are expressed as % of agonist response for each transfected condition. Bars represent ±SD of three independent experiments. NS: Non-stimulated. Data were analyzed with one-way ANOVA within each independent transfection and a Bonferroni's multiple comparisons test. ***p < 0.005, **p < 0.01: Specific antagonists treatment significantly different from 5-HT stimulation.

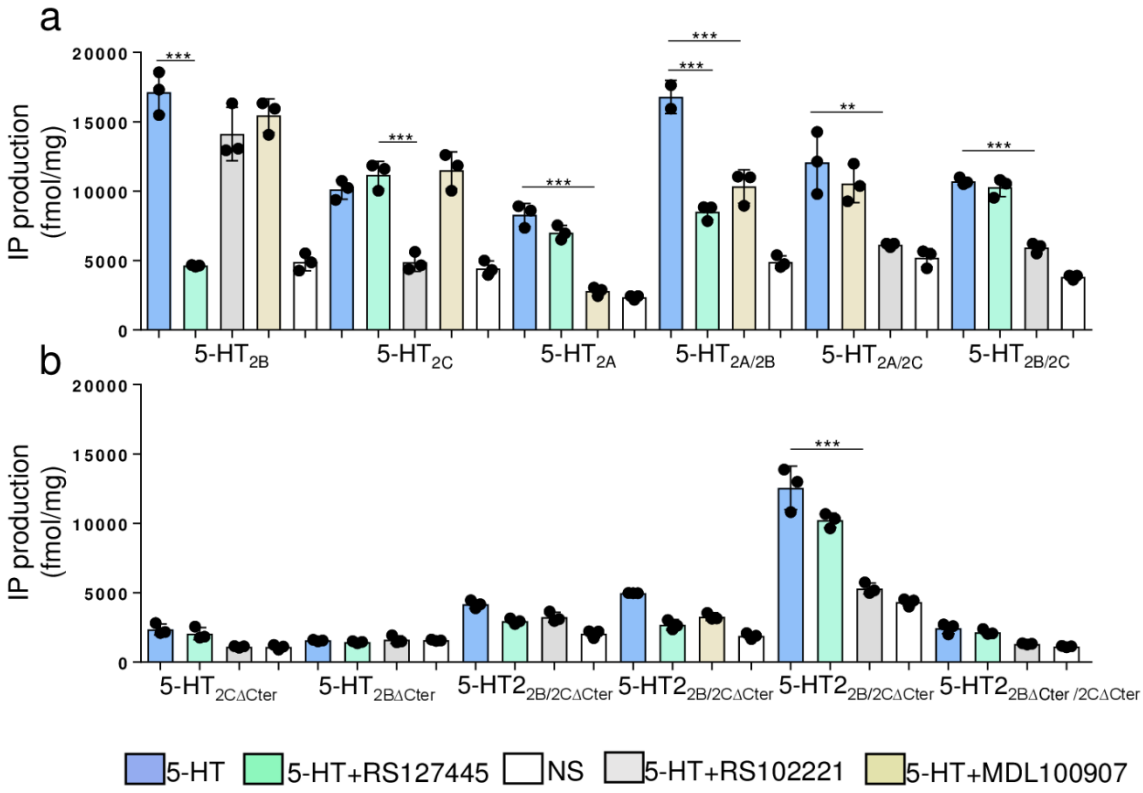


Figure 5: The 5-HT$_{2C}$ receptor blunts 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor signaling. HEK293 cells transiently expressing 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_{2B\Delta Cter}$ and 5-HT$_{2C\Delta Cter}$ receptor alone or in combination, were stimulated with 1 µM of serotonin (5-HT) and IP levels were determined. The selective antagonists (100 nM), 5-HT$_{2B}$ (green-RS127445), 5-HT$_{2C}$ (grey-RS102221) or 5-HT$_{2A}$ (brown-MDL100907) were co-incubated with 5-HT as indicated. **a)** Compared to receptors alone, which produce IP accumulation that can be blocked by the respective selective antagonists, IP accumulation can only be blocked by 5-HT$_{2C}$ receptor antagonist but not the 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor antagonist in co-transfection of either 5-HT$_{2A}$ or 5-HT$_{2B}$ with 5-HT$_{2C}$ receptors. b**)**. Stimulation of 5-HT$_{2B\Delta Cter}$, 5-HT$_{2C\Delta Cter}$ or HT$_{2B\Delta Cter/2C\Delta Cter}$ receptors gives little IP accumulation. Stimulation of 5-HT$_{2A/2C\Delta Cter}$, or HT$_{2B/2C\Delta Cter}$ receptors gives also nearly no IP accumulation, whereas 5-HT$_{2C/2B\Delta Cter}$ leads to IP accumulation that can only be blocked by the 5-HT$_{2C}$ receptor antagonist. Bars represent ± SD of three independent experiments. NS: Non-stimulated. Data were analyzed with one-way ANOVA within each independent transfection and a Bonferroni's multiple comparisons test. ***p < 0.005, **p < 0.01: Specific antagonists treatment significantly different from 5-HT stimulation.

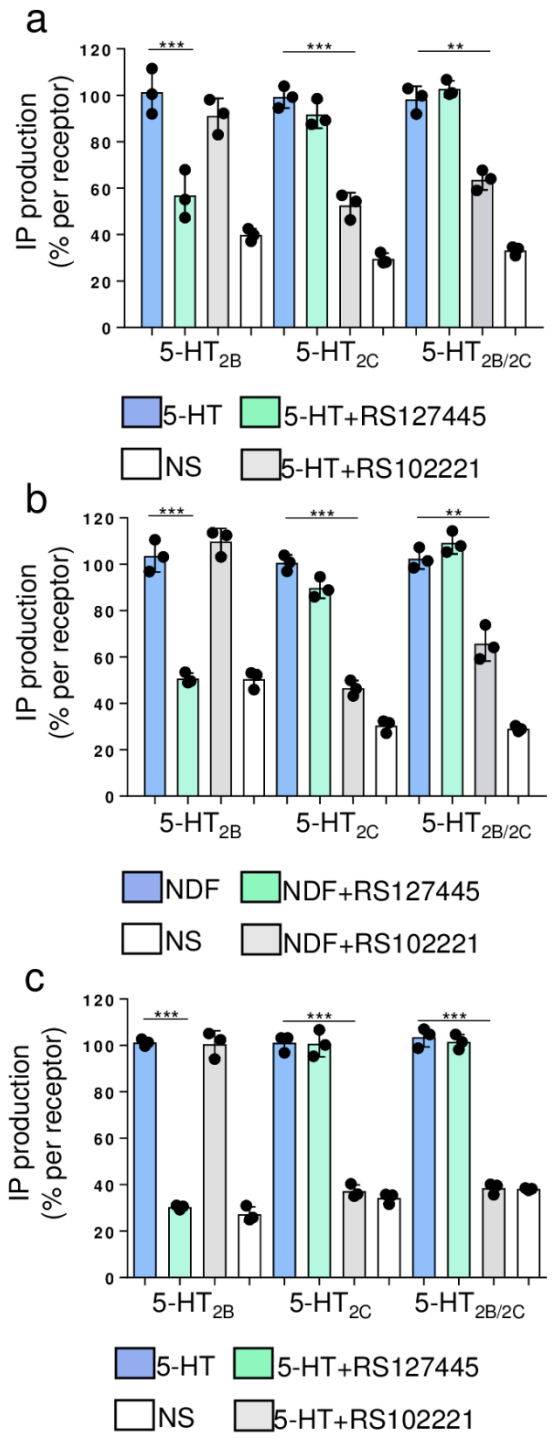


Figure 6: 5-HT$_{2C}$ receptor blunts 5-HT$_{2B}$ receptor signaling independently of the cell types. LMTK$^-$ (**a-b**) or CHO (**c**) cells transiently expressing 5-HT$_{2C}$ and 5-HT$_{2B}$ receptor alone or in combination (5-HT$_{2B/2C}$), were stimulated with 1 µM of the 5-HT$_2$ agonist serotonin (5-HT) or Nor-(+)-Fenfluramine (NDF) and IP accumulation was determined. The selective 5-HT$_{2B}$ (RS127445-green) or 5-HT$_{2C}$ antagonists (RS102221-grey) were co-incubated with agonist in some conditions, as indicated. Data are express as % of maximal agonist response for each transfected condition. Bars represent ±SD of three independent experiments. NS: Non-stimulated. Data were analyzed with one-way ANOVA for each graph and a Bonferroni's multiple comparisons test. ***$p < 0.005$, **$p < 0.01$: Specific antagonists treatment significantly different from 5-HT stimulation.

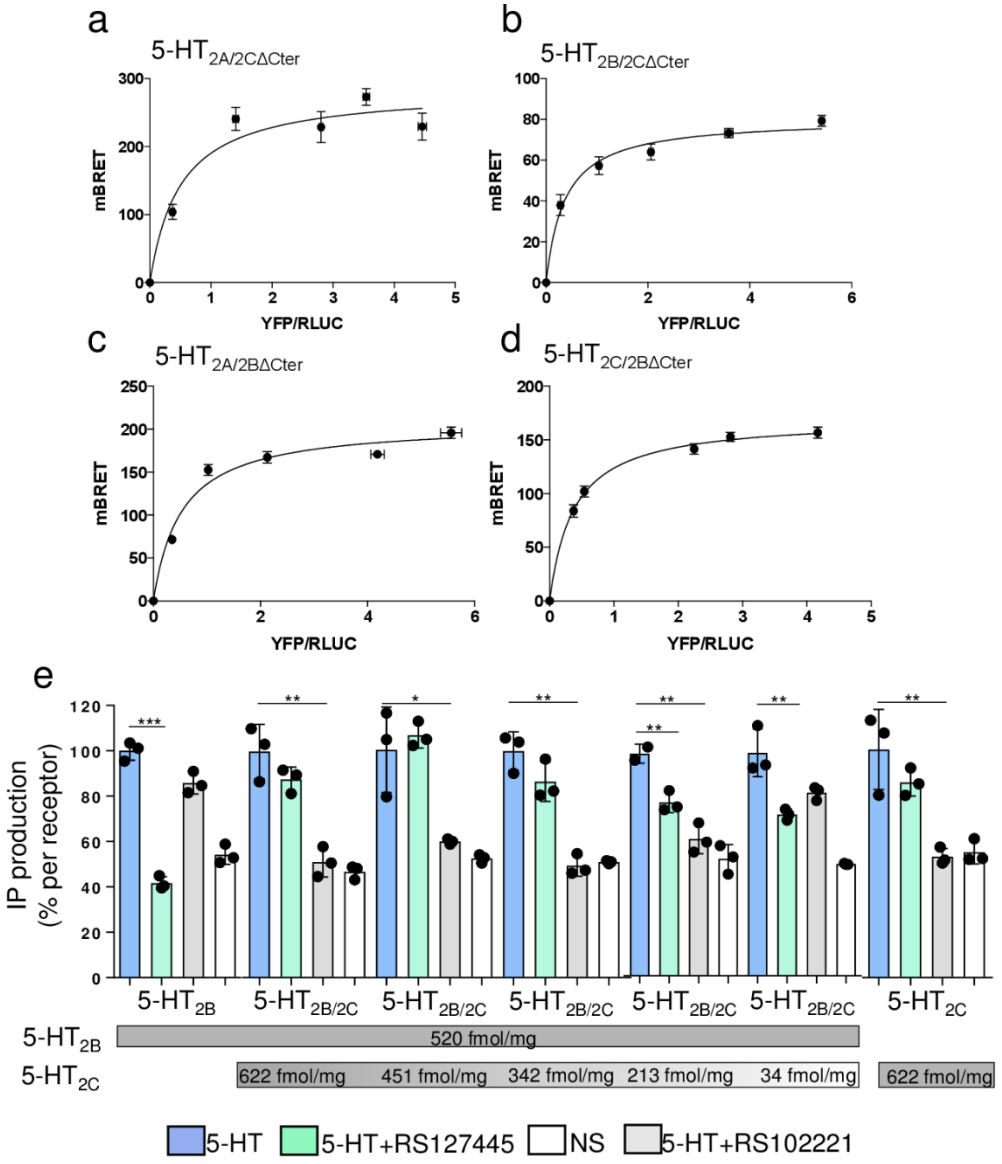


Figure 7: a-d Constitutive heterodimerization of 5-HT$_2$ receptors with 5-HT$_{2B\Delta Cter}$ and 5-HT$_{2C\Delta Cter}$ constructs in living HEK293 cells. HEK-293 cells were co-transfected with plasmids coding for a constant amount of RLuc-5-HT$_{2A}$, RLuc-5-HT$_{2B}$ or RLuc-5-HT$_{2C}$ (BRET donors) and increasing concentrations of YFP tagged ΔCter constructs (the BRET acceptor). Energy transfer was measured after addition of membrane permeable luciferase substrate coelenterazine-h. The BRET signal was determined by calculating the ratio of light emitted at 530 nm and that emitted at 485 nm, as described in "experimental procedures". Error bars indicate SEM of specific BRET-ratio values obtained from triplicates determination. BRET values (Bmax, BRET$_{50}$) were obtained from 3 independent experiments. Plots were established using Graphpad software. a) 5-HT$_{2A/2C\Delta Cter}$: BRETmax 284±27, BRET$_{50}$ 0.49±0.22; b) 5-HT$_{2B/2C\Delta Cter}$ BRETmax 80±3 BRET$_{50}$ 0.36±0.07; c) 5-HT$_{2A/2B\Delta Cter}$ BRETmax 207±12 BRET$_{50}$ 0.52±0.13; d) 5-HT$_{2C/2B\Delta Cter}$ BRETmax 170.0±2.5 BRET$_{50}$ 0.37±0.02. **e) Dose dependency of 5-HT$_{2C}$ blunting effect.** Progressive reduction of the 5-HT$_{2C}$ receptor cDNA transfection (from 5 µg of DNA corresponding to 622 fmol/mg to 0,1µg of DNA corresponding to 34 fmol/mg; as determined by radioligand binding assay) revealed the sensitivity of IP production to 5-HT$_{2B}$ receptor antagonism (starting from 213 fmol/mg). The 5-HT$_{2B}$ receptor expression was constant for each condition (5 µg of DNA corresponding to 520 fmol/mg). Data are expressed as % of maximal agonist response for each transfected condition. Bars represent ±SD of three independent experiments. NS: Non-stimulated. Data were analyzed with one way ANOVA within each independent transfection and a Bonferroni's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001: significantly different from 5-HT stimulation.

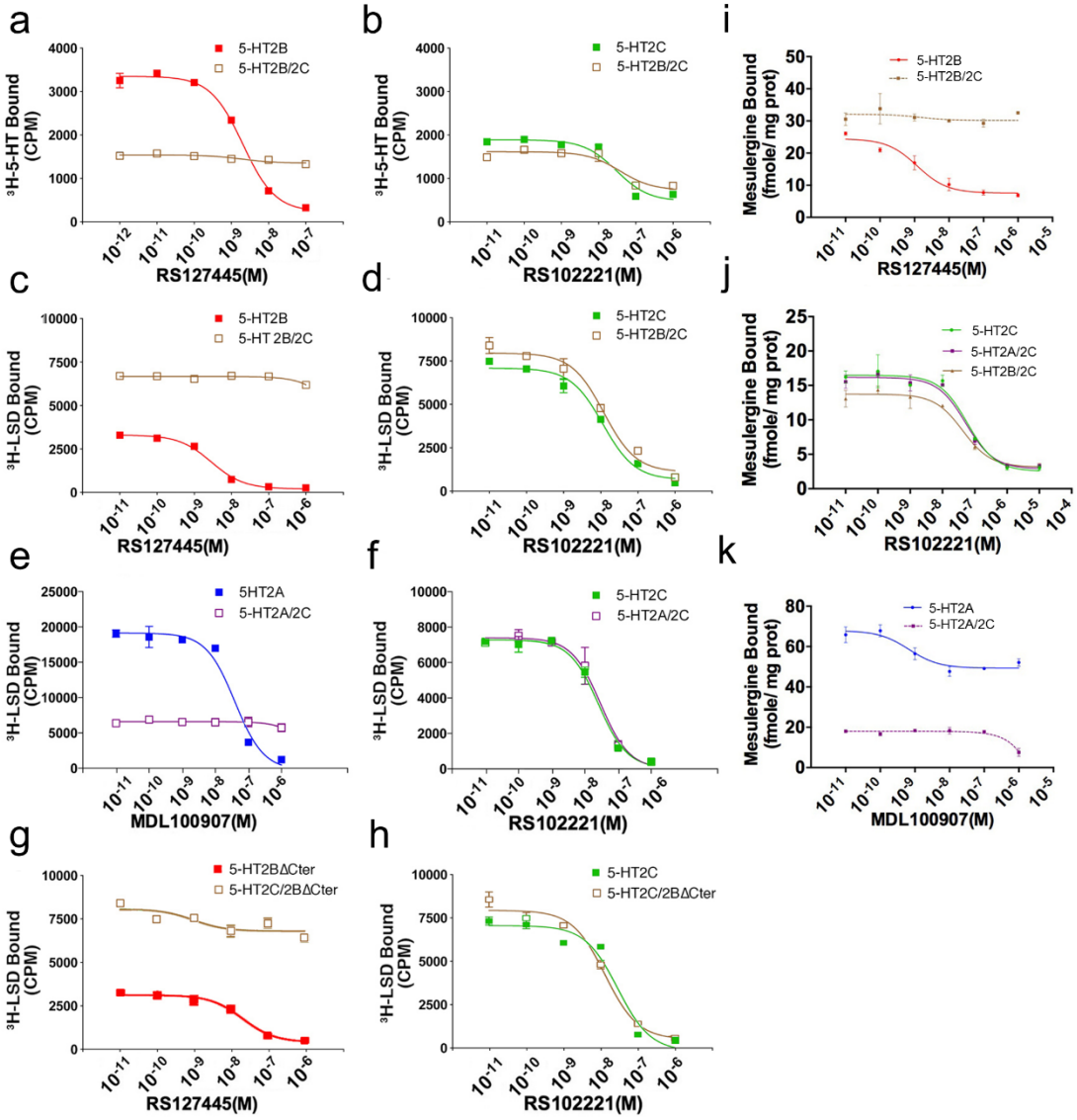
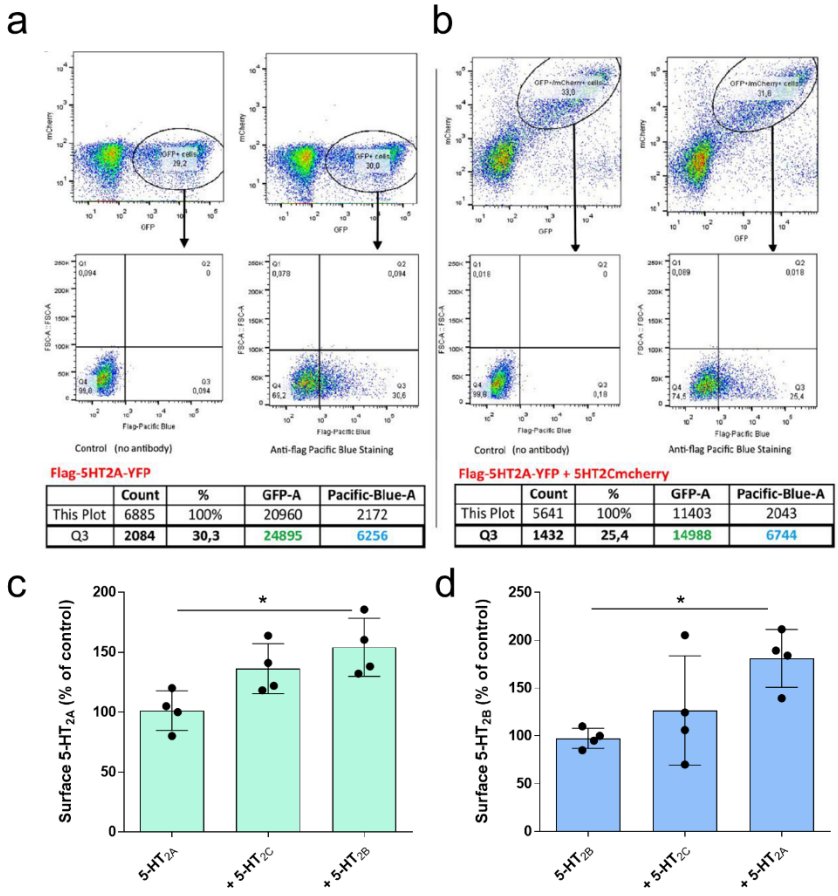


Figure 8: The co-expression of 5-HT$_{2C}$ receptors blunts 5-HT$_{2A}$ and 5-HT$_{2B}$ ligand binding. Transiently transfected cells were incubated with **a-b)** ^{3}H-5-HT, **c-h)** ^{3}H-LSD or **i-k)** ^{3}H-Mesulergine and increasing concentrations of the 5-HT$_{2A}$ or 5-HT$_{2B}$ antagonist or 5-HT$_{2C}$ antagonist. **a-b).** 5-HT$_{2C}$ receptor transfection with 5-HT$_{2B}$ receptors abolishes the ^{3}H-5-HT competition by 5-HT$_{2B}$ antagonist. **c-f).** 5-HT$_{2C}$ receptor transfection with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors abolishes the ^{3}H-LSD competition by 5-HT$_{2A}$ or 5-HT$_{2B}$ antagonists. **g-h).** 5-HT$_{2C}$ receptor transfection with 5-HT$_{2B\Delta Cter}$ receptors abolishes the ^{3}H-LSD competition by the 5-HT$_{2B}$ antagonist, but not the 5-HT$_{2C}$ antagonist. **i-j-k)**. 5-HT$_{2C}$ receptor transfection with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors abolishes the ^{3}H-mesulergine competition by 5-HT$_{2A}$ or 5-HT$_{2B}$ antagonists. These are representative curves of at least 3 independent experiments performed in duplicates and error bars indicate SEM.



	Count	%	GFP-A	Pacific-Blue-A
This Plot	6885	100%	20960	2172
Q3	2084	30,3	24895	6256

	Count	%	GFP-A	Pacific-Blue-A
This Plot	5641	100%	11403	2043
Q3	1432	25,4	14988	6744

Figure 9. Receptor cell-surface export of 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors is not affected by the co-expression with the 5-HT$_{2C}$ protomer. Surface 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors in YFP-Cherry positives non-permeabilized cells, were quantified by cytometry using anti-Flag primary antibody conjugated to Pacific blue dye. **a,b)** Example of cell population analyzed for 5-HT$_{2A}$ receptor GFP and 5-HT$_{2C}$ receptor mCherry expression. Double transfected cells (GFP+/mCherry+) were selected. Flag-Pacific Blue staining (surface receptor expression) was analyzed on GFP+/mCherry+ cells. Q3 shows % of Pacific Blue positive cells. Ratio of cell surface receptors over total were obtained (Mean of pacific-blue signal/Mean of GFP) for each conditions and normalize as percentage of 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor alone (single transfection) **c)** Flag-5-HT$_{2A}$-YFP alone or co-expressed with 5-HT$_{2B}$-Cherry or 5-HT$_{2C}$-Cherry. **d)** Flag-5-HT$_{2B}$-YFP alone or co-expressed with 5-HT$_{2A}$-Cherry or 5-HT$_{2C}$-Cherry. Cell-surface 5-HT$_{2A}$ and 5-HT$_{2B}$ expression is shown by the histograms: bars indicate SD. from three independent experiments. Data were analyzed with one-way ANOVA and a Bonferroni's multiple comparisons test. *$p < 0.05$ significantly different from control (receptor alone).

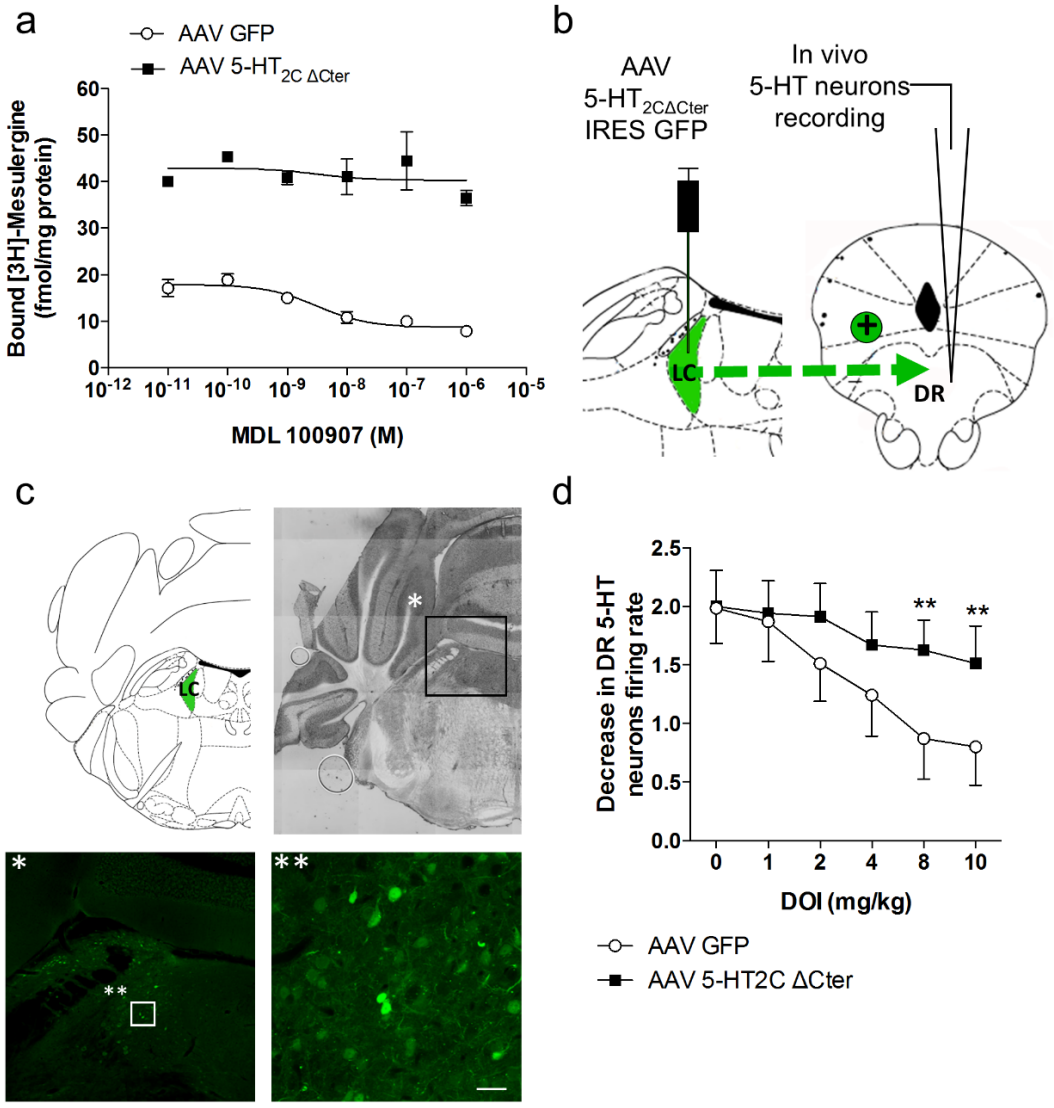


Figure 10: The 5-HT$_{2C\Delta Cter}$ inactive protomer prevents 5-HT$_{2A}$ ligand binding in the PFC and reduces DOI-induced 5-HT$_{2A}$ receptor dependent decrease of 5-HT neurons firing rate.
a) Exogenous 5-HT$_{2C\Delta Cter}$ expression in PFC, upon bilateral infection with adeno-associated viruses carrying a 5-HT$_{2C\Delta Cter}$ construct (AAV-5-HT$_{2C\Delta Cter}$) was associated with a dramatic reduction of endogenous 5-HT$_{2A}$-dependant ligand binding compared to AAV mediated GFP expression only. Representative example of ^{3}H-mesulergine binding (heterologous competition displacement with a selective 5-HT$_{2A}$ antagonist MDL100907) experiments performed on membrane preparation from one PFC. Bars represent ± SEM from triplicates. Binding curve was done using Graphpad software. **b)** WT mice were bilaterally injected with AAV 5-HT$_{2C\Delta Cter}$ IRES GFP in the locus ceruleus (LC) sending excitatory projections (green arrow) into the dorsal raphe nucleus (DR) as shown in the diagram. After one-month recovery and transgene expression, *in-vivo* recording of serotonin (5-HT) neurons in the DR was done. **c)** Mouse brains were examined to verify the distribution of adenovirus-encoded 5-HT$_{2C\Delta Cter}$ and GFP in the LC. Fluorescence imaging of GFP in LC neurons is shown at increasing magnification. The Box * and **, corresponds to magnified areas (black and white square). **d)** Effect of the 5-HT$_{2A}$ receptor agonist DOI on the firing rate of DR 5-HT neurons in WT mice. DOI was administered using a cumulative dosing regimen, that is, all mice received 1, 2, 4, 8 and 10 mg/kg (s.c.) with a 3-min interval between each injection. Data presented as means ± SEM of basal firing rate in mice injected with the control AAV (open circle) or AAV 5-HT$_{2C\Delta Cter}$ (dark square). (n = 7 mice per group). Data were analyzed with two way ANOVA and a Bonferroni's multiple comparisons test. **p < 0.01: significantly different from sham AAV control injected mice. Scale bars: 20 µm.