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Heterodimers of serotonin receptor subtypes 2 are driven by 5-HT_{2C} protomers

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Running title: Dimerization among 5-HT₂ 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 Gα_q-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-HT₂ 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} Gα_q-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-HT₂ 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-HT₂ 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-HT₂ 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-HT₂), 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-HT₂ subfamily consists in three Gα_q/Gα₁₁-coupled receptors, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which mediate excitatory neurotransmission (3). Interestingly, 5-HT₂

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-HT₂ 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-HT₄, and 5-HT₇ receptor subtypes in heterologous expression systems (13,17-20). In addition, there are observations suggesting that the 5-HT₂ 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, D₂-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-HT₇ 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 G α i and GIRK channel activity, without affecting 5-HT₇-receptor-mediated signaling, indicating a unidirectional dominant effect of the 5-HT₇ 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-HT₂ 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-HT₂ 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 G α q-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} Δ 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-HT₂ receptors

The putative formation of heterodimers between 5-HT₂ 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-HT₂ receptors can form heterodimers in intact cells. BRET₅₀ values (values of YFP/RLuc for half-maximal BRET) reflect the propensity of association between the investigated proteins. Interestingly, BRET₅₀ values for heterodimeric association are significantly lower than those measured for homodimeric association of 5-HT₂ subtypes, suggesting that in native cells expressing more than one 5-HT₂ 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-HT₂ receptors on signaling

We next examined the consequence of 5-HT₂ receptors heterodimerization on agonist-induced-Gαq activation. The 5-HT₂ receptors consistently activate the PLC-β pathway in native tissues and heterologous cells (3,12). We first determined whether 5-HT₂ receptor signaling was altered when expressed in the presence of other 5-HT₂ receptors (Fig. 2). Dose-response curves of 5-HT₂-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/K_A) (32) was found among the groups, supporting a lack of major difference in coupling efficiency between

individual 5-HT₂ receptors to Gαq activation and no modification of this coupling efficiency by heterodimers.

In the absence of highly selective, subtype specific 5-HT₂ agonists, we next determined whether agonist-induced 5-HT₂ 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-HT₂-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-HT₂ 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-HT₂ 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_{2CACTer}) (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/2CACTer} and 5-HT_{2B/2CACTer} heterodimers, IP accumulation was dramatically reduced (Fig. 5b). In addition, co-expressing 5-HT_{2C} receptors with 5-HT_{2BΔCTer}, 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_{2CACTer} and 5-HT_{2BΔCTer} receptor constructs compared to the respective WT form, using a radioligand-binding assay on nonpermeabilized cells. Expression of 5-HT_{2B}, 5-HT_{2BΔCTer}, 5-HT_{2C} or 5-HT_{2CACTer} 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 expressed properly at the cell membrane. Moreover, BRET assay confirms 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 cells 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-HT₂ 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 (³H-5-HT- or ³H-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 ³H-5-HT (Fig. 8a) or ³H-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 ³H-5-HT (Fig. 8b) or ³H-LSD (Fig. 8d) binding in cells expressing either 5-HT_{2C} receptors alone or 5-HT_{2B/2C} receptors. Similar findings were obtained for 5-HT_{2A} receptors (Fig. 8e,f). Noteworthy, ³H-5-HT or ³H-LSD binding was comparable in cells expressing 5-HT_{2C} alone or 5-HT_{2C} receptor and another 5-HT₂ 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_{2BΔCTer} receptor (Fig. 8g,h) or with radiolabeled antagonist (³H-Mesulergine) binding experiments (Fig. 8i-k) supporting that 5-HT_{2C} protomers somehow masked the ligand binding site of the other protomer in 5-HT₂ 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-HT₂-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_{2CΔCter} expression in PFC, upon infection with adeno-associated viruses carrying a 5-HT_{2CΔCter} construct (AAV-5-HT_{2CΔCter}) was associated with an increase of [³H]-Mesulergine able to bind to endogenous 5-HT_{2A} and exogenous 5-HT_{2CΔCter} 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 [³H]-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_{2CΔCter} protomer in adrenergic *Locus ceruleus* (LC) neurons. Previous studies reported that the 5-HT₂ 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-HT_{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_{2CΔCter} protomer (Fig. 10b-d). Bilateral stereotaxic injections of AAV- 5-HT_{2CΔCter} 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_{2CΔCter} 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_{2CΔCter} injected mice (Fig. 10d), consistent with an impaired signaling of 5-HT_{2A} receptors. These data indicate that 5-HT_{2CΔCter} 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_{2CΔCter}, which lacks the capacity of activating Gαq proteins, yet inhibits both Gαq coupling and ligand binding of associated 5-HT₂ 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-HT₂ 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 BRET₅₀ 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-HT₂ 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-HT₂ 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_{2CACter} 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_{2CACter} 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-HT₇ 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-HT₇ 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 G_{aq} pathway between membranes expressing 5-HT₂ receptors alone and membranes expressing 5-HT₂ heterodimers. However, heterodimerization may affect 5-HT₂ receptors signaling in many other ways: in addition to the activation of G_{aq}, G_{ai/o} or G_{α_{12/13}} proteins, activation of phospholipase A₂, phospholipase D (ERK_{1/2} or β-arrestin-dependent pathways) (52,53) can be mediated by 5-HT₂ 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 G_q 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-HT₂ 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% penicillin/streptomycin (Sigma, St Louis, MO, USA), in 9-cm dishes (Falcon). Cells were incubated at 37°C in a 5% CO₂ 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}ACter expression.

Adeno-associated virus (AAV9)-expressing GFP (7.5x10¹² virus molecules/ml) and 5-HT_{2CΔCter} (IRES) GFP (8.8 x10¹² virus molecules/ml) under the control of the synapsin promoter (UNC Vector Core, Dr. R. Jude Samulski, Chapel Hill)

were stereotactically 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). Stereotactically 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 mg/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.

[³H]Radioligands and drugs

[³H]Myo-inositol (51.0 Ci/mmol), [³H]-Mesulergine (99 Ci/mmol), [³H]-5-Hydroxytryptamine (80.0 Ci/mmol) (5-HT), [³H]-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).

[³H]Inositol Phosphate (IP) Accumulation Assay

Twenty-four hours before the experiment, cells were incubated in 24-well plates overnight with 20 nM of [³H]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 CaCl₂, 1.2 mM NaH₂PO₄, 1.2 mM MgSO₄, 10 mM Hepes, 10 mM glucose, pH 7.4) supplemented with 20 mM LiCl to prevent IP₁ 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, IP₁ accumulated from IP₃ and IP₂ hydrolysis, was released from lysed and fixed cells. The accumulated IP₁ was eluted on anion exchange columns (Bio-Rad AG-1X8, BioRad Laboratories, Hercules, CA, USA) with 0.2 M ammonium formate in 0.1 M formic acid. Scintillation cocktail (Ultima Gold XR, Perkin Elmer) was added to the eluted [³H]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 3 µg 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 IP₁ degradation (NaCl 146 mM, KCl 4.2mM, MgCl₂ 0.5mM, CaCl₂ 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:

$$\text{Response } A = [A]^n \tau^n E_m / [A]^n \tau^n + ([A] + K_A)^n$$

E_m = maximal response

n = slope

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

τ = efficacy

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

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 MgCl₂ (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 [³H]mesulergine (PerkinElmer Life) and increasing concentration of MDL100907 for 60 min at room temperature. [³H]mesulergine ligand choice was based on his selective 5-HT₂ 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 MgCl₂, 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 [³H] 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_{2BΔCter}, 5-HT_{2C} or 5-HT_{2CΔCter} 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 ³H-mesulergine were diluted in Krebs-Ringer-Hepes buffer at a final concentration between half the K_d and the K_d 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-HT₂ receptors. After forty eight hours, cells were washed in PBS, sonicated and solubilized in lysis buffer [75 mM Tris, 2 mM EDTA, 12 mM MgCl₂, 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 10⁵ per well of a 6-well plates) were transfected with 30-100 ng plasmid DNA coding for the BRET donor (5-HT₂-Luc) and increasing amounts of BRET acceptor plasmids (5-HT₂-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; 10⁵ 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-HT₂-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 \pm 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 $p < 0.05$.

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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.

Conducted experiments: S Doly, B Guiard, E Quentin and I Moutkine.

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.

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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 (Δ Cter);

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-methylpyridin-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)

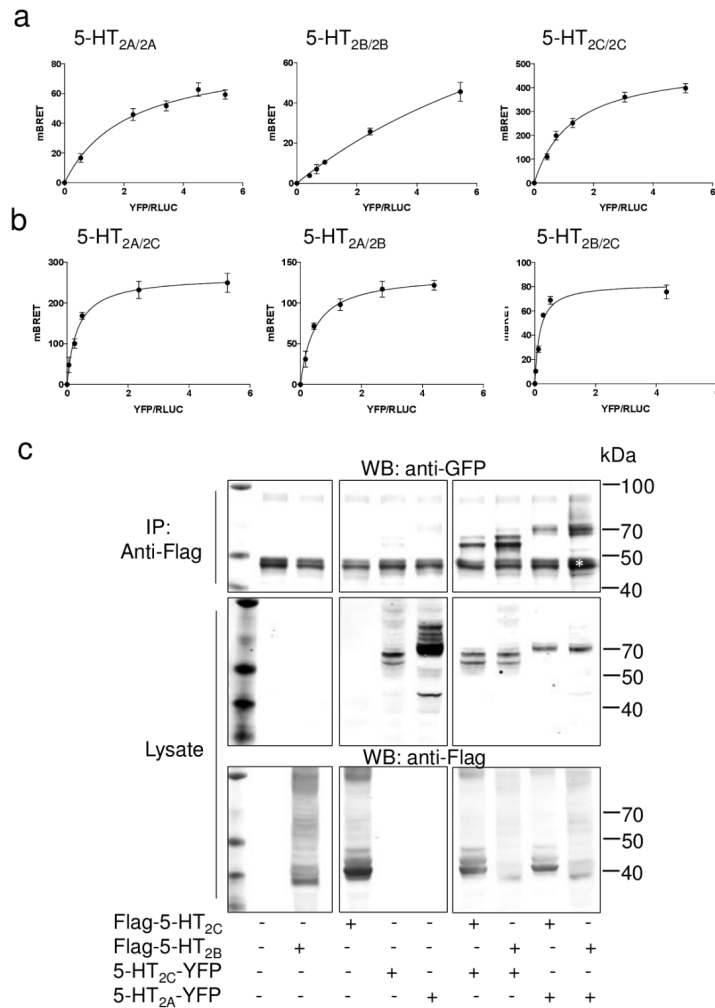


Figure 1: Constitutive heterodimerization of 5-HT₂ receptors and co-immunoprecipitation in living HEK293 cells. **a)** BRET proximity assays between homodimers of 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors. HEK-293 cells were co-transfected with plasmids coding for a constant amount of RLuc-5-HT_{2A}, RLuc-5-HT_{2B} or RLuc-5-HT_{2C} (BRET donors) and increasing concentrations of the corresponding YFP tagged 5-HT₂ 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-HT₂ 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, BRET₅₀) were obtained from 6 independent experiments. Plots were established using Graphpad software. 5-HT_{2A/2A}: BRETmax 76±16, BRET₅₀ 2.4±0.5; 5-HT_{2B/2B} Ambiguous; 5-HT_{2C/2C} BRETmax 434±32 BRET₅₀ 1.2±0.4; 5-HT_{2A/2C} BRETmax 266±24 BRET₅₀ 0.33±0.04; 5-HT_{2A/2B} BRETmax 90±32 BRET₅₀ 0.19±0.07; 5-HT_{2B/2C} BRETmax 85±6 BRET₅₀ 0.16±0.04. **c)** Interactions of 5-HT₂ 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-HT₂-GFP and 5-HT₂-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.

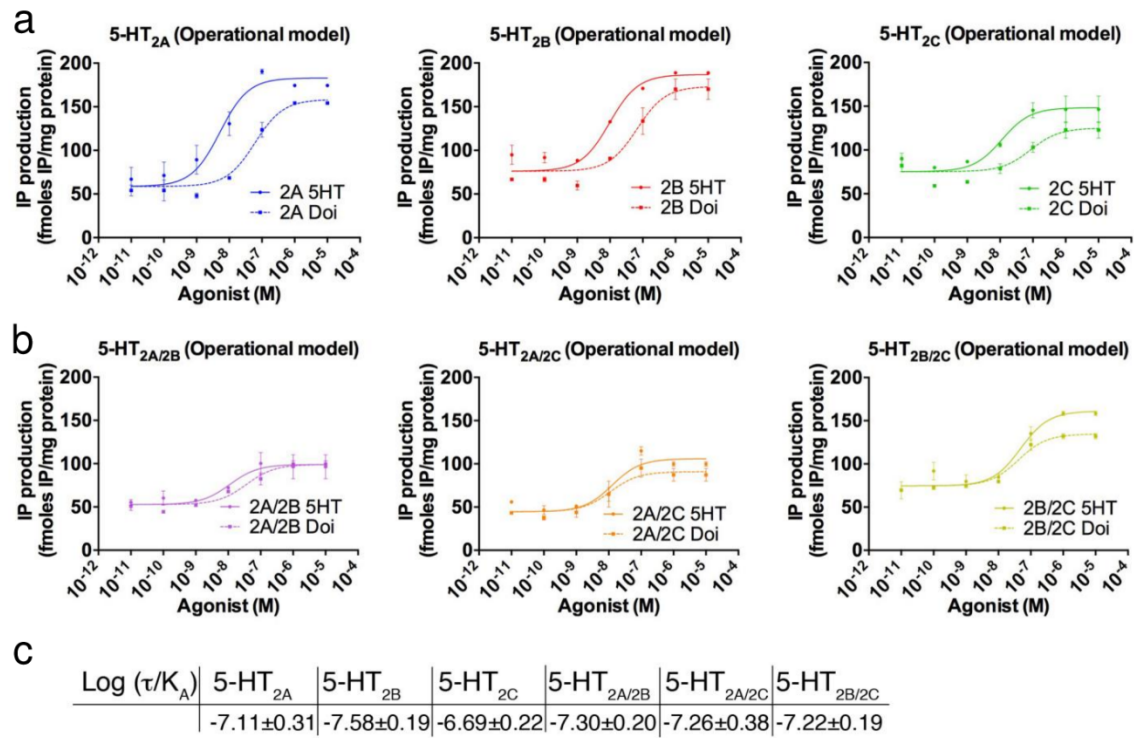


Figure 2: IP production induced by stimulation by a full (5-HT) and a partial agonist (DOI)

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 modeled 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 \pm SEM.

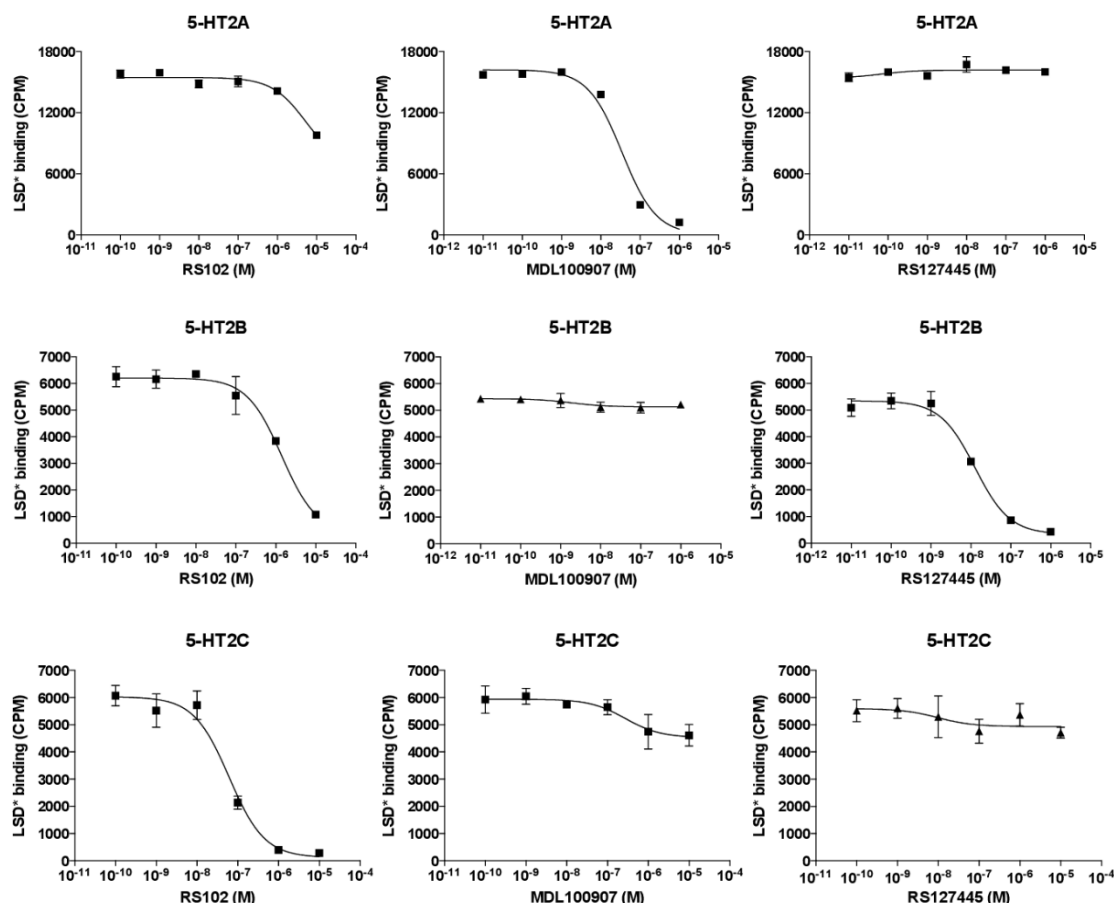


Figure 3: Affinity and selectivity of antagonists used in this study. Affinity and selectivity of antagonists were tested to define the optimal concentration and avoid cross-reactivity. Representative examples of ³H-LSD radioligand binding heterologous competition experiments performed on membrane preparation. HEK293 cells transiently expressing 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors were incubated with ³H-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 \pm SEM from triplicates. Binding curves were done using Graphpad software.

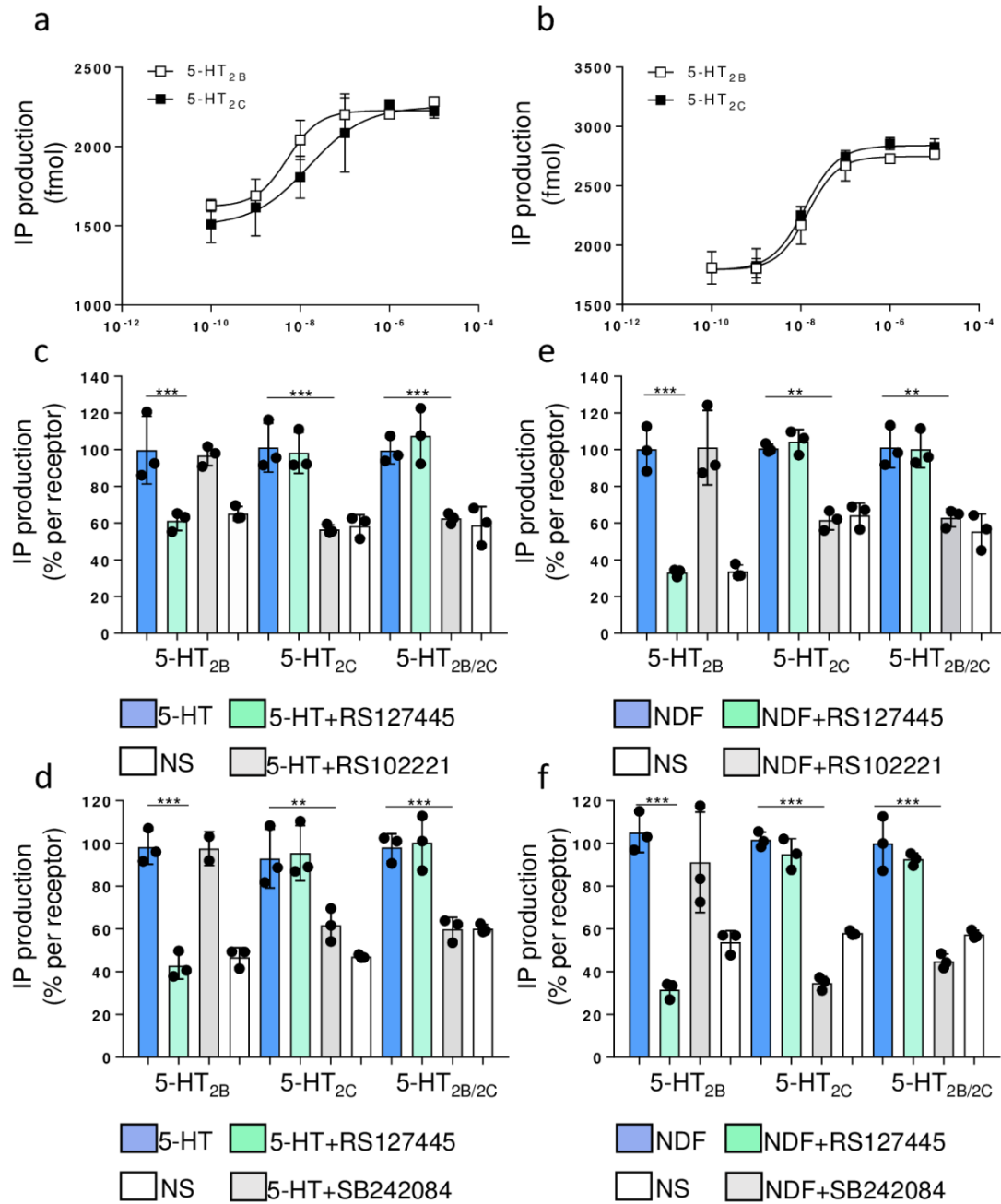


Figure 4: 5-HT_{2C} receptor expression blocks 5-HT_{2B} receptor signaling. **a)** Agonist concentration-induced IP response. Serotonin (5-HT) and Nor-(+)-Fenfluramine (NDF) dose-response curves for stimulation of IP production in cells expressing the same amount of 5-HT_{2B} (white box) or 5-HT_{2C} (dark box) receptors were used to obtain the minimal agonist concentration-induced maximal IP response (1 μ M for both agonists). Bars represent \pm 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 μ M of the 5-HT₂ 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 \pm 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.

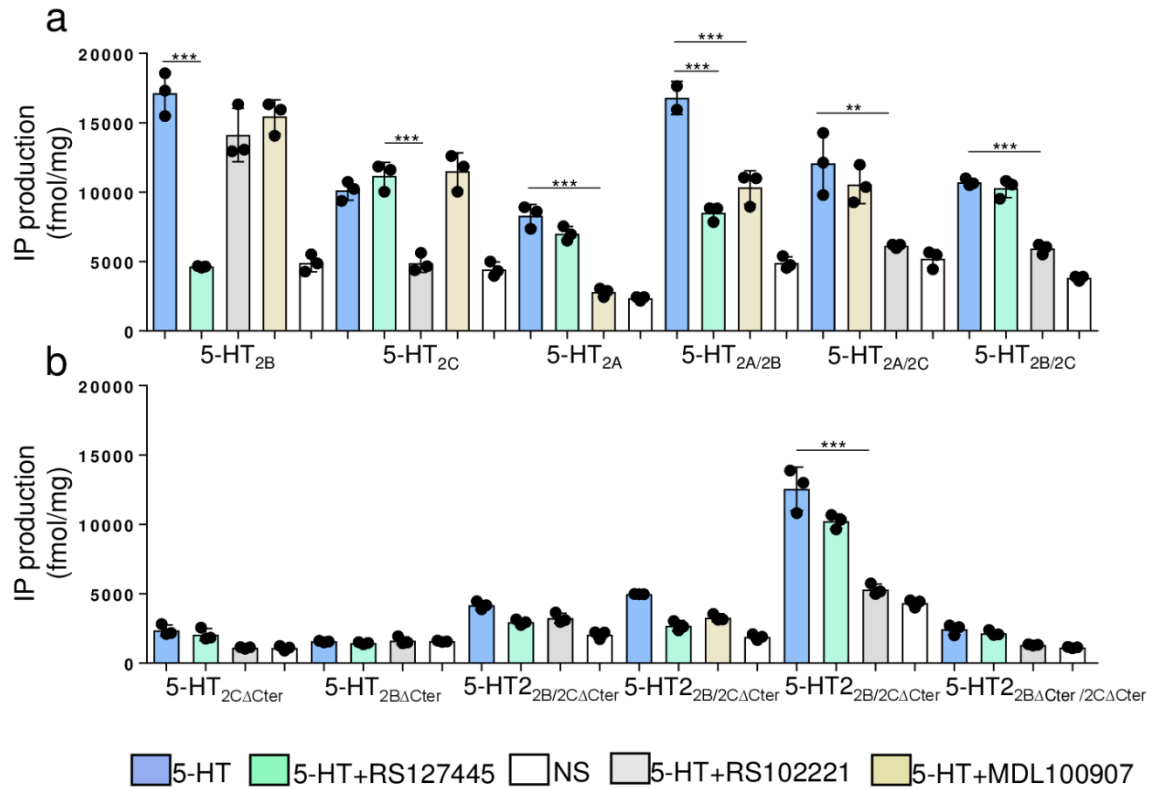


Figure 5: The 5-HT_{2C} receptor blunts 5-HT_{2A} and 5-HT_{2B} receptor signaling. HEK293 cells transiently expressing 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{2B/Cter} and 5-HT_{2C/Cter} receptor alone or in combination, were stimulated with 1 μ M of serotonin (5-HT) and IP levels were determined. The 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/Cter}, 5-HT_{2C/Cter} or 5-HT_{2B/Cter/2C/Cter} receptors gives little IP accumulation. Stimulation of 5-HT_{2A/2C/Cter}, or 5-HT_{2B/2C/Cter} receptors gives also nearly no IP accumulation, whereas 5-HT_{2C/2B/Cter} leads to IP accumulation that can only be blocked by the 5-HT_{2C} receptor antagonist. Bars represent \pm 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.

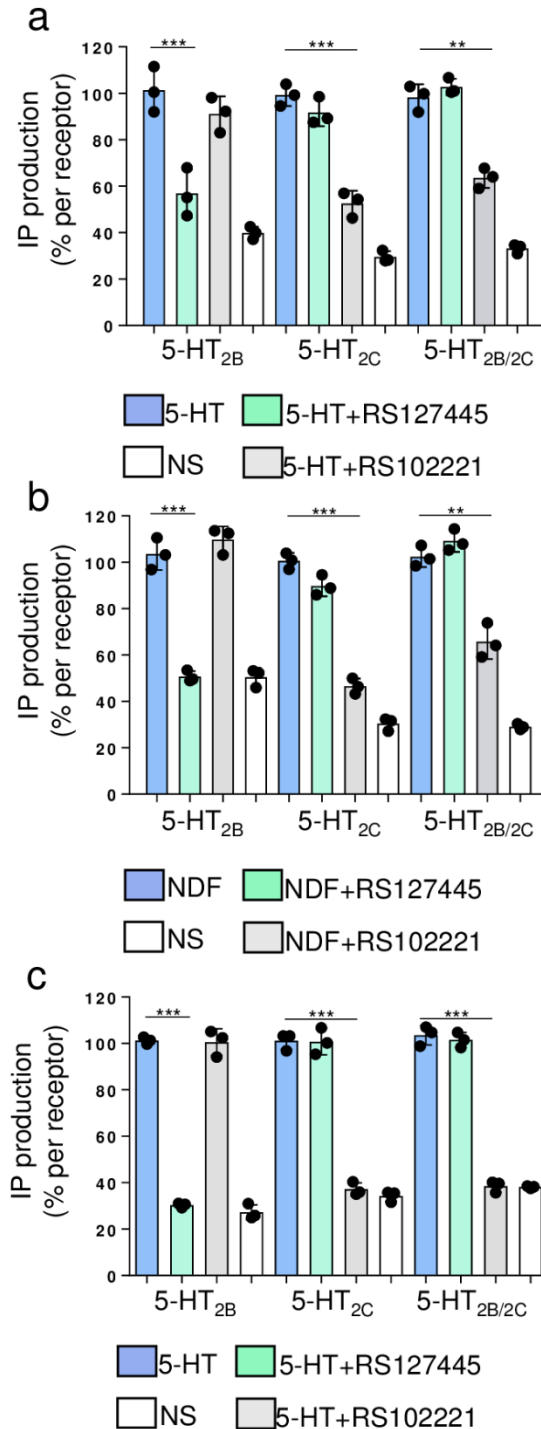


Figure 6: 5-HT_{2C} receptor blunts 5-HT_{2B} receptor signaling independently of the cell types. LMTK⁻ (a-b) or CHO (c) cells transiently expressing 5-HT_{2C} and 5-HT_{2B} receptor alone or in combination (5-HT_{2B/2C}), were stimulated with 1 μ M of the 5-HT₂ agonist serotonin (5-HT) or Nor-(+)-Fenfluramine (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 \pm 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.

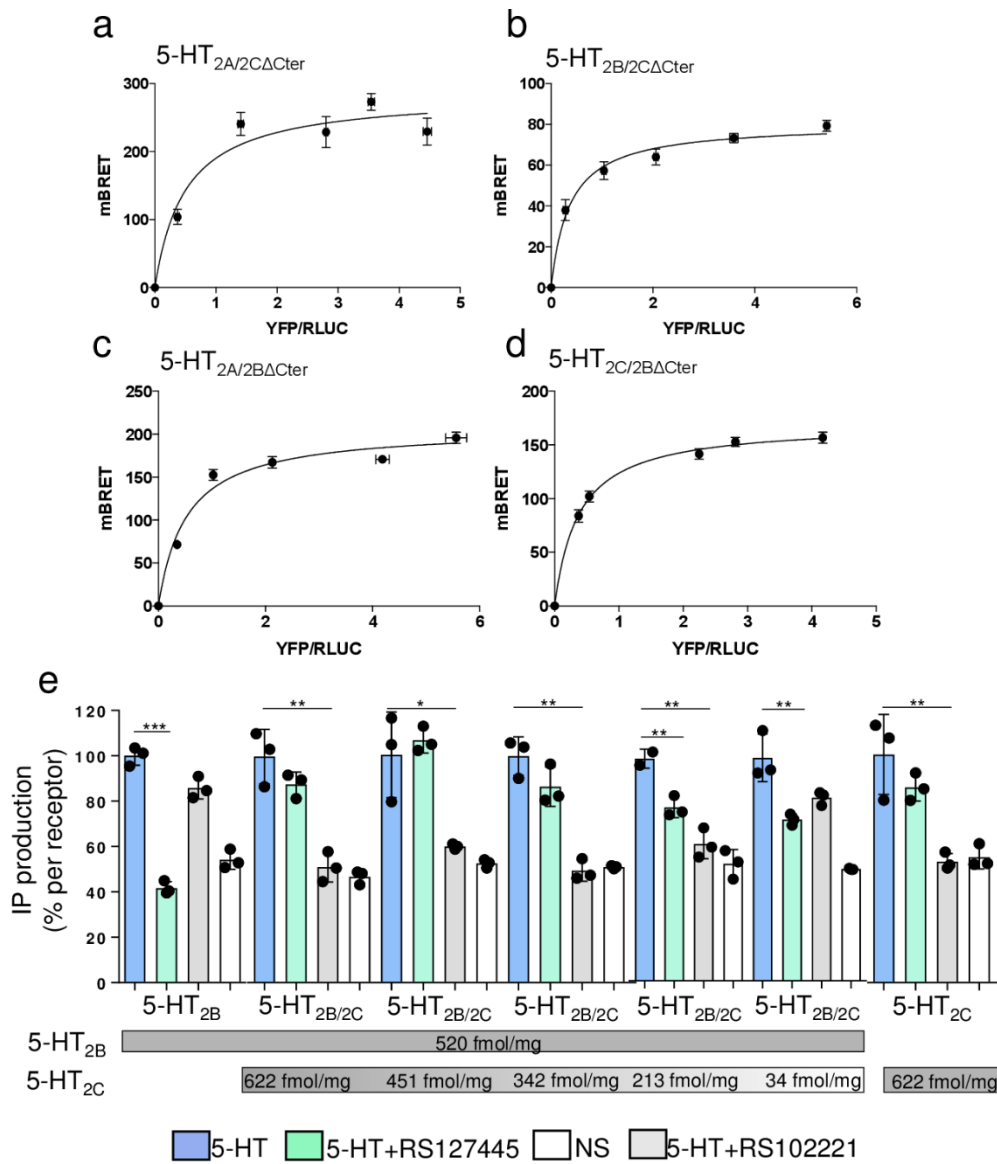


Figure 7: a-d Constitutive heterodimerization of 5-HT₂ receptors with 5-HT_{2B}ΔCter and 5-HT_{2C}ΔCter constructs in living HEK293 cells. HEK-293 cells were co-transfected with plasmids coding for a constant amount of RLuc-5-HT_{2A}, RLuc-5-HT_{2B} or RLuc-5-HT_{2C} (BRET donors) and increasing concentrations 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, BRET₅₀) were obtained from 3 independent experiments. Plots were established using Graphpad software. a) 5-HT_{2A/2C}ΔCter: BRETmax 284±27, BRET₅₀ 0.49±0.22; b) 5-HT_{2B/2C}ΔCter BRETmax 80±3 BRET₅₀ 0.36±0.07; c) 5-HT_{2A/2B}ΔCter BRETmax 207±12 BRET₅₀ 0.52±0.13; d) 5-HT_{2C/2B}ΔCter BRETmax 170.0±2.5 BRET₅₀ 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.

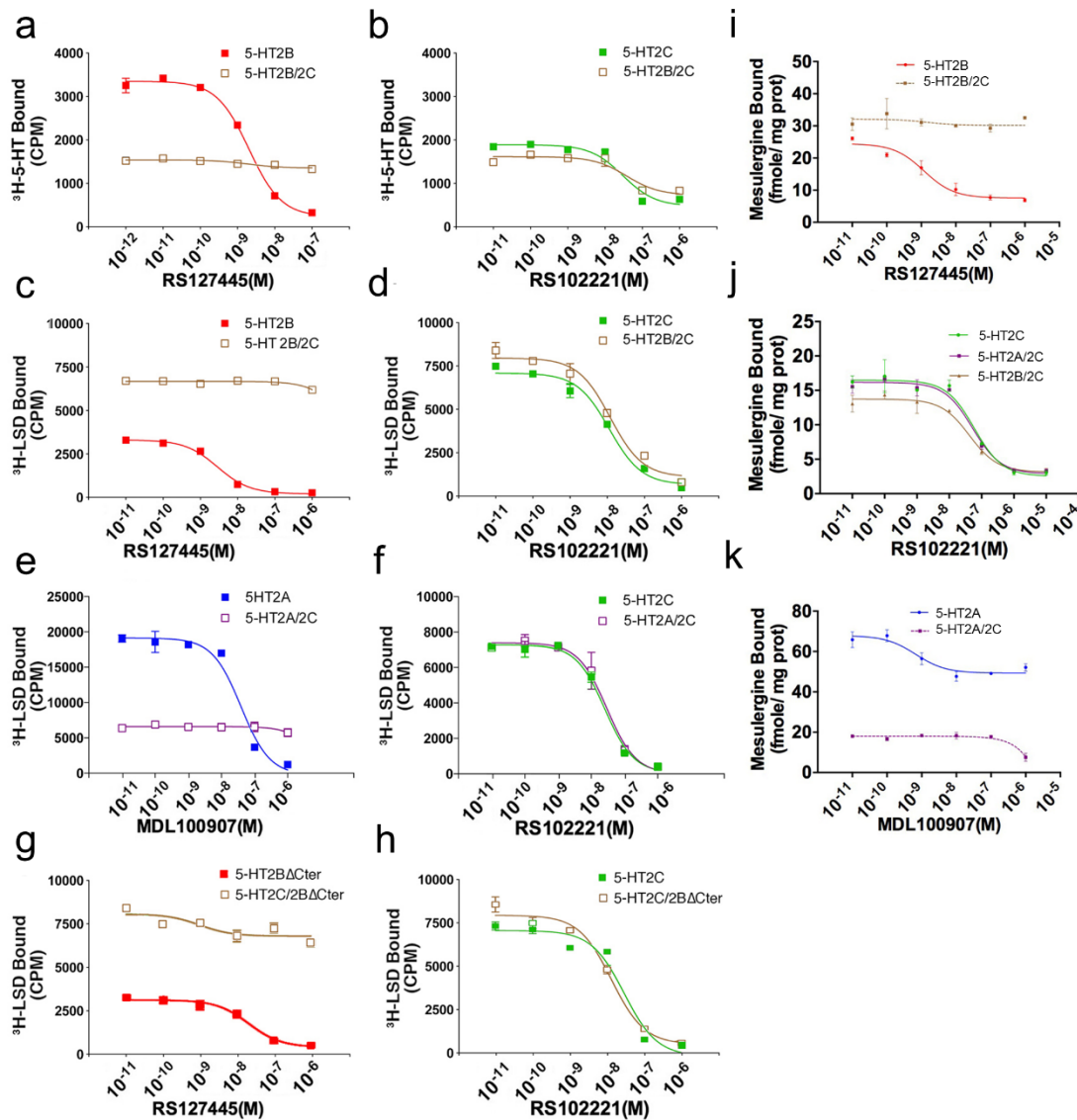


Figure 8: The co-expression of 5-HT_{2C} receptors blunts 5-HT_{2A} and 5-HT_{2B} ligand binding. Transiently transfected cells were incubated with **a-b)** ³H-5-HT, **c-h)** ³H-LSD or **i-k)** ³H-Mesulergine and increasing concentrations of the 5-HT_{2A} or 5-HT_{2B} antagonist or 5-HT_{2C} antagonist. **a-b).** 5-HT_{2C} receptor transfection with 5-HT_{2B} receptors abolishes the ³H-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 ³H-LSD competition by 5-HT_{2A} or 5-HT_{2B} antagonists. **g-h).** 5-HT_{2C} receptor transfection with 5-HT_{2BΔCter} receptors abolishes the ³H-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 ³H-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.

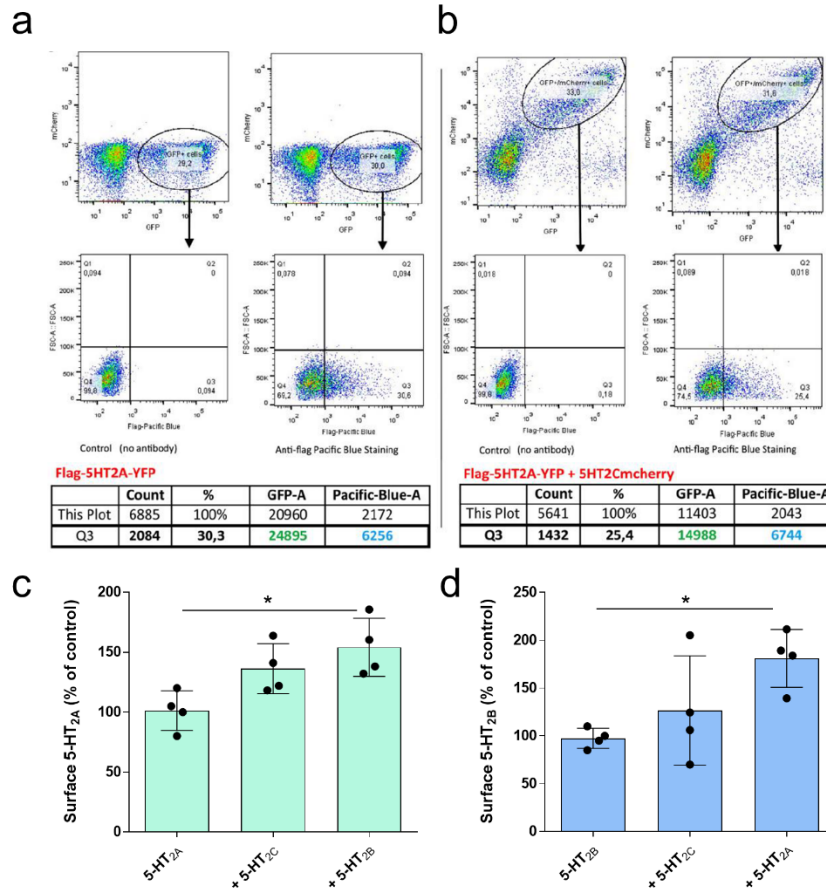


Figure 9. Receptor cell-surface export of 5-HT_{2A} and 5-HT_{2B} receptors is not affected by the co-expression with the 5-HT_{2C} protomer. Surface 5-HT_{2A} or 5-HT_{2B} receptors in YFP-Cherry positives non-permeabilized cells, were quantified by cytometry using anti-Flag primary antibody conjugated 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).

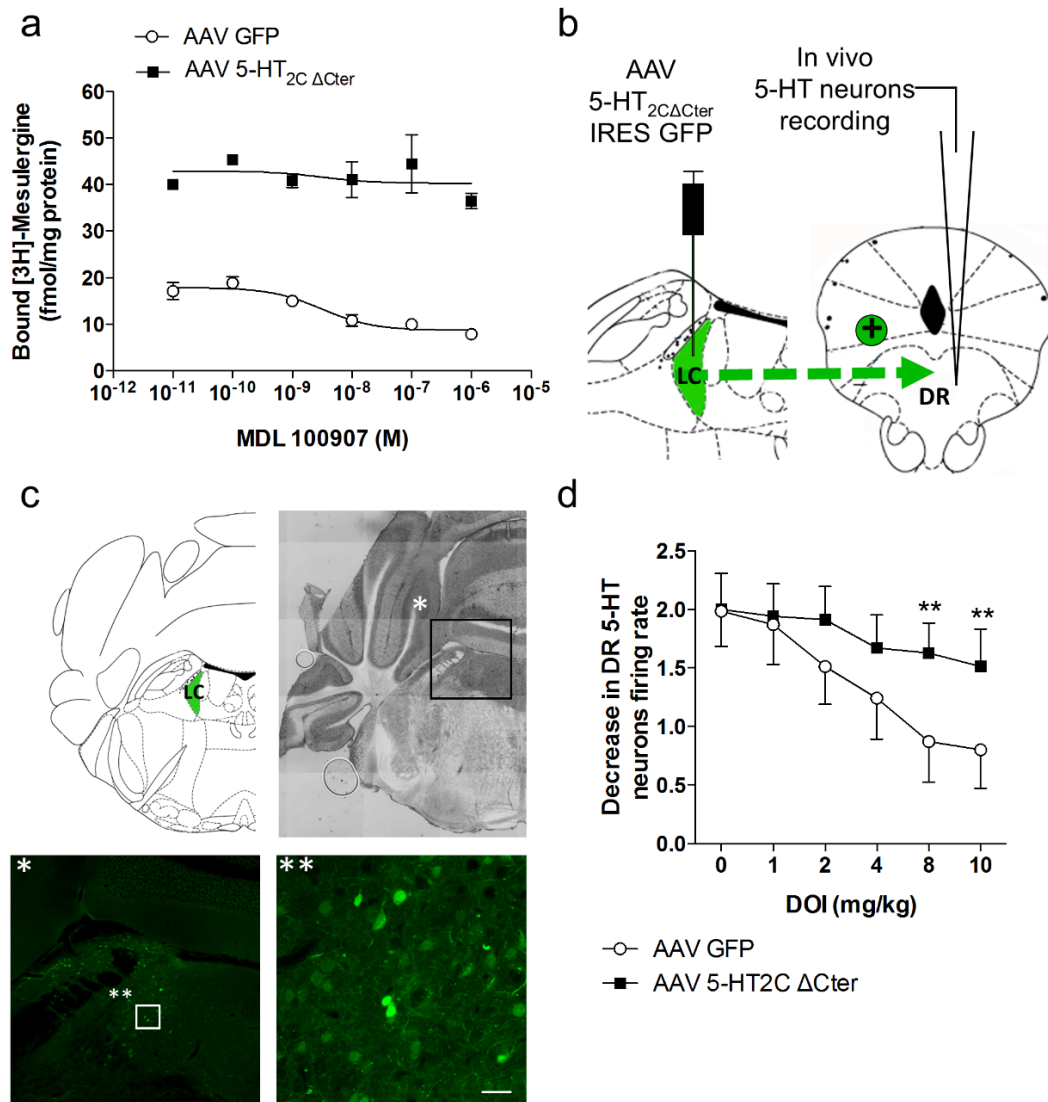


Figure 10: The 5-HT_{2C}ΔCter inactive protomer prevents 5-HT_{2A} ligand binding in the PFC and reduces DOI-induced 5-HT_{2A} receptor dependent decrease of 5-HT neurons firing rate.

a) Exogenous 5-HT_{2C}ΔCter expression in PFC, upon bilateral infection with adeno-associated viruses carrying a 5-HT_{2C}ΔCter construct (AAV-5-HT_{2C}ΔCter) was associated with a dramatic reduction of endogenous 5-HT_{2A}-dependant ligand binding compared to AAV mediated GFP expression only. Representative example of ³H-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}ΔCter 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}ΔCter 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}ΔCter (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.