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DOPAMINE D2 AND 5-HYDROXYTRYPTAMINE 5-HT$_{2A}$ RECEPTORS ASSEMBLE INTO FUNCTIONALLY INTERACTING HETEROMERS

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Abstract

In view of the co-distribution of dopamine $D_{2L}R$ and 5-hydroxytryptamine 5-HT_{2A} receptors ($D_{2L}R$ and 5-$HT_{2A}R$, respectively) within inter alia regions of the dorsal and ventral striatum and their role as a target of antipsychotic drugs; in this study we assessed the potential existence of $D_{2L}R$-5-$HT_{2A}R$ heteromers in living cells and the functional consequences of this interaction. Thus, by means of a proximity-based bioluminescence resonance energy transfer (BRET) approach we demonstrated that the $D_{2L}R$ and the 5-$HT_{2A}R$ form stable and specific heteromers when expressed in HEK293T mammalian cells. Furthermore, when the $D_{2L}R$-5-$HT_{2A}R$ heteromeric signaling was analyzed we found that the 5-$HT_{2A}R$-mediated phospholipase C (PLC) activation was synergistically enhanced by the concomitant activation of the $D_{2L}R$ as shown in a NFAT-luciferase reporter gene assay and a specific and significant rise of the intracellular calcium levels were observed when both receptors were simultaneously activated. Conversely, when the $D_{2L}R$-mediated adenylyl cyclase (AC) inhibition was assayed we showed that costimulation of $D_{2L}R$ and 5-$HT_{2A}R$ within the heteromer led to inhibition of the $D_{2L}R$ functioning, thus suggesting the existence of a 5-$HT_{2A}R$-mediated $D_{2L}R$ trans-inhibition phenomenon. Finally, a bioinformatics study reveals that the triplet amino acid homologies LLT (Leu-Leu-Thr) and AIS (Ala-Ile-Ser) in TM1 and TM3, respectively of the D_2R-5-$HT_{2A}R$ may be involved in the receptor interface. Overall, the presence of the $D_{2L}R$-5-$HT_{2A}R$ heteromer in discrete brain regions is postulated based on the existence of $D_{2L}R$-5-HT_{2A} receptor-receptor interactions in living cells and their codistribution inter alia in striatal regions. Possible novel therapeutic strategies for treatment of schizophrenia should be explored by targeting this heteromer.

Keywords: dopamine D_2R receptor; serotonin 5-HT_{2A} receptor; heteromerization; G protein-coupled receptors; allosteric modulation; protein-protein interactions.

Introduction

Blockade of the D_2R especially in the limbic system likely mediates the antipsychotic actions of D_2R antagonists in treatment of schizophrenia [1; 2; 3; 4]. The following D_2R containing heteromers have been discussed in relation to schizophrenia; D_2R-adenosine A_{2A} receptor ($A_{2A}R$), D_2R-metabotropic glutamate receptor type 5 ($mGlu_5R$), D_2R-cannabinoid receptor type 1 (CB_1R), D_2R-neurotensin receptor type 1 (NTS_1R) and D_2R-D_3R [4]. The D_2R regulated accumbal-ventral pallidal-mediodorsal-prefrontal circuit is of special interest since the D_2R in the nucleus accumbens produces a reduction in the prefrontal glutamate drive via the mediodorsal thalamic nucleus. The 5-$HT_{2A}R$ is also a target for antipsychotic drugs since the postjunctional 5-HT receptor mediating the hallucinogenic actions of d-lysergic acid diethylamide (d-LSD) [5; 6; 7] as well as other hallucinogens of the indolalkylamine type [8; 9]. In view of the co-distribution of D_2R and 5-$HT_{2A}R$ immunoreactivity within inter alia regions of the dorsal and ventral striatum [3; 10; 11] it is of substantial interest to explore if these two receptors can form heteromers, which would represent a novel target for drug development in schizophrenia. In this work we demonstrate the existence of $D_{2L}R$/5-$HT_{2A}R$ heteromers in living HEK293T cells. Interestingly, substantial functional $D_{2L}R$-5-$HT_{2A}R$ receptor-receptor allosteric interactions have been demonstrated by determining the AC and PLC activation via gene reporter assays and by intracellular Ca^{2+} measurements.

Materials and Methods

Plasmid constructs

The constructs presented herein were made using standard molecular biology techniques employing PCR and fragment replacement strategies. The 5-HT$_{2A}$R^{GFP2} and D$_{2L}$R^{Rluc} were constructed by ligating the green fluorescence protein 2 (GFP^2) and the *Renilla* luciferase (*R*luc) to the C-terminal tail of each receptor subtype. In brief, the human 5-HT$_{2A}$R coding sequences without their stop codons were amplified from 3xHA-5HT$_{2A}$R-pcDNA vectors using sense and antisense primers harboring unique NheI and AgeI sites. The fragments were then subcloned in-frame into humanized pGFP^2-N1 vectors (PerkinElmer, Waltham, MA, USA). On the other hand, the cDNA encoding the D$_{2L}$R without its stop codon was subcloned in p*R*luc-N3 (Perkin-Elmer, Madrid, Spain) and pEYFP-N1 (Clontech, Heidelberg, Germany) (D$_{2L}$R^{Rluc} and D$_{2L}$R^{YFP} respectively). The reading frame and PCR integrity of all cloned constructs were confirmed by DNA sequencing.

Cell culture, transfection and confocal microscopy

HEK293T cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2. Cells were plated in 6-well dishes at a concentration of 1×10^6 cells/well or in 75cm^2 flasks and cultured overnight before transfection. Cells were transiently transfected either using linear PolyEthylenImine reagent (PEI) (Polysciences Inc., USA) or TransFectin (Bio-Rad, USA). Transiently transfected HEK293T cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS containing 20 mM glycine and mounted with Vectashield immunofluorescence medium (Vector Laboratories, UK). Microscope observations were performed with a Leica TCS-SL confocal microscope (Leica, USA).

BRET^2 assay

Forty-eight hours after transfection, HEK293T cells transiently transfected with constant (1 µg) or increasing amounts (0.25-9 µg) of plasmids encoding for $D_{2L}R^{Rluc}$ and $5\text{-}HT_{2A}R^{GFP2}$ respectively, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 µg protein) were distributed in duplicate into the 96-well microplate black plates with a transparent bottom (Corning 3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination. For $BRET^2$ measurement, coelenterazine-400a also known as *DeepBlue*™C substrate (VWR, Sweden) was added at a final concentration of 5 µM, and readings were performed 1 min after using the POLARstar Optima plate-reader (BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings [410 nm (with 80 nm bandwidth) and 515 nm (with 30 nm bandwidth)]. The $BRET^2$ ratio is defined as previously described [12].

Luciferase reporter gene assay

We used a dual luciferase reporter assay to indirectly detect variations of cAMP levels or activation of PLC-PKC pathway in transiently transfected cell lines treated with different compounds in a range of concentrations (typically 10 nM to 100 µM)[13; 14]. For luciferase assays, 24 h before transfection, cells were seeded at a density of 1×10^6 cells/well in 6-well dishes and transfected with PEI. Cells were co-transfected with plasmids corresponding to three constructs as follows (per 6-well): 1 µg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p or pGL4-NFAT-luc2p; Promega, Stockholm, Sweden), 1 µg of $D_{2L}R$ plus $5\text{-}HT_{2A}R$ expression vectors and 50 ng *R*luc-encoding internal control plasmid (phRG-B; Promega). Approximately 36 h post transfection, after the cells were incubated with appropriate ligands and harvested with passive lysis buffer (Promega), the luciferase activity of cell extracts was determined using a luciferase assay system according to the manufacturer's protocol in a POLARstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) using a 535 nm excitation filter with a 30-nm bandwidth. Firefly luciferase was measured as firefly luciferase luminescence over a 15 s reaction period. The luciferase values were normalized against *R*luc luminescence values.

Fluorescent measurement of intracellular Ca^{2+} concentration

Calcium mobilization in transiently cotransfected HEK293T cells was measured using the Fluo-4 Non Wash Calcium Assay kit from Molecular Probes (Invitrogen, Sweden) [15; 16]. HEK293T cells were seeded overnight at a density of 1×10^5 cells/well in a 96-well black wall/clear bottom Costar® plate (VWR, Sweden). After removal of medium, cells were incubated with 100 µL probenecid-containing dye-loading solution (2 mM probenecid, 10 mM HEPES in HBSS) for 30 min at 37°C, and then another 20 minutes at room temperature, followed by agonist/vehicle addition at different concentrations. The responses were measured at room temperature in a POLARstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) using 485/10 excitation filter and a 530/10 emission filter. For each experiment, the baseline fluorescence was read for 20 s, following agonist addition fluorescence was then continuously monitored at 1-s intervals for 2 min.

Bioinformatics prediction of the heteromer interface interaction

Based on a bioinformatics approach, a set of amino acid triplet homologies have been deduced in receptor heterodimers that may be responsible for receptor–receptor interactions [17]. It has been indicated how such triplets of amino acid residues and their 'teams' may be utilized to construct a kind of code that determines (and/or predicts) which receptors should or should not form heterodimers. In this study the D_{2L}R-5-HT_{2A}R heterodimer has been analyzed for the existence of a basic set of common triplets in the two participating receptors that may be responsible for the receptor-receptor interactions.

Statistical analysis

The number of samples (*n*) in each experimental condition is indicated in Fig. legends. For statistical evaluation of the biochemical data, unless otherwise specified, statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant.

Results

Subcellular distribution of $D_{2L}R$ and 5-$HT_{2A}R$ in HEK293 cells

We first studied the association of $D_{2L}R$ and 5-$HT_{2A}R$ by means of immunocytochemistry experiments and confocal microscopy analysis. Thus, we determined the subcellular distribution of both $D_{2L}R^{YFP}$ and 5-$HT_{2A}R^{GFP2}$ when expressed alone and in combination. Interestingly, when these two receptors were coexpressed in HEK293T cells an overlap in the distribution in both at the plasma membrane level and in intracellular compartments was confirmed (Supplementary, Fig. 1B). Interestingly, it is important to note that the cell surface expression of $D_{2L}R$ and 5-$HT_{2A}R$ was more evident when cells were cotransfected with these two receptors (Supplementary, Fig. 1A).

Study of $D_{2L}R$ and 5-$HT_{2A}R$ hetromerization by BRET assays

Although the use of biochemical approaches to demonstrate protein-protein interactions has been widely used, it might have some disadvantages since the cellular structure is destroyed by detergent treatment. Thus, in order to assess $D_{2L}R$ and 5-$HT_{2A}R$ form oligomers in living cells, biophysical approaches were performed in cells transiently transfected with receptor constructs that carry appropriate fluorophore pairs in their C-terminus. Thus, the possible direct receptor-receptor interaction was studied by BRET experiments. The BRET assay was performed on HEK293T cells co-transfected with a constant amount of $D_{2L}R^{R\text{luc}}$ construct while increasing the concentrations of a 5-$HT_{2A}R^{GFP2}$ plasmid. As a positive control, cells expressing a GFP^2-*R*luc tandem fusion protein were used. A positive BRET signal was obtained for the transfer of energy between $D_{2L}R^{Rluc}$ and $5\text{-}HT_{2A}R^{GFP2}$. Upon co-expression of the $5\text{-}HT_{2A}R^{GFP2}$ and $D_{2L}R^{Rluc}$ cDNA, a significantly higher $BRET^2$ signal was observed in comparison to the $BRET^2$ signal obtained from a mixture of $D_{2L}R^{Rluc}$ singly expressing cells and $5\text{-}HT_{2A}R^{GFP2}$ singly expressing cells (Fig. 1A). The BRET signal as seen from the $BRET^2$ ratio increased as a hyperbolic function of the concentration of the 5- $HT_{2A}R^{GFP2}$ fusion construct. Thus, as the negative control led to a quasi-linear curve, the specificity of the saturation (hyperbolic) assay for the $D_{2L}R^{Rluc}$ and $5\text{-}HT_{2A}R^{GFP2}$ pair could be established. On the other hand, co-transfected of a constant amount of $D_{2L}R^{Rluc}$ construct with increasing concentrations of a $5\text{-}HT_{1A}R^{GFP2}$ plasmid does not increased as a hyperbolic function of the concentration of the 5- $HT_{1A}R^{GFP2}$ (Fig. 1A). Furthermore, the specificity of this interaction is also indicated on Fig. 1B where the co-transfection of increasing concentrations of $D_{2L}R$ and $5\text{-}HT_{2A}R$ leads to a concentration dependent disappearance of the $BRET^2$ signal, while co-transfection of pcDNA3.1+ or $5\text{-}HT_{1A}R$ in the same concentration range failed to diminish the signal. Taken together, these results support the notion that the $5\text{-}HT_{2A}R$ directly and specifically interacts with $D_{2L}R$ in living cells and forms a $D_{2L}R$-5- $HT_{2A}R$ heteromer.

Functional consequences of $D_{2L}R$/5- $HT_{2A}R$ heteromerization

To explore the implications of the dopamine and $5\text{-}HT_{2A}R$ receptor interactions in cotransfected cells on second messenger signaling cascades (i.e. cAMP, IP_3 and Ca^{2+}), we examined the 5-$HT_{2A}R$ and $D_{2L}R$ agonist-mediated signaling changes as measured by different luciferase reporter assays in heterologous HEK293T cells coexpressing the tagged $D_{2L}R$ and tagged 5-$HT_{2A}R$ receptors.

Determination of AC modulation by a CRE-luciferase reporter assay

The concentration response curve obtained with the $D_{2L}R$ like agonist quinpirole to inhibit forskolin-mediated CRE-Luc induction was shifted to the right by 5-HT (1 μM), showing a reduced potency of this agonist at the $D_{2L}R$ receptor (Fig. 2A). The forskolin-induced increase of luciferase activity by a direct activation of AC is significantly reduced by 100 nM of quinpirole, a concentration around the EC_{50} value (Fig. 2B).The inhibition by quinpirole of the CRE-Luc induction was blocked by the D2R like antagonist raclopride (1 μM) and partly counteracted by 5-HT (0.5μM) and the 5-HT_{2A}R agonist TCB2 (50 nM).The 5-HT_{2A}R antagonist ketanserine (1 μM) blocked the actions of 5-HT and TCB2.

Determination of PLC modulation by a NFAT-luciferase reporter assay

Next, we explored the functionality of the heteromer and the Gq/11 mediated signaling of the 5-HT_{2A} receptor and its modulation by quinpirole (50-100 nM) using NFAT-luciferase reporter assay under the control of the receptor-receptor heteromer (Fig. 3A). Quinpirole shifted the concentration-response curve of 5-HT to the left showing an increased potency of 5-HT to activate the 5-HT_{2A} receptor (Fig. 3B).The pharmacological analysis of this interaction was made with 5-HT (0.5 μM) and TCB2 (50 nM) in concentrations around the EC_{50} value (Fig. 3B).

In line with the results in Fig. 3A, quinpirole enhanced the 5-HT and TCB2 mediated NFAT-Luc induction and ketanserine (1μM) counteracted the effects of the two agonists at the 5-HT2A receptor (Fig. 3B).

Intracellular Ca^{2+} responses in cotransfected cells

The Gq/11 mediated 5-HT_{2A}R responses were also studied using intracellular Ca^{2+} measurements (Fig. 4). It is demonstrated that the concentration dependent rise of intracellular Ca^{2+} levels by 5-HT is shifted to the left by quinpirole. Again an increased potency of 5-HT at the 5-HT_{2A}R receptor is demonstrated in the presence of D_{2L}R activation by quinpirole, Taken together, this suggests that the 5-HT_{2A}R signaling over Gq/11 becomes increased in the heteromer by quinpirole induced activation of the D_{2L}R protomers, while the D_2R signaling over Gi/o in the heteromer becomes reduced by 5-HT induced activation of the 5-HT2A protomer (Fig. 4).

The triplets LLT and AIS may be crucial for D_{2L}R-5-HT2A heterodimer formation.

Based on a bioinformatics approach, Tarakanov and Fuxe [17] have deduced a set of triplet homologies that may be responsible for receptor-receptor interactions. This set consists of two nonintersecting subsets: 'pro-triplets' and 'contra-triplets'. Any pro-triplet appears as a homology in at least one heterodimer but does not appear as a homology in any non-heterodimer. Just the reverse, any contra-triplet appears in at least one non-heterodimer but does not appear in any heterodimer. For example, apart from D_{2L}R-5-HT2A heterodimer, the triplet of amino acid residues LLT (Leu-Leu-Thr) appears as a homology in seven receptor heterodimers: D_5R-GABAA, CCR2-CCR5, CCR2-CXCR4, D_2R-NTS1, mGluR2-5-HT_{2A}R, MT1-MT2, and TLR1-TLR2, whereas the triplet AIS (Ala-Ile-Ser) appears as a homology in five heterodimers: D_5R-GABAA, CCR2-CCR5, D_1R-D_2R, D_1R-D_3R, and D_2R-D_3R. Thus, both triplets LLT and AIS appear together as the homologies in three heterodimers: D_2R-5-HT_{2A}R, GABAA-D_5R, and CCR2-CCR5 (Supplementary, Fig. 2A-B). Moreover, the triplet LLT can be found also in chemokines (e.g., CCL7 and CCL22), whereas the triplets LLT and AIS are collocated in the chemokine receptors CCR5 and CXCR6 (Supplementary, Fig. 2C). In addition, there are no triplets LLT and AIS in TM1 and TM3 of the receptor 5-HT1A (Supplementary, Fig. 2D). Taking together with the BRET experimental results, these bioinformatics predictions suggest the existence of a basic set of common triplets in the two participating receptors that may participate in the receptor-receptor interactions interfaces.

Discussion

The results of this study gives for the first time evidence for the existence of functionally interactive D_{2L}R-5-HT_{2A}R heteromers in cotransfected cell lines based on the demonstration of a specific and strong $BRET^2$ signal using $BRET^2$ competition assays, of colocalization of the two receptors in the transiently cotransfected cells and of indications of allosteric receptor-receptor interactions modulating 5-HT_{2A}R and D_{2L}R signaling in this heteromers. The specificity is best shown by the failure of 5-HT$_{1A}$R expression to reduce the BRET^2 signal from the D$_{2L}$R-5-HT$_{2A}$R heteromer.

The forskolin-induced CRE-luciferase reporter gene assay suggested the existence of antagonistic 5-HT$_{2A}$R-D$_{2L}$R receptor-receptor interactions in these heteromers. Thus, quinpirole showed a reduced potency to activate the Gi/o signaling of the D$_2$R receptor upon coactivation of the 5-HT$_{2A}$R with 5-HT or TCB2. The NFAT-luciferase reporter gene assay and the intracellular calcium measurements in turn indicated that the existence of facilitatory D$_{2L}$R-5-HT$_{2A}$R receptor-receptor interactions in these heteromers. In fact, 5-HT and the 5-HT$_{2A}$R agonist TCB2 showed an increased potency to produce Gq/11 mediated responses at the 5-HT$_{2A}$R upon coactivation of the D$_{2L}$R receptor with quinpirole.

In view of above it seems possible that there exists within the D$_{2L}$R-5-HT$_{2A}$R heteromer a receptor-receptor interaction which may lead to the development of inhibitory allosteric feedback mechanisms over the receptor interface upon activation of the D$_2$R receptor. Thus, under these conditions via transfer of allosteric information the 5-HT$_{2A}$R agonists at the 5-HT$_{2A}$R protomers may show an increased potency to activate Gq/11 mediated 5-HT$_{2A}$R signaling. This event may also reflect an increased ability of the 5-HT$_{2A}$R to exert its trans-inhibitory action on the Gi/o mediated signaling of the D$_2$R protomers of the heteromer as indicated in our analysis, in this way producing the postulated inhibitory feedback (Supplementary, Fig. 3).

It is possible that the D$_{2L}$R-5-HT$_{2A}$R heteromer exists in the ventral striato-pallidal GABA pathway. If so, the antagonistic 5-HT$_{2A}$R-D$_{2L}$R receptor-receptor interaction would help to reestablish the glutamate drive from the mediodorsal thalamic nucleus to the prefrontal cortex leading to an anti-schizophrenic potential [4].There exist strong indications that A$_{2A}$-D$_2$R heteromers with antagonistic A$_{2A}$-D$_2$R interactions also exist in the ventral striato-pallidal GABA neurons and A$_{2A}$ agonists possess antipsychotic properties [4].Their possible colocalization and interactions with putative $D_{2L}R$-5-$HT_{2A}R$ heteromers in the ventral striato-pallidal GABA neurons remain to be investigated.

Based on a bioinformatics approach indications have been obtained that TM1 and TM3 may participate in the $D_{2L}R$-5-$HT_{2A}R$ interface by the demonstration of the protriplets LLT and AIS amino acid homologies in TM1 and TM3, respectively. In support of this view these two protriplet homlogies do not exist in the non-heteromer $D_{2L}R$-5-$HT_{1A}R$ pair. This can now be experimentally tested in a mutational analysis.

Further work is certainly necessary to analyze the receptor-receptor interactions in these novel $D_{2L}R$-5-$HT_{2A}R$ heteromers, especially their role in modulating the Gi/o signaling of the 5-HT_{2A} receptors which mediates the hallucinogenic 5-$HT_{2A}R$ actions [8; 9] and to demonstrate their existence in the brain. However, the discovery made in this work already opens up a new perspective on the interactions of antipsychotic $D_{2L}R$ receptor and 5-$HT_{2A}R$ antagonists in the brain which may include a common target the $D_{2L}R$-5-$HT_{2A}R$ heteromer exhibiting distinct allosteric receptor-receptor interactions that may be pathologically altered in schizophrenia [18; 19; 20]. Already a brain mGluR2-5-HT_{2A} receptor heteromer has been indicated to exist especially in the cerebral cortex which is likely to play a significant role in schizophrenia [9].

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Figure and Legends

Fig. 1. BRET^2 studies of $D_{2L}R$ and 5-$HT_{2A}R$ heteromerization in HEK293T cells. (A) BRET^2 saturation curves for the $D_{2L}R$-5-$HT_{2A}R$ hetero-oligomers at increasing expression levels of the GFP^2 tagged receptor. Cell individually expressing $D_{2L}R^{Rluc}$ were mixed prior exposition to h-coelenterazine with cells individually expressing 5-$HT_{2A}R^{GFP2}$ as a negative control. Plotted on the X-axis is the fluorescence value obtained from the GFP2, normalized with the luminescence value of $D_{2L}R$-Rluc expression 10 min after h-coelenterazine incubation. Mean ± S.E.M.; *n* = 8, in triplicate. (B) BRET^2 competition experiment for the $D_{2L}R$-5-$HT_{2A}R$ hetero-oligomers. At a fixed ratio (1:2) of expression levels of the $D_{2L}R$-Rluc/5-$HT_{2A}R^{GFP2}$ tagged receptor in presence of increasing concentration of wild-type receptors. Plotted on the X-axis is the concentration of cDNA transfected per competitor. Mean ± S.E.M.; *n* = 8 in triplicate. ***: Significantly different compared to 5-$HT_{1A}R$ in the range from 3-8 μg cDNA) ($P<0.001$) by Two-way analysis of variance (ANOVA).

Fig. 2. Agonist-induced 5-$HT_{2A}R$ and $D_{2L}R$ receptor activation in a forskolin-induced CRE-luciferase reporter gene assay. (A) Dose-response curves by quinpirole in $D_{2L}R$-5-$HT_{2A}R$-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 1 μg firefly

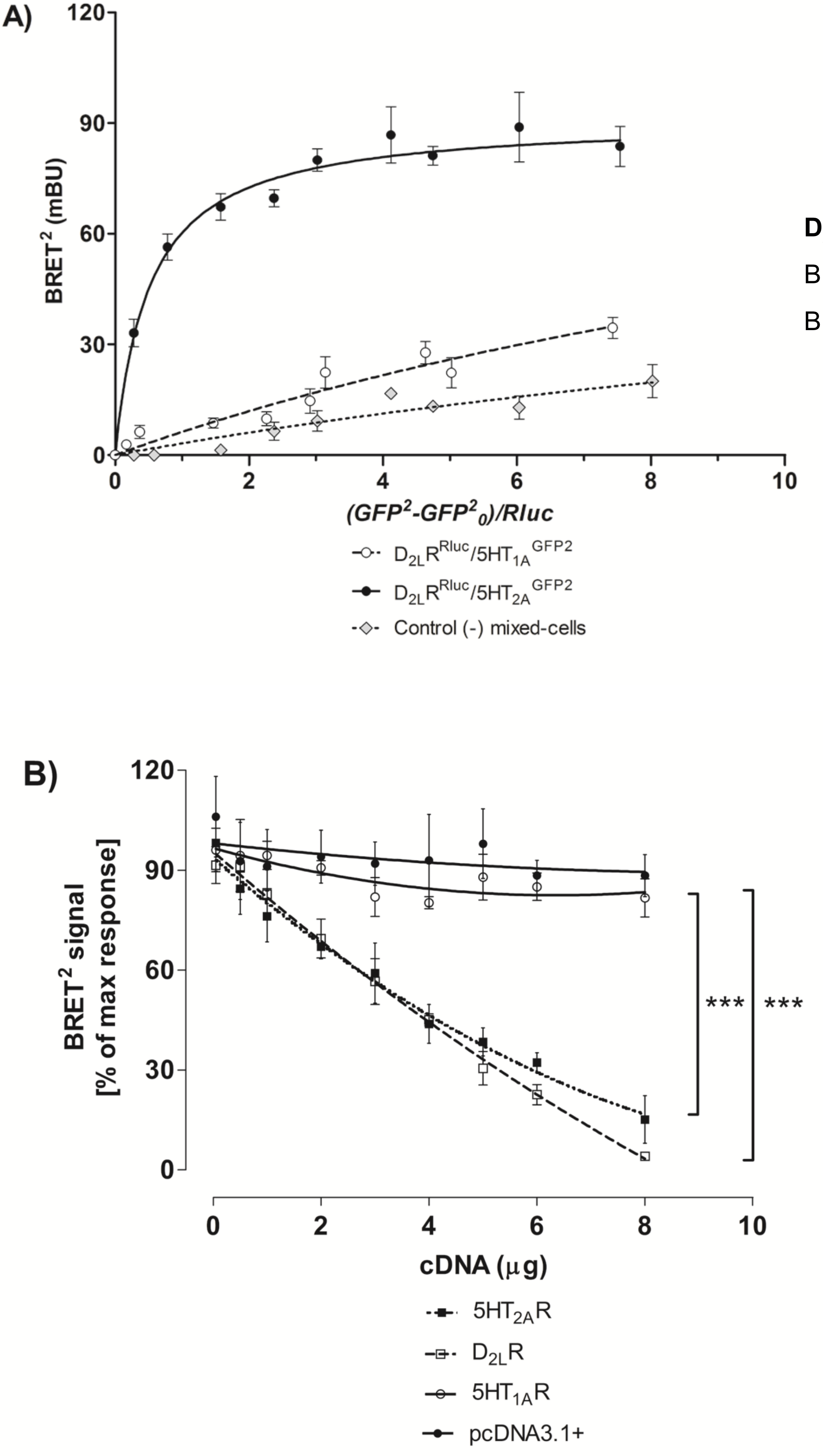
luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 µg of both (5-HT_{2A}R and D_{2L}R) expression vectors and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). After 4 hours of cells incubation with respective agonists with or without 5-HT 1µM (in presence of 1 µM forskolin, sub-maximal concentration value) luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. This figure is representative of one experiment performed at least three times, each in duplicate (EC_{50} = 90.9nM without 5-HT incubation and EC_{50} = 228.4nM with 5-HT incubation). (B) HEK293T cells were transiently co-transfected with 1 µg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 1 µg of both (5-HT_{2A}R and D_{2L}R) expression vectors and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). After 4 hours of cells incubation with respective agonists with or without the respective antagonist (in presence of 1 µM forskolin, sub-maximal concentration value) luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the means ± S.E.M. of three independent experiments performed in triplicate. +++: Significantly different compared to control ($P<0.001$); *** and *: Significantly different compared to quinpirole 100nM ($P<0.001$ and $P<0.05$); #: Significantly different compared to 5-HT 0.5µM + quinpirole 100nM ($P<0.05$). Forskolin (1µM); quinp, quinpirol (100nM); 5-HT, 5-hydroxytryptamine (0.5µM); racl, raclopride (1µM), 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB2) (50nM) and Ket, ketanserin (1 µM).

Fig. 3. NFAT reporter assay response after agonist-induced 5-HT_{2A}R and D_{2L}R activation. (A) Dose-response curves by 5-HT in D_{2L}R-5-HT_{2A}R-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 1 µg firefly luciferase-encoding experimental plasmid (pGL4-NFAT-luc2p), 1 µg of both (5-HT_{2A}R and D_{2L}R) expression vectors and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). After 6 hours of cells incubation with

respective agonists with or without quinpirole (50 or 100nM) luciferase activity was measured. Light emission is expressed as a percentage of maximal response value. This figure is representative of one experiment performed at least five times, each in triplicate (EC_{50} = 33.5nM without quinpirole incubation, EC_{50} = 17.9nM for 50nM quinpirole incubation and EC_{50} = 20.4nM for 100nM quinpirole incubation). (B) HEK293T cells were transiently co-transfected with 1 µg firefly luciferase-encoding experimental plasmid (pGL4-NFAT-luc2p), 1 µg of both ($D_{2L}R$ and 5-$HT_{2A}R$) expression vectors and 50 ng *Renilla* luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 6 hours with agonist or antagonist (in presence of agonist). The data represent the mean ± S.E.M. of three independent experiments performed in triplicate. +++: Significantly different compared to control ($P<0.001$); *** and **: Significantly different compared to 5-HT 0.5µM ($P<0.001$ and $P<0.01$); ###: Significantly different compared to TCB2 (50nM) ($P<0.001$). quinp, quinpirol (100nM); 5-HT, 5-hydroxytryptamine (0.5µM); racl, raclopride (1µM), 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB2) (50nM) and Ket, ketanserin (1 µM).

Fig. 4. Intracellular Ca^{2+} responses after agonist-induced 5-HT_{2A} and D2R activation. Cotransfected HEK293T cells were stimulated with 5-HT over the indicated concentration ranges in presence or absence of quinpirole (50 and 100nM). Relative Fluorescence (ΔF) values were determined with the Fluo-4 Non Wash Calcium Assay kit and expressed as a percentage of maximal response value. This figure is representative of one experiment performed at least six times, each in triplicate (EC_{50} = 10.9nM without quinpirole incubation, EC_{50} = 4.1nM for 50nM quinpirole incubation and EC_{50} = 1.7nM for 100nM quinpirole incubation).

Figure 1. Borroto-Escuela et al. 2010



D$_{2L}$R^{Rluc}-5-HT$_{2A}$R^{GFP2}

BRET$_{max}$ = 91.1 ± 2.9

BRET$_{50}$ = 0.51± 0.07

Figure 2. Borroto-Escuela et al. 2010

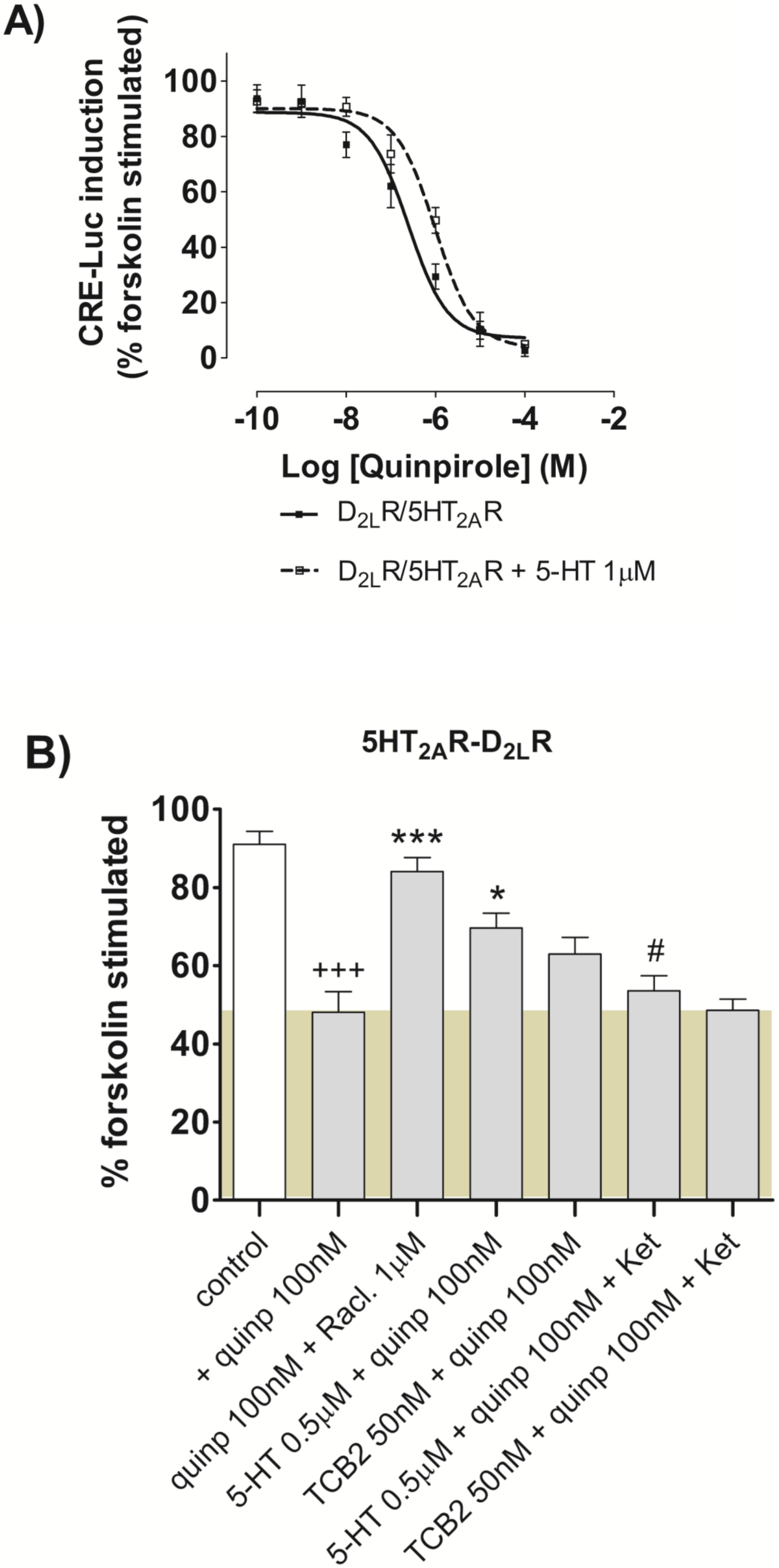


Figure 3. Borroto-Escuela et al. 2010

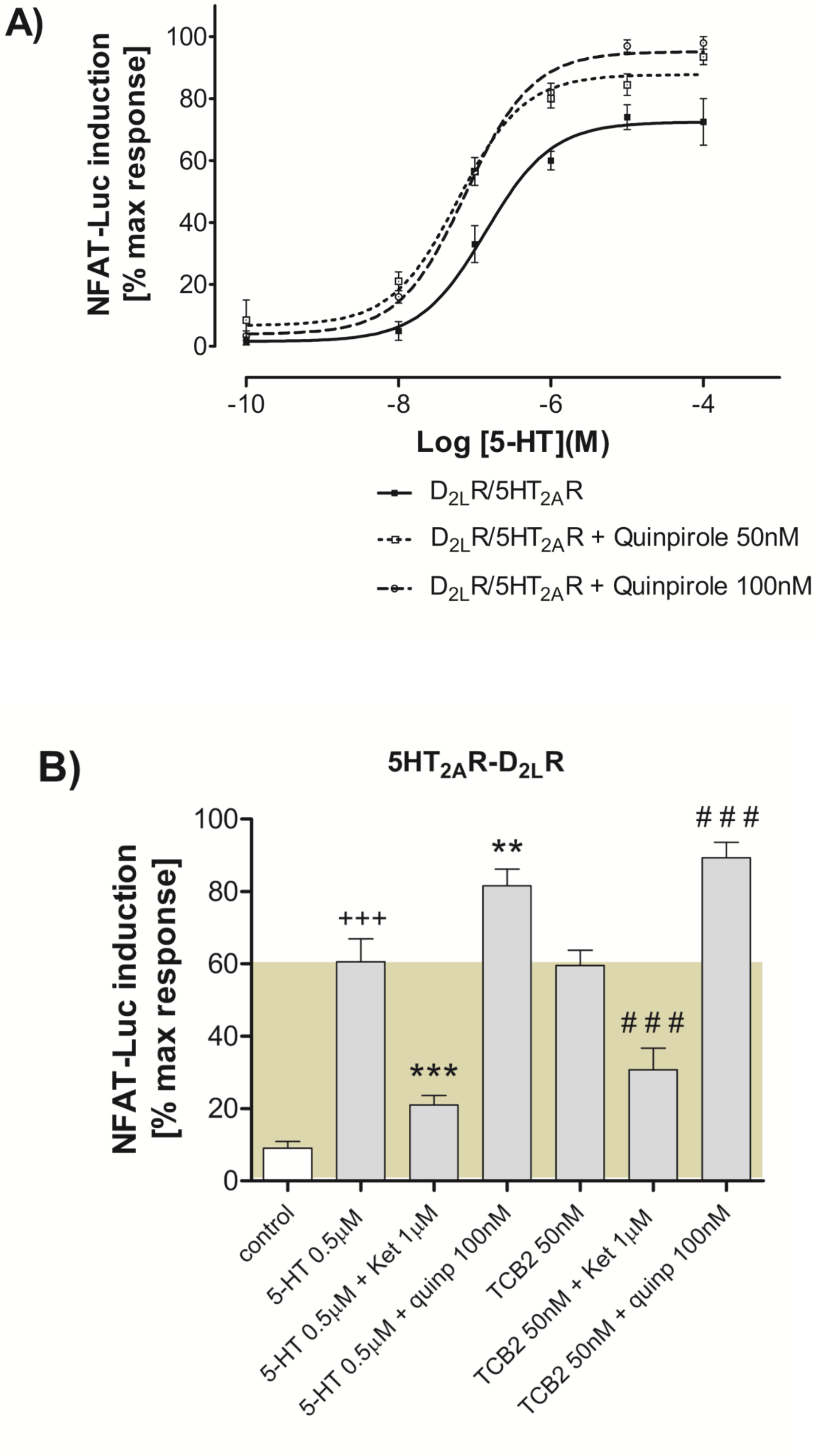
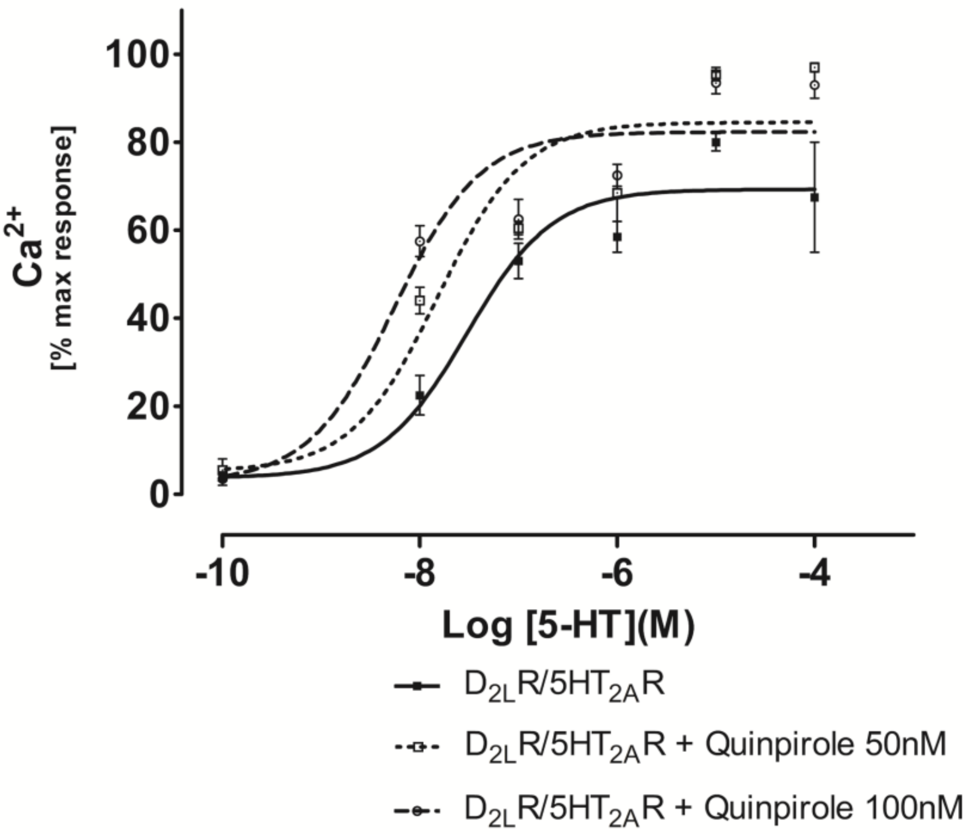


Figure 4. Borroto-Escuela et al. 2010



SUPPLEMENTARY DATA

Fig. 1. 5-$HT_{2A}R$ and $D_{2L}R$ receptor co-localize in HEK293T cells.

Fig. 2.

A and B: Triplet homologies LLT and AIS (grey-shaded) in the transmembrane and cytoplasmic domains of the receptor heterodimers $D_{2L}R$-5-$HT_{2A}R$, D_5R-GABAA, and CCR2-CCR5

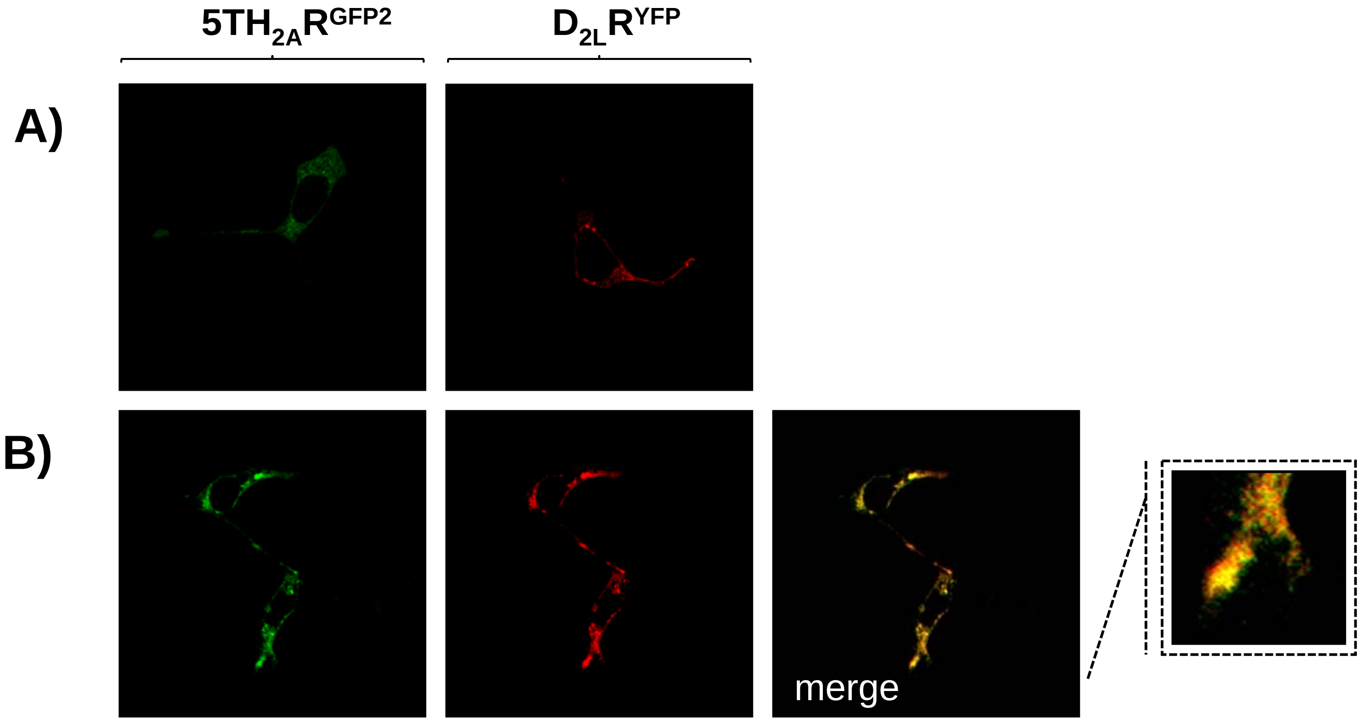
C: Example of the triplet LLT in the chemokine receptors (CCL7 and CCL22) and the triplets LLT and AIS collocated in the chemokine receptors (CCR5 and CXCR6)

D: There are no triplets LLT and AIS (grey-shaded) in TM1 and TM3 of the receptor 5-$HT_{1A}R$. The following amino acid residues are marked by a color code as the basic elements of leucine-rich motifs. Red bold L is leucine (Leu). Orange bold I and V are isoleucine (Ile) and valine (Val) that may also occupy a position of Leu in leucine-rich motifs. Green N and C are asparagine (Asn) and cysteine (Cys). Black bold S and T are serine (Ser) and threonine (Thr) where agonist-regulated phosphorylation may occur. White letters are charged amino acids: negatively (dark blue background) E (Glu), D (Asp) or positively (dark red background) R (Arg), K (Lys), H (His). Color-shaded are two-letter homologies which include leucine and seem rather typical for ligand-receptor interactions: LL (green), LI or IL and LV or VL (blue), LN (pink), together with 'leucine-serine zipper' LS or SL (yellow).

Fig. 3. Schematic cross-talk signalling pathway of 5-$HT_{2A}R$ and $D_{2L}R$ receptors to the firefly luciferase gene regulated by the NFAT/CRE response element.

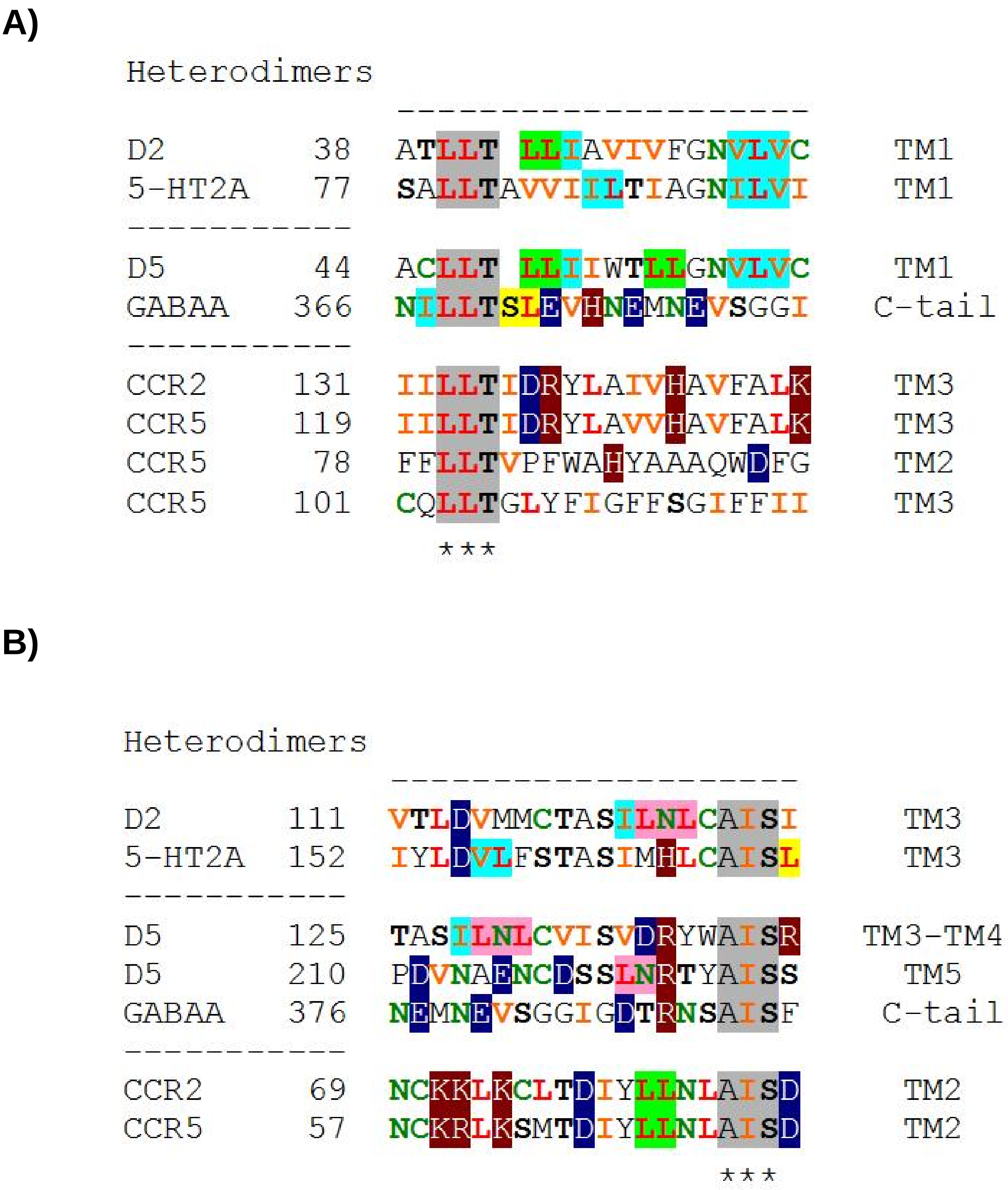
SUPPLEMENTARY DATA

Fig. 1 Borroto-Escuela et al. 2010



SUPPLEMENTARY DATA

Fig. 2 Borroto-Escuela et al. 2010



A)

```
Heterodimers
                   --------------------
D2          38     ATLLT LLIAVIVFGNVLVC     TM1
5-HT2A      77     SALLTAVVIILTIAGNILVI     TM1
-----------
D5          44     ACLLT LLIIWTLLGNVLVC     TM1
GABAA      366     NILLTSLEVHNEMNEVSGGI   C-tail
-----------
CCR2       131     IILLTIDRYLAIVHAVFALK     TM3
CCR5       119     IILLTIDRYLAVVHAVFALK     TM3
CCR5        78     FFLLTVPFWAHYAAAQWDFG     TM2
CCR5       101     CQLLTGLYFIGFFSGIFFII     TM3
                     ***
```

B)

```
Heterodimers
                   --------------------
D2         111     VTLDVMMCTASILNLCAISI      TM3
5-HT2A     152     IYLDVLFSTASIMHLCAISL      TM3
-----------
D5         125     TASILNLCVISVDRYWAISR    TM3-TM4
D5         210     PDVNAENCDSSLNRTYAISS      TM5
GABAA      376     NEMNEVSGGIGDTRNSAISF     C-tail
-----------
CCR2        69     NCKKLKCLTDIYLLNLAISD      TM2
CCR5        57     NCKRLKSMTDIYLLNLAISD      TM2
                                   ***
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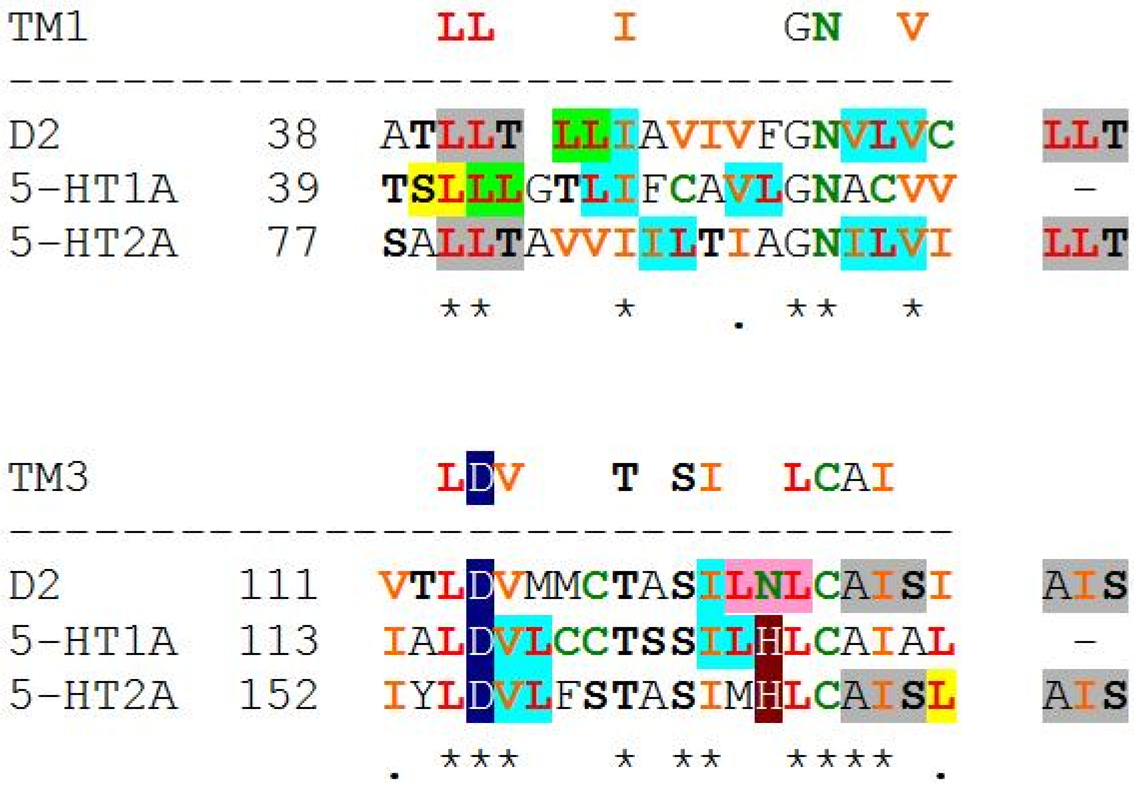
C)

```
Chemokines
             --------------------
CCL7        9  CLLLTAAAFSPQGLAQPVGI   N-terminal
CCL22      65  VVLLTFRDKEICADPRVPWV     C-tail

Chemokine Receptors
             ---------------------
CCR5       69  LLNLAISDLFFLLTVPFWAHY      TM2
CXCR6     186  AISTVVLATQMTLGFFLPLLT      TM5
```

D)



```
TM1            LL    I      GN  V
---------------------------------
D2         38  ATLLT LLIAVIVFGNVLVC   LLT
5-HT1A     39  TSLLLGTLIFCAVLGNACVV    -
5-HT2A     77  SALLTAVVIILTIAGNILVI   LLT
               **    *   . **  *

TM3            LDV   T SI  LCAI
---------------------------------
D2        111  VTLDVMMCTASILNLCAISI   AIS
5-HT1A    113  IALDVLCCTSSILHLCAIAL    -
5-HT2A    152  IYLDVLFSTASIMHLCAISL   AIS
             . ***   * **  **** .
```

SUPPLEMENTARY DATA

Fig. 3 Borroto-Escuela et al. 2010