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Attenuation of Oxytocin and Serotonin 2A Receptor Signalling through Novel Heteroreceptor Formation.

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3 **Attenuation of Oxytocin and Serotonin 2A Receptor Signalling through Novel**
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5 **Heteroreceptor Formation.**
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ABSTRACT

The oxytocin receptor (OTR) and the 5-hydroxytryptamine 2A receptor (5-HTR_{2A}) are expressed in similar brain regions modulating central pathways critical for social and cognition-related behaviours. Signalling crosstalk between their endogenous ligands, oxytocin (OT) and serotonin (5-hydroxytryptamine, 5-HT) highlights the complex interplay between these two neurotransmitter systems and may be indicative of the formation of heteroreceptor complexes with subsequent downstream signalling changes. In this study, we assess the possible formation of OTR-5HTR_{2A} heteromers in living cells and the functional downstream consequences of this receptor-receptor interaction. First, we demonstrated the existence of a physical interaction between the OTR and 5-HTR_{2A} *in vitro*, using a flow cytometry-based FRET approach and confocal microscopy. Furthermore, we investigated the formation of this specific heteroreceptor complex *ex vivo* in the brain sections using the Proximity Ligation Assay (PLA). The OTR-5HTR_{2A} heteroreceptor complexes were identified in limbic regions (*inter alia* hippocampus, cingulate cortex, and nucleus accumbens), key regions associated with cognition and social-related behaviours. Next, functional cellular-based assays to assess the OTR-5HTR_{2A} downstream signalling crosstalk showed a reduction in potency and efficacy of OT and OTR synthetic agonists, carbetocin and WAY267464 on OTR-mediated Gαq signalling. Similarly, the activation of 5-HTR_{2A} by the endogenous agonist, 5-HT, also revealed attenuation in Gαq-mediated signalling. Finally, altered receptor trafficking within the cell was demonstrated, indicative of co-trafficcking of the OTR/5-HTR_{2A} pair. Overall, these results constitute a novel mechanism of specific interaction between the OT and 5-HT neurotransmitters via OTR-5HTR_{2A} heteroreceptor formation and provide potential new therapeutic strategies in the treatment of social and cognition-related diseases.

KEYWORDS: OTR, 5-HTR_{2A}, heteroreceptor complexes, fcFRET, PLA, Gαq-mediated signalling crosstalk

INTRODUCTION

Oxytocinergic signalling represents one of the major neuroendocrine systems in mammals ¹. Oxytocin (OT), a nine-amino acid peptide hormone, is produced in paraventricular (PVN) and supraoptic nuclei of the hypothalamus. Peripherally-secreted OT is mainly known to stimulate uterine smooth muscle contractions associated with parturition and milk ejection during lactation ²⁻⁵. The known actions of OT are mediated through the OT receptor (OTR), which belongs to the largest subclass of the rhodopsin- β adrenergic receptor family (class A) of G-protein coupled receptors (GPCRs) ^{6,7}. Detection of OTR in kidney, thymus, bone cells, osteoblasts, cardiomyocytes, vascular endothelial cells and different types of cancer cells highlights the vast spectrum of peripheral OT functionalities from fertility regulation to controlling the immune and cardiovascular systems, bones and muscles formation, and growth of certain cancer cells ⁸⁻¹⁰.

Within the central nervous system, OT is released in a number of brain regions, including limbic structures and sensory processing areas, where this peptide functions as a neurotransmitter ^{11,12}. Central OT has been found to primarily modulate complex social and cognitive behaviours, such as; social memory, recognition and reward, attachment, cooperation, exploration, motivation, as well as anxiety and aggression ¹³⁻¹⁶. Furthermore, dysfunction in the OT system is associated with several mental disorders characterized by social impairments, including autism spectrum disorders (ASD), social anxiety disorder and schizophrenia ^{17,18}. Many pre-clinical and clinical data have shown beneficial effects of OT administration on social cognition and prosocial behaviour ^{14,19,20}. The promising therapeutic potential of exogenous OT on social related behaviours was demonstrated across multiple studies in healthy subjects as well as in patients with ASD and schizophrenia ^{21,22}. However, an increasing number of studies is also showing evidence of inconsistent effects of exogenous OT administration between subjects, especially in patients with ASD and schizophrenia ²³⁻²⁵. These variable OT responses, highlight that the efficacy of the OT administration in human has not yet been firmly established ²⁶⁻²⁸. The differences in effects between subjects may be explained by the complex nature of the OT signalling system, as well as different basal levels of neurotransmitters in specific brain regions. This may suggest that the OT system is able to interact with multiple

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3 neurotransmitters, modulating social related behaviours through different
4 mechanisms, which influence the final functional outcome ^{29–31}.

7 The ability of OT to modulate the serotonin (5-hydroxytryptamine, 5-HT) system have
8 been demonstrated across multiple studies ^{31–34}. Serotonergic projections innervate
9 almost every brain region, and 5-HT represents the key neurotransmitter involved in
10 several behaviours including, mood, stress, and social behaviours ^{35,36}. Interestingly,
11 OT administration has been shown to significantly increase 5-HT axon length and
12 density in the amygdala and hypothalamus of prairie vole males during development,
13 demonstrating OT-mediated modulation of 5-HT innervation in early life ³³. The OT
14 neuropeptide is also known to influence 5-HT synthesis and release from 5-HT
15 neurons in the midbrain raphe nuclei, leading to a reduced anxiety-like behaviour in
16 mice ³². The modulation of 5-HT release by the OT peptide is driven through OTR and
17 serotonin 2A/2C receptors (5-HTR_{2A/2C}), which are both expressed on 5-HT neurons.
18 Interestingly, 5-HT receptors, including the 5-HTR_{2A/2C}, have been shown to mediate
19 OT secretion from the PVN of the hypothalamus ^{37–39}. In addition, several studies
20 have demonstrated the impact of elevated plasma 5-HT, as seen in one-third of ASD
21 patients, on OT system dysregulation in a developmental hyperserotonergic model.
22 Hyperserotonergic rodents were characterized by a decreased number of OT
23 expressing cells in the PVN, which was correlated with significantly lower expression
24 of the 5-HTR_{2A} on these neurons ^{40–42}. Recent studies have yielded very exciting
25 results, demonstrating that the coordinated activity of OT and 5-HT neurotransmitters
26 in the nucleus accumbens of mice is crucial for the rewarding properties of social
27 interactions ³¹. This specific interaction between OT and 5-HT systems was then
28 validated in nonhuman primates and in humans, where OT administration influenced
29 5-HT signalling in the amygdala, insula, hippocampus, dorsal raphe nucleus, and
30 orbitofrontal cortex, key limbic regions implicated in the control of stress, mood, and
31 social behaviours ^{29,34}. Although the OTR and 5-HTRs have been suggested to be
32 involved in behaviours listed above, the precise mechanism of the neurotransmitter
33 system interaction has not yet been fully elucidated. Nevertheless, evidence is
34 emerging highlighting a specific crosstalk between the OT and 5-HT neurotransmitter
35 systems, with a particular role for the OTR and 5-HTR_{2A}.

36 Similarly to the OTR, the 5-HTR_{2A} also belongs to class A GPCRs, being primarily
37 coupled to Gαq proteins following its activation ^{43,44}. Both GPCRs are critical signal
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3 transducers in the brain and have received much attention as promising therapeutic
4 targets for social and cognition related disorders ^{45–47}. In addition, both OTR and 5-
5 HTR_{2A} are well known to crosstalk with other GPCRs through formation of
6 heteroreceptor complexes ^{48–51}. Oligomerization of GPCRs is known to modulate their
7 downstream signalling and exert a significant impact on receptor physiology and
8 function ^{48,52–54}. Interestingly, the changes in the formation and function of GPCRs
9 heterodimers are associated with many neuropsychiatric disorders ^{55–58}. For example,
10 formation of an OTR/dopamine 2 receptor (D2R) heteroreceptor complex has been
11 shown to affect downstream signalling of both receptors and modulate the OT-
12 mediated anxiolytic effect ^{49,53,59}. Downstream signalling of the 5-HTR_{2A} in complexes
13 with the D2R and the metabotropic 2 receptor (mGluR2) have also been shown to be
14 affected ^{48,57}. Furthermore, the formation of such specific 5-HTR_{2A} heteroreceptor
15 complexes has been implicated in the mechanism of antipsychotic and hallucinogenic
16 drugs actions ^{55,60,61}. The mechanism of atypical antipsychotics is also a good example
17 that compounds interacting simultaneously with multiple GPCRs are clinically more
18 effective, compared to drugs specific for one receptor ⁶². Identification and
19 characterization of novel crosstalk and heteromerization between different GPCRs are
20 poised to yield promising future pharmacotherapeutic strategies for the development
21 of novel drug with high efficacy and specificity for many central nervous system (CNS)
22 disorders, especially for those with a multifactorial and polygenic aetiology.
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38 Based on the reciprocal interaction of the OT and 5-HT systems, and the fact that both
39 the OTR and 5-HTR_{2A} are involved in social and cognition related behaviours with an
40 overlapping central expression profiles within brain regions associated with these
41 processes, we hypothesize that the OTR and 5-HTR_{2A} may form heteroreceptor
42 complexes. In this study, we first evaluate the possible formation of OTR-5HTR_{2A}
43 heteroreceptor complexes *in vitro* using a flow cytometry-based FRET (fcFRET)
44 approach and confocal microscopy. Next, the formation of these specific
45 heteroreceptor complexes is investigated *ex vivo*, in rat brain sections with the use of
46 Proximity Ligation Assay (PLA) ⁶³. Finally, functional cellular-based assays, including
47 intracellular calcium mobilization, IP-One accumulation and ligand-mediated
48 trafficking ^{54,64} are used to demonstrate the significant changes in the Gαq-dependent
49 signalling and trafficking of both receptors. Overall, these data show for the first time
50 convincing evidence for the formation of the OTR-5HTR_{2A} functional heteroreceptor
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3 complex, which may represent a novel molecular mechanism underpinning the OT
4 and 5-HT signalling system crosstalk in the brain.
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RESULTS AND DISCUSSION

Flow cytometry-based FRET demonstrates a physical interaction between the OTR and 5-HTR_{2A}.

The interaction between the OTR and 5-HTR_{2A} was assessed in human embryonic kidney (HEK293A) cells expressing the receptors under investigation using flow cytometry-based FRET (fcFRET). The fcFRET analysis allows the evaluation of the physical interactions between receptors in a large population of cells, providing statistically more robust and reliable data compared to confocal microscopy. In addition, this method classifies the population of cells and elucidates the difference in fcFRET efficiency with other cellular parameters, such as; viability, size and granulation⁵¹. Indeed, fcFRET is a non-invasive, sensitive and quantitative method that has been successfully used to assess protein-protein interactions, including the physical interaction between GPCRs^{66–68}.

To optimize an assay for quantitative measurements of fcFRET signal HEK293A cells with the expression of fluorescent protein tags (tGFP and tRFP), without any GPCRs were first analysed. Analysis of fcFRET signal was performed on the gated population of single, live, and successfully transduced cells. As expected, cells containing both fluorescent proteins (HEK293A-tGFP-Lv-tRFP) did not show a significant fcFRET signal and neither did cells expressing each tag separately or HEK293A cells without tags (Figure 2, supplementary data). This result indicates that the fcFRET signal detected between receptors under investigation is not due to an overexpression, random collision or dimerization of fluorescent proteins. Next, we demonstrated a significantly higher fcFRET signal in cells co-expressing the OTR tagged with tGFP and OTR tagged with tRFP (HEK293A-OTR-tGFP-Lv-OTR-tRFP) ($28.8 \pm 1.5\%$) when compared to cells expressing either the OTR tagged with tGFP (0%), or OTR tagged with tRFP ($0.2 \pm 0.2\%$) (Figure.1 supplementary data). In addition, cells with the expression of the donor construct and the control acceptor construct (HEK293A-OTR-tGFP-control-tRFP) were used for quantification of nonspecific fcFRET signal ($1.4 \pm 0.4\%$)⁶⁶. These results confirmed the ability of the OTR to form constitutive homodimers^{50,69} and showed the reliability of the experimental settings.

Next, analysis of fcFRET signal between the OTR and 5-HTR_{2A} receptors was performed. A significant increase in the percentage of fcFRET positive cells was observed for cells co-expressing 5-HTR_{2A}-EGFP and OTR-tRFP (HEK293A-5HTR_{2A}-

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3 EGFP-Lv-OTR-tRFP) ($23.3 \pm 3.1\%$) compared to cells solely expressing the donor
4 construct (HEK293A-5HTR_{2A}-EGFP) ($0.3 \pm 0.03\%$) or acceptor construct (HEK293A-
5 Lv-OTR-tRFP) ($0.3 \pm 0.03\%$) and compared to cells expressing the 5-HTR_{2A}-EGFP
6 with control-tRFP construct ($3.2 \pm 0.6\%$) (Figure 1A and 1C). Furthermore, fcFRET
7 signal analysed as median fluorescence was also significantly higher in cells co-
8 expressing the OTR/5-HTR_{2A} pair (48 ± 1.6 RFU) compared to cells with the
9 expression of the donor ($15,3 \pm 5.2$ RFU) or acceptor ($18,7 \pm 10.3$ RFU) construct only
10 and compared to cells expressing the 5-HTR_{2A}-EGFP with the control-tRFP construct
11 (14.2 ± 6.9 RFU) (Figure 1B and 1D). Taking together, the above results highlighted
12 the constitutive and specific association between the OTR and 5-HTR_{2A} within the cell,
13 indicating the formation of a heteroreceptor complex between these receptors *in vitro*,
14 in intact HEK293A cells.
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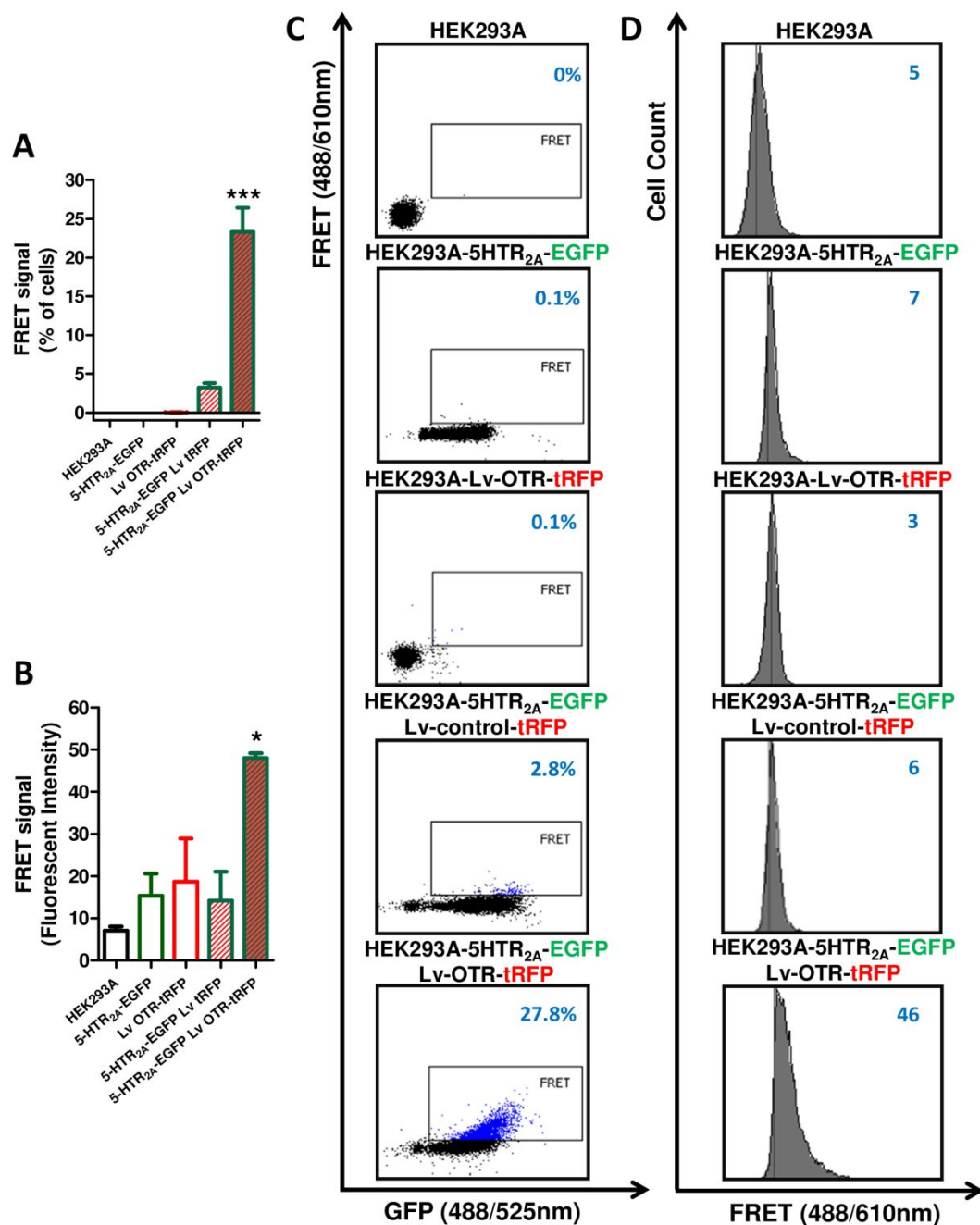


Figure 1. fcFRET between the OTR and 5-HTR_{2A}. The fcFRET signal is presented as a percentage of cells (A,C) and as median fluorescence (B,D) in wild-type HEK293A cells, cells stably expressing the 5-HTR_{2A} tagged with EGFP (donor), cells transiently transduced with lentiviral vector expressing OTR tagged with tRFP (acceptor), cells expressing 5-HTR_{2A} tagged with EGFP and the control-tRFP, and cells co-expressing 5-HTR_{2A} tagged with EGFP and OTR tagged with tRFP. Graphs represent mean \pm SEM from three independent experiments (A,B). Statistical significance of fcFRET signal in cells co-expressing both receptors compared to cells expressing donor with the control acceptor constructs is denoted as * for $p < 0.05$ and *** for $p < 0.001$. Dot plots (C) show percentage of cells with fcFRET signal (FRET vs EGFP plots), histograms (D) demonstrate median fluorescence of fcFRET signal (Cell count vs FRET signal). Dot plots and histograms are representative of three independent experiments.

Cellular colocalization of the OTR and 5-HTR_{2A}.

Cellular localization of the receptors was investigated using a confocal microscopy in intact living HEK293A cells co-expressing the OTR and 5-HTR_{2A}. The 5-HTR_{2A} was mainly found within the cell membrane which was shown by the green fluorescence signal from 5-HTR_{2A} fused with EGFP (Figure 2A). The red fluorescence signal from OTR fused with tRFP was shown on the cell membrane, as well as in the intracellular space (Figure 2B). The ubiquitous expression of the OTR-tRFP within the cell may be explained by constitutive (ligand-independent) activity and internalization of the OTR, as well as the previously described low rate of recycling back to the cell membrane^{70,71}. Moreover, a similar pattern of OTR expression is observed in a number of heterologous expression systems as well as endogenously in different type of tissues^{72–75} (also see Figure 3 in supplementary data). Interestingly, an overlap between green and red fluorescence as indicated by the yellow signal demonstrated colocalization of both receptors on the cell membrane and within the cytoplasm of cells (Figure 2C, merged picture). The colocalization of the OTR/5-HTR_{2A} pair within the same confocal plane is evidence for the potential formation of OTR-5-HTR_{2A} heteromers and reinforces the observed fcFRET signal (Figure 1). What is more interesting colocalization between the OTR and 5-HTR_{2A} observed intracellularly may indicate possible co-trafficking of both receptors within the cell, which was previously observed in the case of other GPCRs heterodimer pairs^{64,76,77}.

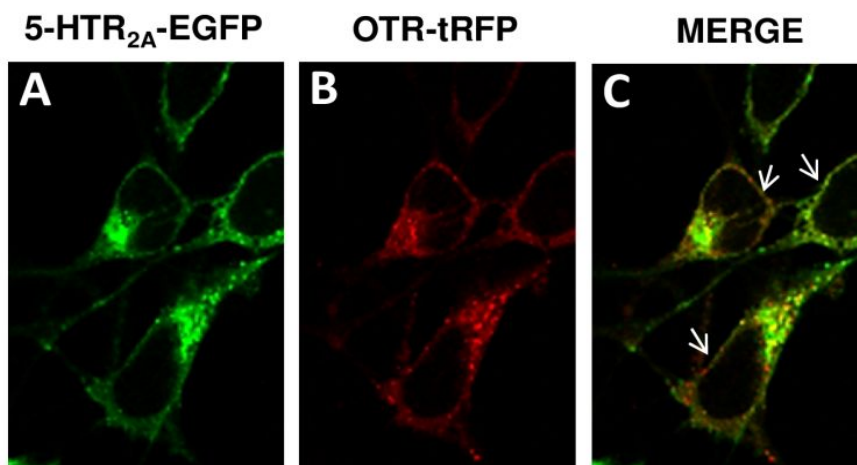


Figure 2. Cellular colocalization of the OTR/5-HTR_{2A} pair. HEK293A cells stably expressing the 5-HTR_{2A} tagged with EGFP (green) (A) were transiently transduced with lentiviral vector expressing OTR tagged with tRFP (red) (B). Merged picture (yellow/orange) shows colocalization of the two receptors within the cell (C).

The OTR and 5-HTR_{2A} form heteroreceptor complexes in rat brain regions.

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3 In the dorsal hippocampus of WT SD rats (Bregma -3.6 mm) a high density of PLA
4 positive OTR-5HTR_{2A} heteroreceptor complexes clusters was found in the pyramidal
5 cell layer of the CA2 and CA3 regions, while only a few were found in the stratum
6 oriens and radiatum of these areas. This was similar to the background found in
7 negative controls and the myelinated bundles of the crus cerebri (CC) (Figure 3A). A
8 multiple z-scan (20) confocal microscopy photograph corresponding to the CA3 region
9 with higher magnification of the high-density PLA positive clusters is shown in Figure
10 3A. The quantitative data present the number of PLA clusters (blobs) per nucleus per
11 sampled field (30X30 μm). They range mainly from 8-13 PLA clusters in the CA2 and
12 CA3 regions to 4-6 PLA clusters in the polymorphic layer of the dentate gyrus (PoDG)
13 and it shows the high density in the pyramidal cell layer. A very low density of the PLA
14 clusters is found in the granular cell layer of the dentate gyrus (gDG). The molecular
15 cell layer of the dentate gyrus (mDG) contains densities similar to the densities and
16 values found in negative controls.

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18 In the cingulate cortex (Bregma 1.2 mm) a PLA positive OTR-5HTR_{2A} heteroreceptor
19 complexes clusters is found in layers II and III, shown in low and high magnifications
20 (Figure 3B). Also, a PLA positive clusters was found in the nucleus accumbens shell
21 and core (Bregma 1.2 mm) (Figure 3B). In the dorsal striatum these receptor
22 complexes could not be clearly observed.

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24 A PLA positive signal validates the *in vitro* results and demonstrates the formation of
25 OTR-5HTR_{2A} heteroreceptor complexes in rat brain sections under endogenous
26 expression levels of both receptors. Moreover, specific distribution pattern of OTR-
27 5HTR_{2A} heteroreceptor complexes indicate their potential role in distinct cortical and
28 subcortical limbic regions. The formation of these receptor complexes in the CA2 and
29 CA3 regions of the hippocampus and cingulate cortex may be involved in modulation
30 of OTR dependent social recognition and memory²⁰ as well as 5HTR_{2A} driven social
31 exclusion processing⁷⁸. It is also tempting to hypothesize that OTR-5HTR_{2A}
32 heteromers identified in nucleus accumbens can be partially responsible for the
33 crosstalk between OT and 5-HT neurotransmitters shown to be crucial for rewarding
34 properties of social interactions³¹. In conclusion, the above results underlie the
35 potential role of OTR-5HTR_{2A} heteroreceptor complexes in distinct limbic circuits
36 relevant to social interactions.

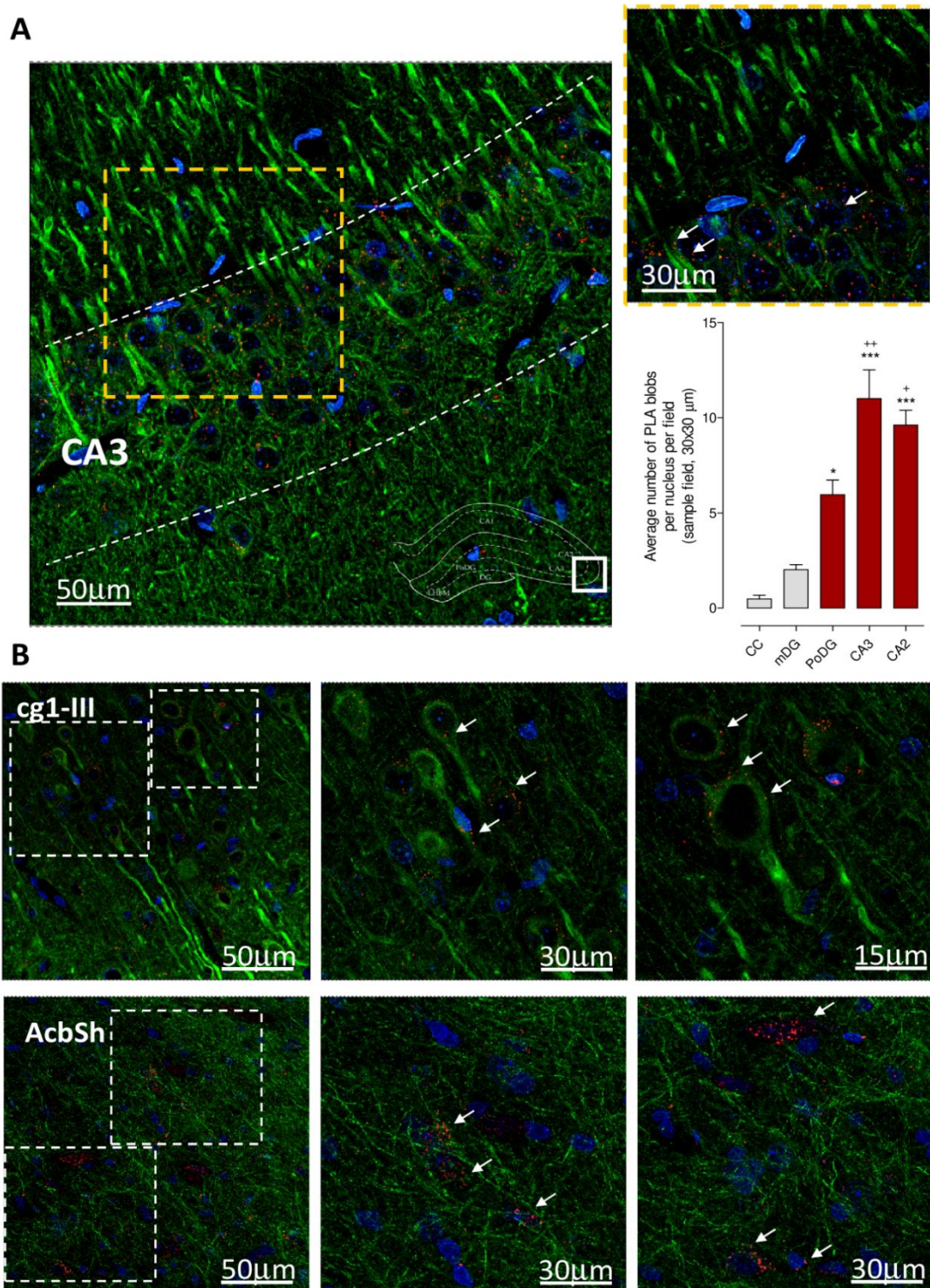


Figure 3. Illustration of the OTR-5HTR_{2A} heteroreceptor complexes in the dorsal hippocampus and nucleus accumbens of rat brain. (A) Microphotographs from transverse sections of the rat dorsal hippocampus (Bregma level: -3.6 mm) show the distribution of the OTR-5HTR_{2A} heteroreceptor complexes in CA3 using the *in situ* proximity ligation assay (*in situ* PLA) technique. The square outlines the CA3 area from which the picture was taken. Receptor complexes are shown as red PLA blobs (clusters) found in high densities per cell in a large number of nerve cells in the pyramidal cell layer using confocal laser microscopy. No specific PLA blobs were found in the stratum moleculare and radiatum of the CA3-CA2 regions (*cornu ammonis*). The nuclei are shown in blue by DAPI staining and the neuronal marker in green

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3 (Neuro-Chrom™ Pan neuronal marker antibody-Alexa488 conjugated, ABN2300A4). In the
4 higher right panel the PLA blobs are presented in higher magnification in the pyramidal cell
5 layer. In the lower right part of the figure the density (per nucleus per sampled field) of the PLA
6 positive complexes in PoDG (polymorph layer of the dentate gyrus), CA3, and CA2 are highly
7 significantly different (***) from the density found in crus cerebri (CC) and the granular cell layer
8 of the dentate gyrus (gDG). The density is also significantly higher in the CA2 (+) and CA3 (++)
9 versus PoDG (Mean ± SEM, 4 rats per group). (B) The upper panel of B show representative
10 examples of these PLA receptor complexes from transverse sections of the rat cingulate cortex,
11 area 1 (Bregma level: 1.2 mm). They present the distribution of OTR-5HTR_{2A} heteroreceptor
12 complexes. They are shown as red PLA blobs (clusters) in layers II and III. Layer III represents
13 the external pyramidal cell layer where large PLA positive clusters are found and appear to be
14 located on the surface of many pyramidal cells. Higher magnifications of the two squares
15 outlined in left panel are shown in the two right panels. The nerve cell bodies and apical
16 dendrites are seen in green (neuronal marker). The lower panel in B is taken from nucleus
17 accumbens shell (AcbSh). The neuronal marker (Neuro-Chrom™ Pan neuronal marker
18 antibody-Alexa488 conjugated, ABN2300A4) shows the neurite network. A number of nerve
19 cell bodies are associated with a PLA positive blobs representing OTR-5HTR_{2A} heteroreceptor
20 complexes that may also have an intracellular location through trafficking. The outlined squares
21 in the left panel are shown in higher magnifications in the two right panels (B).

22 23 24 **Ligand-mediated internalization of the OTR and 5-HTR_{2A}.**

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27 Desensitisation and subsequent internalization of GPCRs provides an important
28 physiological mechanism that protects cells against overstimulation^{73,79,80}. Most
29 GPCRs, including the OTR and 5-HTR_{2A} are rapidly internalized following agonist
30 treatment and efficiently recycle to the cell surface after agonist removal^{71,79–81}. It has
31 been documented that the formation of heteroreceptor complexes can affect basal and
32 ligand-mediated internalization of the heterodimer protomers within the cell^{64,76,82}.
33 Thus, we investigated the effect of co-expression of the OTR and 5-HTR_{2A} on their
34 cellular trafficking under basal conditions and following treatment with their respective
35 endogenous ligands, oxytocin (OT) (100 nM) and serotonin (5-HT) (1 μM) (Figure 4).
36 Significant OT-mediated internalization of the OTR tagged with tRFP was observed in
37 cells solely expressing the OTR (HEK293A-Lv-**OTR-tRFP**) (Figure 4A). Similarly,
38 significant internalization of the 5-HTR_{2A} tagged with EGFP was shown following 5-HT
39 treatment in cells solely expressing the 5-HTR_{2A} (HEK293A-**5HTR_{2A}-EGFP**) (Figure
40 4B). Interestingly, in cells co-expressing both receptors (HEK293A-5HTR_{2A}-EGFP-Lv-
41 **OTR-tRFP**), a significant increase in basal internalization of the OTR was observed.
42 Further increase in OT or 5-HT-mediated internalization compared to control
43 conditions although small also was noted in these cells (Figure 4A, blue bars with
44 stripes). Moreover, we observed that the basal internalization of the 5-HTR_{2A} in cells
45 co-expressing both receptors (HEK293A-**5HTR_{2A}-EGFP-Lv-OTR-tRFP**) was
46 consistently increased compared to cells solely expressing the 5-HTR_{2A} (Figure 4B).

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3 Interestingly, when both receptors were co-expressed (HEK293A-5HTR2A-EGFP-Lv-
4 OTR-tRFP) a small, albeit insignificant increase in 5-HTR_{2A} internalization was
5 consistently observed following OT treatment compared to the control condition
6 (untreated cells) (Figure 4B).
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10 Changes in basal trafficking properties of the OTR and 5-HTR_{2A} following their co-
11 expression in mammalian cells may at least partially explain the colocalization of both
12 receptors observed not only on the subcellular membrane but also intracellularly
13 (Figure 2C). Moreover, these observations are similar to what was shown for the 5-
14 HTR_{2A} and the metabotropic glutamate receptor 2 (mGluR2)⁶⁰. These receptors
15 demonstrated to form stable 5-HTR_{2A}-mGluR2 heterodimers in HEK293 cells, which
16 significantly increased their intracellular presence under basal conditions. This
17 indicates that both receptors are assembled as heterocomplexes at an early stage,
18 during maturation and trafficking to the cell membrane⁷⁷. Several other studies have
19 shown that GPCRs are indeed secreted to the cell surface as oligomerized complexes
20 ^{50,83,84}. Thus, the significant intracellular presence of the OTR and 5-HTR_{2A} following
21 their co-expression in cells may suggest that OTR and 5-HTR_{2A} also form constitutive
22 heteromers during maturation and trafficking from endoplasmic reticulum to the cell
23 membrane. Alternatively, the above results may indicate an increase in basal activity
24 of both receptors and a subsequent higher internalization rate as previously shown for
25 cannabinoid CB1 and orexin OX₁ receptor complexes⁷⁶. Noteworthy, the increased
26 internalization of the 5-HTR_{2A} after treatment with OT may support the hypothesis that
27 the 5-HTR_{2A} is co-internalized along with the OTR, from the cell membrane to
28 membranes of the endosomal compartment as previously demonstrated for the 5-
29 HTR_{2A}-mGluR2 and the 5-HTR_{2C}-GHSR1a heteromers^{60,64}. The OT-induced changes
30 in cellular trafficking of the 5-HTR_{2A} are also consistent with the formation of stable
31 OTR-5-HTR_{2A} heteromer complexes demonstrated in Figure 1.
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34 Finally, the changes in basal and ligand-mediated cellular receptor trafficking may also
35 lead to alterations in the downstream signalling pathways of each protomer. This may
36 be particularly relevant for increased signalling over the β -arrestin pathway, which not
37 only leads to an increased receptor internalization⁸⁵ but also directly affects the G
38 protein-dependent downstream signalling pathways of GPCRs⁸⁶.
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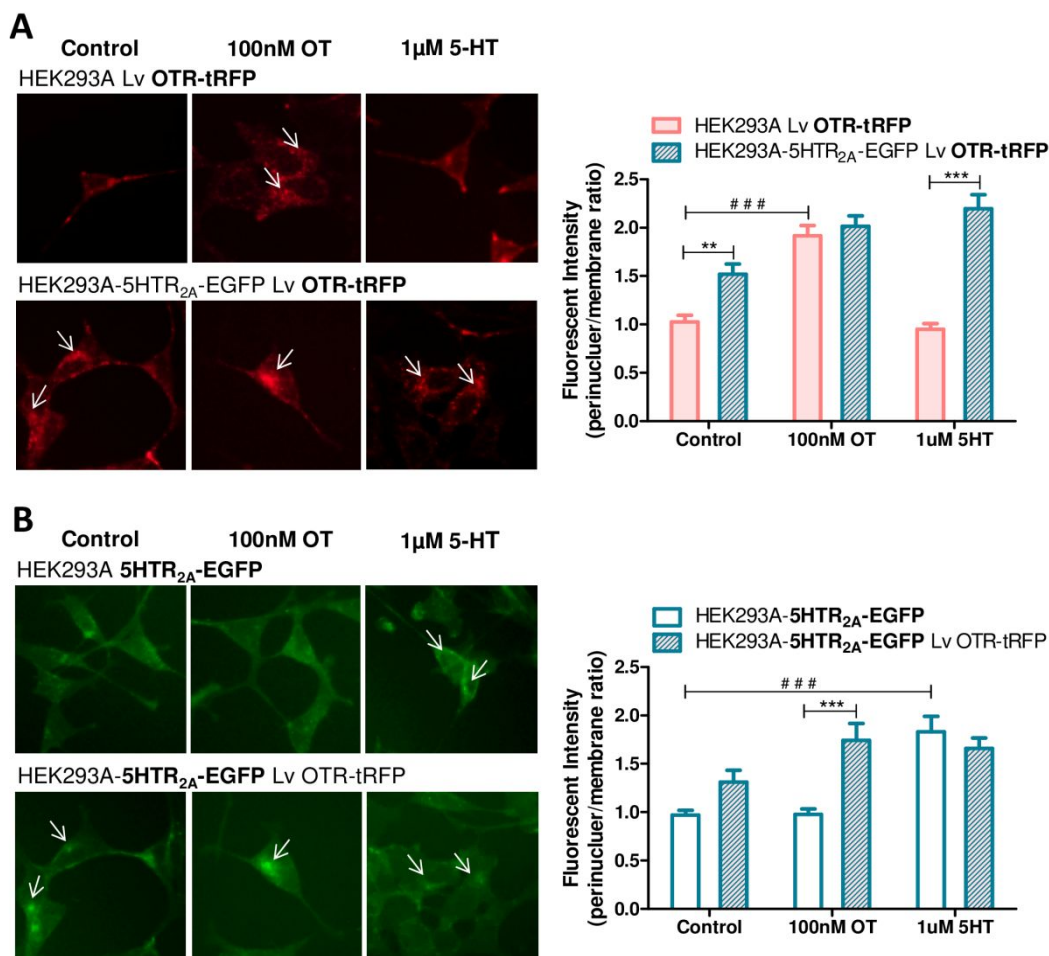


Figure 4. Cellular trafficking of the OTR and 5-HTR_{2A}. Representative images (left panel) and quantitative analysis (right panel) of ligand-mediated internalization of OTR tagged with tRFP (A) and 5-HTR_{2A} tagged with EGFP (B) versus cells co-expressing both receptors. Graphs represent mean \pm SEM from three independent experiments run in triplicate. Statistical significance of cells co-expressing both receptors compared to cells solely expressing the corresponding receptor is denoted as; ** indicating $p < 0.01$; or *** indicating $p < 0.001$. Statistical significance of cells following OTR or 5-HT treatment compared to the control condition is denoted as; ### indicating $p < 0.001$.

Downstream signalling changes following the OTR and 5-HTR_{2A} co-expression in cells.

Next, the downstream signalling consequences following co-expression of the OTR and 5-HTR_{2A} were investigated. The OTR and 5-HTR_{2A} are known to mainly signal through the G α q-mediated pathway, where activation of the G α q protein leads to generation of the second messenger, D-myoinositol 1,4,5-triphosphate (IP₃), causing subsequent intracellular calcium release from the endoplasmic reticulum into the cytoplasm^{3,87}. Therefore, we assessed the ligand-mediated changes in intracellular calcium mobilization in HEK293A cells solely expressing the OTR or the 5-HTR_{2A} and

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3 cells co-expressing both receptors. The cellular response was detected following the
4 addition of endogenous receptor ligands, OT and 5-HT. The potency of OT ($EC_{50} =$
5 0.12 ± 0.01 nM) in cells solely expressing the OTR (HEK293A Lv OTR) and potency
6 of 5-HT ($EC_{50} = 12.6 \pm 0.7$ nM) in cells solely expressing the 5-HTR_{2A} (HEK293A-5-
7 HTR_{2A}) were consistent with literature data (Figures 5A and 5B), which confirms the
8 functionality of the receptors expressed in the heterologous expression system^{79,88,89}.
9 Interestingly, the intracellular calcium release following an increasing concentration of
10 OT was significantly reduced in cells co-expressing both the 5-HTR_{2A} and OTR
11 (HEK293A-5-HTR_{2A} Lv OTR) compared to cells expressing only the OTR (Figure 5A).
12 The concentration-response curve of OT was characterized by a significantly lower
13 potency ($EC_{50} = 1.0 \pm 0.4$ nM) and efficacy ($E_{max} = 71.1\% \pm 6.7$) in cells co-expressing
14 both receptors compared to cells solely expressing the OTR ($EC_{50} = 0.1 \pm 0.01$ nM,
15 $E_{max} = 130.1\% \pm 15.1$) (Figure 5A). Similarly, the concentration-response curve of 5-
16 HT was characterised by a lower potency ($EC_{50} = 67.5 \pm 19.6$ nM) but no significant
17 changes in efficacy ($E_{max} = 84.3\% \pm 3.2$) in cells co-expressing both receptors
18 compared to cells solely expressing the 5-HTR_{2A} ($EC_{50} = 12.6 \pm 0.7$ nM, $E_{max} = 72.0\%$
19 ± 6.1) (Figure 5B).

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33 The transient release of calcium into the cytosol is also mediated by IP₃. Therefore,
34 we evaluated the ligand-mediated changes in the production of this second
35 messenger. The concentrations of OT (10 nM and 1 nM) and 5-HT (100 nM and 10
36 nM) were chosen based on the calcium assay results (Figures 5A and 5B). As
37 expected, OT-mediated IP-One (inositol monophosphate) accumulation was
38 significantly decreased in cells co-expressing the 5-HTR_{2A}/OTR pair compared to cells
39 solely expressing the OTR, depicted as a significant increase in percentage IP-One
40 values of control (Figure 5C). The analogous results were obtained for 5-HT-mediated
41 IP-One accumulation in cells co-expressing both receptors (Figure 5D), validating the
42 observed changes in Gαq-mediated signalling in calcium accumulation assay (Figures
43 5A and 5B).

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52 In addition, the observed attenuation in ligand-mediated Gαq signalling in cells co-
53 expressing the OTR and 5-HTR_{2A} was shown to be independent of changes in the
54 expression level of both receptors. Flow cytometry analysis of EGFP and tRFP
55 assessed before each experiment showed no changes in the expression level of both
56 receptors between cell lines. The transient transduction with OTR-tRFP did not affect
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3 the 5-HTR_{2A}-EGFP expression level in cells co-expressing both receptors (HEK293A-
4 5HTR_{2A}-EGFP-Lv-OTR-tRFP) compared to non-transduced cells solely expressing
5 the 5-HTR_{2A}-EGFP. Similarly, the level of OTR-tRFP expression following transient
6 transduction did not differ in cells co-expressing both receptors compared to cells
7 solely expressing the OTR-tRFP (Figure 4, supplementary data). Control experiments
8 measuring OTR-mediated calcium release between cells stably expressing the OTR-
9 tGFP and cells transiently expressing the OTR-tRFP (following lentiviral transduction)
10 demonstrated no significant differences (Figure 6A, supplementary data). This clearly
11 shows no effect of the gene delivery mode (stable expression versus transient lentiviral
12 transduction) or different fluorescent tags (EGFP or tGFP) on the OTR-mediated Gαq
13 signalling. Moreover, additional control experiments performed using non-transfected
14 and non-transduced HEK293A cells as well as HEK293A cells stably expressing tGFP
15 didn't show any unspecific activation of Gαq signalling (Figure 5, supplementary
16 material). Finally, there were no changes in the OTR-mediated calcium response
17 between cells solely expressing the OTR-tGFP and cells co-expressing the OTR-tGFP
18 with OTR-tRFP (following lentiviral transduction), again showing no effect of the
19 lentiviral transduction protocol, nor the OTR overexpression on Gαq-mediated
20 signalling (Figure 6B, 6C, 6D, supplementary data).

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34 The above results highlight a significant attenuation in the OTR and 5-HTR_{2A}-mediated
35 Gαq signalling, which appears to be dependent on the specific interaction between the
36 two receptors, rather than on changes in their expression level, fluorescent tags or
37 gene delivery mode.
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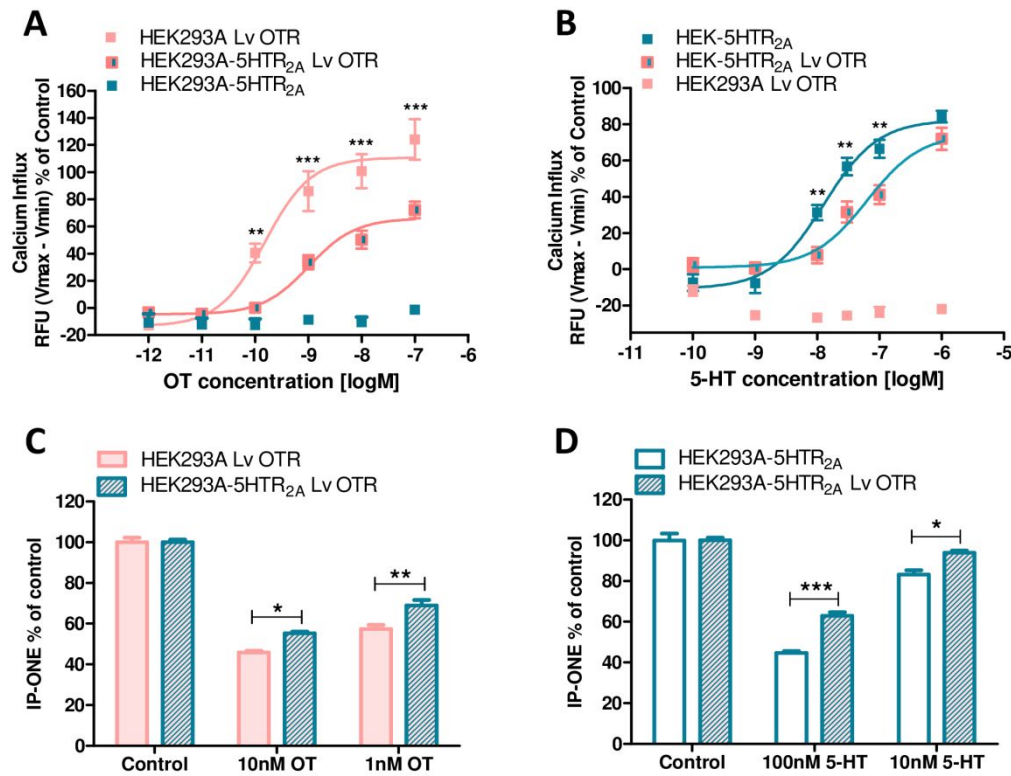


Figure 5. Co-expression of the OTR and 5-HTR_{2A} attenuates Gαq-dependent signalling of both receptors. Intracellular calcium release induced by increasing concentration of OT (A) and 5-HT (B) in HEK293A cells stably expressing the 5-HTR_{2A} tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. Intracellular calcium mobilization is presented as a percentage of maximal calcium response elicited by the control (3% FBS). Graphs represent means ± SEM from at least three independent experiments run in triplicates. IP-One production induced by 10 nM and 1 nM OT (C), and 100 nM and 10 nM 5-HT (D) in HEK293A cells stably expressing 5-HTR_{2A} tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. IP-One production is presented as a percentage of control (100% for non-stimulated cells). Graphs represent means ± SEM from experiments run in triplicate. Statistical significance of cells co-expressing both receptors compared to cells solely expressing one receptor is denoted as * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.001$.

Next, further experiments were performed to investigate if specific antagonists of the OTR and 5-HTR_{2A} could affect the observed attenuation in the Gαq-dependent downstream signalling pathway. Two antagonists of the OTR (Atosiban and L-371-257) and two antagonists of the 5-HTR_{2A} (M100907 and Eplivanserin) were used (Figure 6). As expected, both 5-HTR_{2A} antagonists used in 1 μM concentration were able to inhibit 5-HT-induced calcium mobilization in cells solely expressing the 5-HTR_{2A} and cells co-expressing both receptors (Figures 6B and 6D). Moreover, the lack of a non-specific interaction between OT and 5-HTR_{2A} antagonists was demonstrated in cells solely expressing the OTR (Figures 6A and 6C). Although a weak inhibition in OT-induced calcium mobilization after co-administration with M100907 can be

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3 observed, it is not statistically significant (Figure 6A). The lack of non-specific
4 interaction between these two ligands in cells solely expressing the OTR was also
5 confirmed in additional experiments with various concentrations of OT and M100907
6 (data not shown). Importantly, none of the 5-HTR_{2A} antagonists significantly
7 modulated OT-mediated calcium release in cells co-expressing the OTR and 5-HTR_{2A}
8 (Figures 6A and 6C). These observations for 5-HTR_{2A} antagonists were confirmed
9 following their pre-treatment with cells co-expressing both receptors (Figure 7,
10 supplementary data). Treatment with OTR antagonists (Atosiban and L-371-257)
11 yielded similar results as observed for 5-HTR_{2A} antagonists. Both, Atosiban and L-
12 371-257 used in 1 μ M concentration inhibited OT-induced calcium mobilization in cells
13 solely expressing this receptor (Figures 6F and 6H) but did not affect 5-HT-induced
14 calcium signalling in cells co-expressing the OTR and 5-HTR_{2A} (Figures 6E and 6G).
15 Taking together, none of the antagonists used in the experiment were able to modulate
16 the attenuation in G α q-dependent signalling observed in HEK293A cells co-
17 expressing the OTR-5-HTR_{2A} pair (Figure 5).
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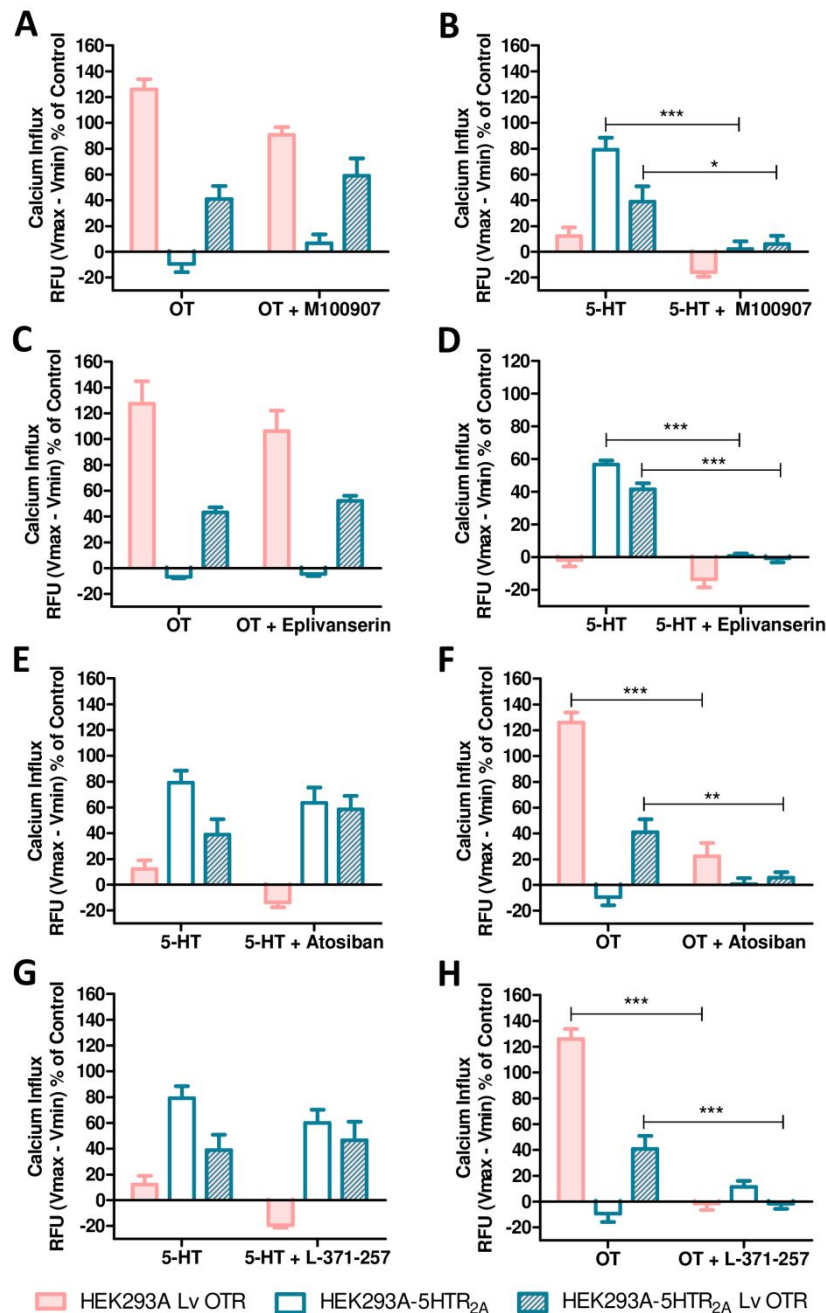


Figure 6. Pharmacological inhibition of the OTR or 5-HTR_{2A} does not affect the OTR-5-HTR_{2A} heterocomplex specific G_q-dependent signalling. Intracellular calcium release in cells solely expressing OTR, cells solely expressing 5-HTR_{2A}, and cells co-expressing both receptors induced by 10 nM OT alone and in the presence of 1 μ M 5-HTR_{2A} antagonists; M100907 (A) and Eplivanserin (C), as well as 1 μ M OTR antagonists; Atosiban (F) and L-371-257 (H). Intracellular calcium release induced by 100 nM 5-HT alone and in the presence of 1 μ M OTR antagonists; Atosiban (E) and L-371-257 (G), as well as 5-HTR_{2A} antagonists; M100907 (B) and Eplivanserin (D). All graphs represent means \pm SEM from at least two independent experiments run in triplicates, demonstrated as percentage of maximum calcium response (3% FBS). Statistical significance of cells co-expressing both receptors compared to cells solely expressing corresponding receptor is denoted as * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.001$.

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3 The effect of the 5-HTR_{2A} on OTR-dependent Gαq signalling induced by OT is more
4 pronounced in our in vitro model compared to the effect of the OTR on 5-HTR_{2A}. Thus,
5 to further investigate pronounced alteration in OTR-mediated Gαq signalling, cells with
6 the co-expression of the OTR and 5-HTR_{2A} were treated with different concentrations
7 of two synthetic OTR agonists; carbetocin and WAY267464^{90,91}. Similar to what we
8 observed for OT, the intracellular calcium release induced by increasing
9 concentrations of carbetocin and WAY267464 was significantly reduced in cells co-
10 expressing the 5-HTR_{2A} and OTR compared to cells solely expressing the OTR
11 (Figure 7). The potency and efficacy of carbetocin was significantly lower ($EC_{50} = 9.4$
12 ± 2.5 nM, $E_{max} = 21.9\% \pm 4.1$) in cells co-expressing both receptors compared to cells
13 solely expressing the OTR ($EC_{50} = 0.5 \pm 0.3$ nM, $E_{max} = 86.0\% \pm 11.0$) (Figure 7A).
14 Intracellular calcium response induced by increasing concentrations of WAY267464
15 was completely abolished in cells co-expressing the OTR and 5-HTR_{2A} ($EC_{50} = nc$,
16 $E_{max} = 2.7\% \pm 1.5$) compared to cells solely expressing the OTR ($EC_{50} = 11.6$ nM, E_{max}
17 $= 61.2\% \pm 1.2$) (Figure 7B). Nevertheless, pre-treatment of cells with 5-HTR_{2A}
18 antagonist (eplivanserin) did not affect carbetocin (Figure 7C) nor WAY267464 (Figure
19 7D) induced calcium response in cells co-expressing both receptors. In the same
20 experimental setup, eplivanserin was able to inhibit 5-HT-mediated intracellular
21 calcium accumulation in cells solely expressing the 5-HTR_{2A} confirming compound
22 specificity (data not shown).
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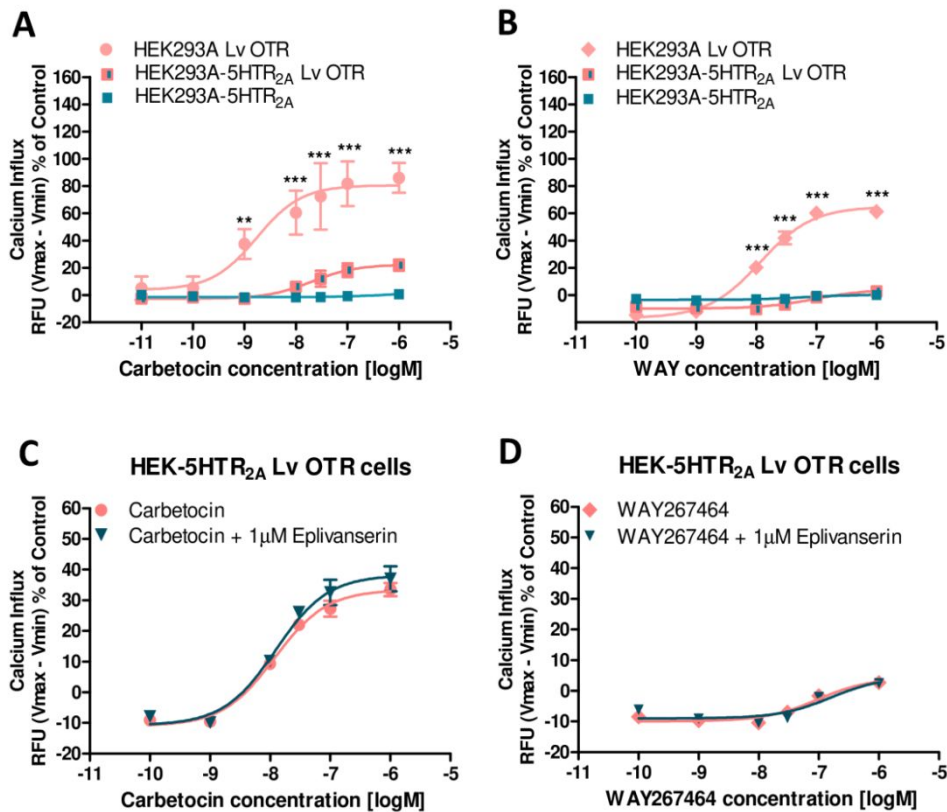


Figure 7. Synthetic OTR ligand-mediated attenuation of $G_{\alpha q}$ -dependent signalling in cells co-expressing the OTR and 5-HTR_{2A}. Intracellular calcium release induced by increasing concentration of Carbetocin (A) and WAY267464 (B) in cells expressing OTR, in cells expressing 5-HTR_{2A}, and in cells co-expressing both receptors. Graphs present mean \pm SEM from at least two independent experiments run in triplicates. Statistical significance of cells co-expressing both receptors compared to cells solely expressing corresponding receptor is denoted as ** for $p < 0.001$, and *** for $p < 0.001$. Intracellular calcium release induced by increasing concentration of Carbetocin (C) and WAY (D) in the presence of 5-HTR_{2A} antagonist; Eplivanserin in cells co-expressing both receptors. Graphs present mean \pm SEM from an experiment run in triplicate. Results are demonstrated as a percentage of maximum calcium response (3% FBS).

The results clearly show that one receptor induces highly reproducible functional attenuation in partner receptor signalling. A significant decrease in 5-HTR_{2A} dependent signalling has been demonstrated upon co-expression with the OTR. The attenuated OTR-mediated signalling is even more evident compared to 5-HTR_{2A}-mediated signalling changes. These interesting observations may be related to conformational rearrangements of one protomer resulting in trans-inhibition of another after agonist binding. Moreover, the lower potency and efficacy of OTR agonists are completely in line with the increasing attenuation of the receptor downstream signalling (Figures 5A, 7A and 7B). This may suggest that the potency of receptor ligands to activate $G_{\alpha q}$ signalling is lower for the OTR-5-HTR_{2A} heteromer complexes than for the corresponding receptor homodimers. Interestingly, current findings are in line with

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3 antagonistic interactions previously observed between the 5-HTR_{2A} and mGluR2
4 which have been established *in vitro* in heterologous expression models and across
5 multiple *ex vivo* and *in vivo* studies ^{57,60}. The formation of 5-HTR_{2A} heteromeric
6 complexes with the D2R has also been demonstrated to result in D2R-mediated Gai
7 signalling attenuation ⁴⁸. In contrast, concomitant activation of the D2R was shown to
8 increase the 5-HTR_{2A}-mediated Gαq signalling. Thus, the existence of a 5-HTR_{2A}-
9 mediated D2R trans-inhibition mechanism was suggested. Considering the above, the
10 5-HTR_{2A} in heteroreceptor complexes with other GPCRs has a tendency to inhibit
11 downstream signalling of the partner receptor. This further supports 5-HTR_{2A}-
12 mediated inhibition of the OTR-dependent signalling observed in the current study.
13 Interestingly, previous findings also demonstrated changes in downstream OTR
14 signalling following the formation of OTR heteroreceptor complexes with other GPCRs
15 ^{50,53}. In the case of OTR-D₂R heterodimers both the Gq/11 and the MAPK pathways
16 activation have been increased ⁵³. By simple analogy we could hypothesize that co-
17 expression of the OTR and 5-HTR_{2A} in HEK293A cells may also affect other signalling
18 pathways including MAPK cascade. However, based on the broader literature and also
19 our own experience specific heteromers are often able to only affect a single G-
20 protein-dependent signalling pathway ⁶⁶. Evidence also suggests that GPCR
21 heteromers can activate signalling cascades which are not activated by the individual
22 protomers not in the complex ⁹². Additionally, different ligands can differently alter
23 signalling pathways mediated by the formation of heteroreceptor complexes (biased
24 signalling) ^{54,93}. Thus, further studies investigating the full spectrum of downstream
25 signalling pathways following modulation by different receptors ligands and their
26 specific effects on the OTR-5-HTR_{2A} heteroreceptor are now warranted.

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28 Taking together the above, previously described heteroreceptor complexes of the 5-
29 HTR_{2A} with mGlu2 and D2R, as well as formation of heteromers between the OTR
30 and D2R, were shown to produce allosteric receptor-receptor interactions between the
31 two protomers ^{48,53,60}. Current results provide evidence that the formation of stable
32 OTR-5-HTR_{2A} heterocomplexes leads to bidirectional antagonistic receptor-receptor
33 interactions with greater 5-HTR_{2A} dominance. However, unlike previous studies, the
34 antagonists of both OTR and 5-HTR_{2A} did not affect the specific signalling driven by
35 OTR-5-HTR_{2A} heterocomplexes. Moreover, there was no synergistic nor additive
36 effect in Gαq signalling when cell co-expressing both receptors were co-treated with
37 their respective endogenous ligands, OT and 5-HT (Figure 8, supplementary data).
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3 Thus, one of the possible mechanisms of this specific receptor-receptor interaction is
4 the physical binding between the two protomers and subsequent conformational
5 changes without a steric hinderance of the binding pockets.
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8 **CONCLUSION**

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10 GPCRs comprise the largest family of cell surface receptors, which are major
11 signalling mediators for many hormones and neurotransmitters involved in diverse
12 physiological functions ranging from glucose metabolism to neurotransmission.
13 GPCRs were originally thought to function as monomers. However, oligomerization of
14 these receptors has now become accepted as a fundamental process in GPCR
15 signalling. Oligomerization of specific GPCR protomers is characterized by an
16 increased receptor signalling diversity and exhibits unique functional and
17 pharmacological properties adding a new dimension to GPCR pharmacology. Since
18 mechanisms that lead to diverse brain pathologies such as social and cognition related
19 disorders involve GPCR signalling, the distinct pharmacological profiles of GPCR
20 assemblies may serve as novel mechanisms, important for the development of more
21 specific pharmacological strategies to modulate cell response and regulate many
22 physiological processes.
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33 This study shows compelling evidence for a functionally relevant formation of a novel
34 heteroreceptor complex between the OTR and 5-HTR_{2A}. Both receptors have been
35 shown to physically interact in living mammalian cells co-expressing both receptors
36 (Figure 1). Confocal microscopy demonstrated that this specific interaction seems to
37 appear at the cell membrane as well as intracellularly (Figure 2). Alterations in the
38 trafficking of both receptors within the cell and their significant intracellular presence
39 in basal conditions (Figure 4) are in line with the colocalized expression and strongly
40 suggests changes in OTR and 5-HTR_{2A} maturation and trafficking to the cell
41 membrane. However, this phenomenon may also be a consequence of increased
42 coupling to β -arrestin and subsequently higher basal activity of the two receptors. In
43 any case both hypothesis support a physical interaction between the OTR and 5-
44 HTR_{2A} within the cell. Noteworthy, a significant attenuation was demonstrated
45 primarily in OTR but also in 5-HTR_{2A}-mediated G α q-dependent signalling (Figure 5
46 and 7) indicating a functional relevant consequence of OTR/5-HTR_{2A} interaction. In
47 conclusion, the current study provides evidence that the OTR-5HTR_{2A} heterocomplex
48 formation leads to bidirectional antagonistic receptor-receptor interactions *in vitro* in
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3 the heterologous system. As the antagonists of both receptors did not affect OTR-
4 5HTR_{2A} heterocomplex specific signalling (Figure 6), it is likely that the physical
5 binding between the two protomers serves as a mechanism for this specific receptor-
6 receptor crosstalk.
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10 Moreover, the formation of OTR-5HTR_{2A} heteroreceptor complexes were
11 demonstrated *ex vivo* in rat brain sections using *in situ* PLA technique (Figure 3). OTR-
12 5HTR_{2A} heteroreceptor complexes were observed in the pyramidal cell layer of CA2-
13 CA3 regions of the hippocampus, the layers II and III of the cingulate cortex and in a
14 number of nerve cell bodies of the nucleus accumbens shell and core. The proximal
15 dendrites of these nerve cells had a low number of PLA clusters located close to them.
16 This specific distribution pattern may indicate the potential role of OTR-5-HTR_{2A}
17 heteroreceptor complexes in distinct cortical and subcortical limbic regions. The
18 formation of these receptor complexes may therefore have special role in distinct
19 limbic circuits of relevance for social salience and memory, bearing in mind the
20 importance of OTR in social interactions.
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24 The existence of novel functional OTR-5HTR_{2A} heteroreceptor complexes constitutes
25 one of the possible mechanisms for intriguing interactions between the OT and 5-HT
26 neurotransmitter systems. It also provides potential novel therapeutic strategies in the
27 treatment of social and cognition-related diseases. Further *in vivo* studies exploring
28 the physiological and behavioural nature of the specific interactions observed between
29 the OTR and 5-HTR_{2A} in limbic regions are now warranted.
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METHODS

Receptors ligands.

Oxytocin (OT) (#O3251), carbetocin (#SML0748), 5-hydroxytryptamine (5-HT) (#H9523), atosiban (#A3480), and M100907 (#M3324) were purchased from Sigma-Aldrich (Wicklow, Ireland). L-371-257 (#2410), WAY267464 (#3933) and eplivanserin hemifumarate (#4958) were purchased from Tocris Bioscience (Ellisville, MO). A 3 mM stocks of compounds were prepared in H₂O (oxytocin, carbetocin, 5-HT, atosiban, M100907, WAY267464, eplivanserin hemifumarate) or in DMSO (L-371-257). All compound stocks were further diluted to the required concentrations in the proper assay buffer.

Cell culture and stable transfection.

Plasmid containing the canonical sequence (transcript variant 1) of the human serotonin 2A receptor (5-HTR_{2A}) (NM_000621) was supplied from University of Missouri–Rolla (#HTR02ATN00). The coding sequence of the receptor lacking its stop codon was amplified using forward 5'-AGCTCGAGACCATGGATATTCTTTGTGAAGAAAATAC-3' and reverse 5'-GAGAGGATCCCACACACAGCTCACC-3' primers containing XhoI and BamHI restriction sites, respectively. The amplified sequence was then subcloned into the multicloning site of the pCMV-EGFP-N1 vector (Clontech #6085-1) to obtain the 5-HTR_{2A} C-terminally tagged with EGFP. The obtained plasmid construct; pCMV-5-HTR_{2A}-EGFP-N1 was verified by restriction analysis, sequencing and then used for stable transfection of Human Embryonic Kidney (HEK293A) cells.

HEK293A cells (Invitrogen, Carlsbad, CA) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, #D5796; Sigma-Aldrich, Wicklow, Ireland) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (#F7524; Sigma-Aldrich) and 1% Non-Essential Amino Acids (NEAA) (#11140035; Gibco Life Technologies, Gaithersburg, MD). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. HEK293A cells were transfected with the plasmid containing human 5-HTR_{2A} sequence fused with EGFP in the presence of Lipofectamine LTX Plus reagent, according to the manufacturer's instructions (#15338100; Invitrogen). 48 h after transfection, the cell media was changed for DMEM supplemented with 500 ng/μl G-418 (#345812; Calbiochem), allowing for the

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3 selection of cells with stably integrated pCMV-5-HTR2A-EGFP-N1 plasmid. The cells
4 with the highest expression of the receptor were selected using flow-assisted cell
5 sorting (FACS AriaII, BD Biosciences), followed by clonal expansion in 96-well plates.
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7 Expression level of 5-HTR_{2A} in generated monoclonal cell line was routinely monitored
8 using an epifluorescence microscope (Olympus IX70) and a flow cytometer
9 (FACSCalibur, BD Biosciences).
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14 **Lentiviral transfection and transduction.**

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16 The coding sequences of the human oxytocin receptor (OTR) was subcloned into the
17 multicloning site of the HIV-based, replication deficient, lentiviral expression vector;
18 pHR-SIN-BX-tRFP. The construct containing the canonical sequence of the human
19 OTR (NM_000916.3), C-terminally tagged with red fluorescent protein (tRFP) was
20 generated by inserting the coding sequence of the receptor lacking its stop codon from
21 pCMV6-AC-OXTR-GFP plasmid (#RG211797; OriGene, Rockville, MD) into the target
22 vector (pHR-SIN-BX-tRFP) with the use of BamHI and XhoI restriction enzymes. The
23 obtained pHR-SIN-BX-OXTR-tRFP plasmid construct was validated by restriction
24 analysis and DNA sequencing. HEK293A cells were then transiently transduced with
25 the obtained lentiviral expression vector using a second generation packaging, gene
26 delivery, viral vector production system, previously described by our group⁶⁴. Briefly,
27 HIV-based lentiviral particles containing the OTR sequence were produced using
28 HEK293T-17 cells, by transient co-transfection of the expression construct; pHR-SIN-
29 BX- OXTR -tRFP, the packaging construct; pCMV ΔR8.91, and the envelope
30 construct; pMD.G-VSV-G. Following this, HEK293A cells were transiently transduced
31 with the OTR-tRFP expressing lentiviral particles diluted in transduction media,
32 consisting of DMEM with 2% FBS, 1% NEAA, and 8 μg/ml polybrene (#H9268; Sigma).
33 The efficiency of transduction was monitored with the use of an epifluorescence
34 microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences)
35 before each experiment.
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50 **Flow Cytometry Fluorescence Resonance Energy Transfer (fcFRET).**

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52 HEK293A cells stably expressing 5-HTR_{2A} tagged with EGFP were transiently
53 transduced with lentiviral OTR sequence tagged with tRFP. Following transduction,
54 cells were washed with PBS and mechanically removed from the wells. Cell
55 suspension was then centrifuged for 4 min at 200 x g, at room temperature. The pellet
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3 of cells was re-suspended in 400 μ l of 2 nM EDTA (#E5134; Sigma) in PBS. Prior to
4 analysis cells were passed through a 100 μ m nylon mesh cell strainer (#10199-658;
5 VWR) and collected in a 5 ml round bottom polystyrene tubes (#352054; Corning).
6 The fcFRET analysis was performed on a FACS Ariall cytometer (BD Biosciences)
7 according to the protocol optimized in our group (Chruścicka et al., 2018; Schellekens
8 et al., 2015). Briefly, EGFP was excited at 488 nm from blue laser and detected with
9 a 525/50 nm bandpass filter, whereas tRFP was excited at 561 nm from yellow/green
10 laser and detected with a 610/20 nm bandpass filter. FRET signal between EGFP and
11 tRFP was measured by excitation at 488 nm from blue laser and detection with a
12 610/20 nm bandpass filter located on the same laser. For the proper separation of
13 EGFP fluorescence and FRET emission from blue laser, a 505 Long Pass (LP)
14 dichroic mirror (DM) was used. Wild-type HEK293A cells were used for initial
15 instrument setup and to differentiate cells based on their size and granulation,
16 according to forward and side scattering plot (FSC/SSC), which allowed to eliminate
17 doublets, dead cells, and debris from further analysis. In the next step, cells expressing
18 donor or acceptor construct only were used to fine tune PMT settings and to perform
19 the proper compensation for spectral bleed through, in particular for EGFP emission
20 in the tRFP-fcFRET detector. In addition, cells co-expressing the donor construct
21 (GPCR-EGFP) with the control acceptor construct (control-tRFP; plasmid with the
22 sequence of tRFP without the receptor) were used to further control unspecific fcFRET
23 signal coming from the cross-excitation. The same number of cells (10^4) was recorded
24 for each sample. Data was analysed using BD FACSDiva (BD Biosciences).
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42 **Colocalization with the use of confocal microscope.**

43 HEK293A stably expressing 5-HTR_{2A}-EGFP were transiently transduced to co-
44 express OTR-tRFP. Following transduction, cells were passaged and seeded on poly-
45 L-lysine-coated (#P4707; Sigma) borosilicate glass slides (#631-0150; VWR
46 International) at the density of 5×10^5 cells per well of 24-well plate, followed by 24 h
47 incubation in the standard culture conditions. Co-localization of the receptors was
48 assessed in living cells using laser scanning confocal fluorescent microscope (FV
49 1000 Confocal System; Olympus). Pictures were taken with 63 x objective lens using
50 Olympus fluoview FV3000 software. Co-localization between 5-HTR_{2A}-EGFP and
51 OTR-RFP was analysed by overlay with the use of ImageJ software (US National
52 Institutes of Health).
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Receptors trafficking assay.

The trafficking of receptors was analysed by monitoring the fluorescent proteins translocation away from the cellular membrane into vesicles within the cytosol. HEK293A cells stably expressing the 5-HTR_{2A}-EGFP and transduced with lentiviral OTR-tRFP, were seeded on 24-well plates (#83.3922.005; Sarstedt) at the density of 5 x 10⁴ cells/well. Cells were then incubated for 48 h at standard culture conditions. 24 h before experiment, media was replaced with serum-free DMEM containing 1% NEAA. To investigate ligand-mediated changes in receptors trafficking, cells were incubated with different concentrations of 5-HTR_{2A} or OTR endogenous agonists for 30 minutes at 37°C. After the treatment, cells were fixed in 4% paraformaldehyde (PFA) for 20 min and washed two times in PBS. Ligand mediated internalization of the receptors was assessed using inverted fluorescence microscope (IX71; Olympus). Fluorescent images were acquired with 20 x objective lens using Olympus cell R software. Results demonstrated in the manuscript represent average from 3 independent experiments each performed in duplicates (two wells for each condition in each experiment). Within each well, three images were captured. For each image 6 cells were quantitatively analysed by two independent researchers. Quantification of the receptors trafficking was assessed by calculating the ratio between subcellular and membrane fluorescent intensity after excluding background fluorescence, with the use of Java image processing program (ImageJ, US National Institutes of Health). The obtained results were depicted using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA).

Animals

All experiments were performed using male Sprague-Dawley rats (SD) (Scanbur, Sweden). The animals were group-housed under standard laboratory conditions (20–22°C, 50–60% humidity). Food and water available *ad libitum*. The rats were 3–4 months of age at the time of experiments. All studies involving animals were performed in accordance with the Stockholm North Committee on Ethics of Animal Experimentation, the Swedish National Board for Laboratory Animal and European Communities Council Directive (Cons 123/2006/3) guidelines for accommodation and care of Laboratory Animals.

In situ proximity ligation assay (*in situ* PLA)

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3 To study the OTR-5HTR_{2A} heteroreceptor complexes the *in situ* proximity ligation
4 assay (*in situ* PLA) was performed as described previously⁹⁴. Adult age-matched male
5 Sprague–Dawley rats (n=4) were anaesthetized and perfused intracardially with 4%
6 (wt/vol) formalin in saline. Brains were removed, post-fixed by immersion overnight in
7 4% formalin in PBS and coronal sections (30 μm) were cut on a cryostat and
8 processed for free-floating *in situ* PLA. Free-floating formalin fixed brain sections
9 (storage at -20°C in Hoffman solution) at Bregma level (-3.6 mm and 1.2 mm) were
10 washed four times with PBS and quenched with 10 mM Glycine buffer for 20 min at
11 room temperature. Then, after three PBS washes, were permeabilized with a
12 permeabilization buffer (10% FBS and 0.5% Triton X-100 or Tween 20 in Tris buffer
13 saline (TBS), pH 7.4) for 30 min at room temperature. Again the sections were washed
14 twice, 5 min each, with PBS at room temperature and incubated with the blocking
15 buffer (0.2% BSA in PBS) for 30 min at room temperature. The brain sections were
16 then incubated with the primary antibodies diluted in a suitable concentration in the
17 blocking solution for 1-2 h at 37°C or at 4°C overnight. The day after, the sections were
18 washed twice, and the proximity probe mixture was applied to the sample and
19 incubated for 1 h at 37°C in a humidity chamber. The unbound proximity probes were
20 removed by washing the slides twice, 5 min each time, with blocking solution at room
21 temperature under gentle agitation and the sections were incubated with the
22 hybridization-ligation solution (BSA (250 g/ml), T4 DNA ligase (final concentration of
23 0.05 U/μl), 0.05% Tween-20, 250 mM NaCl, 1 mM ATP and the circularization or
24 connector oligonucleotides (125-250 nM)) and incubated in a humidity chamber at
25 37°C for 30 min. The excess of connector oligonucleotides was removed by washing
26 twice, for 5 min each, with the washing buffer A (Sigma-Aldrich, Duolink Buffer A (8.8
27 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20 dissolved in 800 ml high purity water, pH to
28 7.4) at room temperature under gentle agitation and the rolling circle amplification
29 mixture was added to the slices and incubated in a humidity chamber at 37°C for 100
30 min. Then, the sections were incubated with the detection solution in a humidity
31 chamber at 37°C for 30 min. In a last step, the sections were washed twice in the dark,
32 for 10 min each, with the washing buffer B (Sigma-Aldrich, Duolink Buffer B (5.84 g
33 NaCl, 4.24 g Tris Base, 26.0 g Tris-HCl. Dissolved in 500 ml high purity water, pH 7.5)
34 at room temperature under gentle agitation. The free-floating sections were put on a
35 microscope slide and a drop of appropriate mounting medium (e.g., VectaShield or
36 Dako) was applied. The cover slip was placed on the section and sealed with nail
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3 polish. The sections were protected against light and stored for several days at -20°C
4 before confocal microscope analysis. The in situ PLA experiments were performed
5 using the following primary antibodies: rabbit monoclonal anti-5-HTR_{2A}
6 (#SAB4301791, 1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, Stockholm, Sweden) and goat polyclonal
7 anti-oxytocin receptor (#ab87312, 5 $\mu\text{g}/\text{ml}$; Abcam, Stockholm, Sweden). As a
8 neuronal marker the Neuro-ChromTM Pan neuronal marker antibody-Alexa488
9 conjugated (#ABN2300A4, Merck/Sigma-Aldrich) was used. The PLA signal was
10 visualized and quantified by using a Leica TCS-SL confocal microscope (Leica, USA)
11 and the Duolink Image Tool software. A range of positive and negative controls have
12 been used to guarantee the specificity of the PLA signal. The negative control consists
13 in the suppression of the species-specific primary antibody corresponding to the 5-
14 HTR_{2A} in the presence of the two PLA probes. As a positive control of the PLA assay,
15 a parallel analysis of the 5-HTR_{1A}-5HTR_{2A} isoreceptor complexes and the D₂R-OTR
16 heteroreceptor complexes have been performed. Detailed quality control analysis for
17 the 5HTR_{2A} and for the OTR antibodies have been reported previously ^{49,63}.
18 Furthermore, both anti-5HTR_{2A} and anti-OTR antibodies were previously validated in
19 our team in terms of their quality (in Western blot in collaboration with Human Atlas
20 project and in HEK293 cells with and without expression of each receptor subtype
21 using confocal analysis). Antibodies were used under optimal conditions, taking into
22 consideration parameters, such as; concentration, targeted epitopes, fixation
23 conditions, and antigen-retrieval ⁹⁴.
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40 **Intracellular calcium mobilization assay.**

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42 Receptor-mediated changes in intracellular calcium (Ca^{2+}) were monitored with the
43 use of automatic fluorescent reader, FLIPR Tetra[®] (Molecular Devices, LLC
44 Sunnyvale, CA) as previously described ^{54,95}. HEK293A cells with the expression of
45 the receptors under investigation were seeded in black 96-well microtiter plates at a
46 density of $3.0 - 4.0 \times 10^4$ cells/well and incubated overnight in standard culture
47 conditions. 24 h prior to the experiment growth media was replaced with serum-free
48 DMEM containing 1% NEAA. At the day of experiment cells were incubated for 90 min
49 with 80 μl of the Ca5 dye diluted in assay buffer containing 1 x Hank's Balanced Salt
50 Solution; HBSS (#14065049; Gibco Life Technologies, Gaithersburg, MD) and 20 mM
51 HEPES (#H0887; Sigma-Aldrich) in the concentration recommended by the
52 manufacturer's protocol (#R8186; Molecular Devices). The addition of receptor ligands
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(40 μ l/well) was performed with the use of automatic pipettor of the FLIPR Tetra®High-Throughput Cellular Screening System. To investigate the effect of receptor antagonists, compounds were co-administered together with agonist or pre-incubated for 90 min with the Ca5 dye. Fluorescent readings were taken for a total of 120 seconds with excitation wavelength of 485 nm and emission wavelength of 525 nm. The relative increase in intracellular calcium [Ca^{2+}] was calculated as the difference between the maximum and baseline fluorescence, and demonstrated as percentage relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained for 3% FBS. Background fluorescence was recorded for non-stimulated cells and subtracted from RFU. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA). The concentration-response curves of receptor ligands were generated using the nonlinear regression. The curves were fitted to a 3-parametric logistic equation, allowing for the determination of EC_{50} values.

HTRF based IP-One accumulation assay.

The detection of IP-One (inositol monophosphate) was performed in HEK293A cells expressing receptors under investigation, with the use of a homogeneous time-resolved fluorescence (HTRF) IP-One assay (#62IPAPEB; Cisbio, Codolet, France). The assay was performed according to the manual's instruction provided by Cisbio with minor modifications. Briefly, 24 h before experiment growth media was replaced with serum-free DMEM containing 1% NEAA. Directly before the experiment cells were scraped and centrifuged for 3 min at 200 x g. The cell pellet was then suspended in assay buffer (146 mM NaCl, 1 mM CaCl_2 , 10mM HEPES, 0.5 mM MgCl_2 , 4.2 mM KCl, 5.5 mM glucose) containing 50 mM LiCl to inhibit degradation of IP-One. For the stimulation step, 35 μ L of cell suspension was pipetted to a flat bottom 96-well plate at the density of 3×10^5 /well (#655075; Greiner Bio-One International) containing the appropriate concentration of compounds. Cells were incubated with compounds for 1 h at 37 °C. Following this step, 15 μ L of IP1-d2 conjugate and 15 μ L of anti-IP1 cryptate conjugate diluted in lysis buffer were added and incubated for 1 h in room temperature. After 1 h of incubation, the fluorescence at 620 nm and 665 nm was read with the use of FlexStation instrument (Molecular Devices, LLC Sunnyvale, CA) and the readout setup recommended by the company (Cisbio, Codolet, France). The results were calculated as the 665-nm/620-nm ratio multiplied by 10^4 and depicted as percentage

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3 of relative fluorescent units (RFU) normalized to maximum response (100% signal)
4 obtained for non-stimulated cells. The specific signal is inversely proportional to the
5 concentration of endogenous IP-One in the sample. Data were analysed using
6 GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA).
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10 **Statistical analysis.**

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12 Data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software
13 Inc., San Diego, CA). The concentration-response curves of receptors ligands were
14 generated using the nonlinear regression. The curves were fitted to a 3-parametric
15 logistic equation, allowing for the determination of EC_{50} and E_{max} values. Statistical
16 comparison of the concentration-response curves parameters (EC_{50} and E_{max})
17 between cells co-expressing both receptors and cells solely expressing the
18 corresponding receptor, were performed using the Student's test. Moreover, statistical
19 comparison of each compound concentration used in calcium mobilization, IP-One
20 accumulation, and ligand-mediated internalization assays between cells expressing
21 the OTR, 5HTR_{2A} and cells co-expressing both receptors was performed using Two-
22 way ANOVA with Bonferroni's multiple comparison tests. Statistical analysis of fcFRET
23 was performed using One-way ANOVA with Bonferroni's multiple comparison tests.
24 Statistical analysis of In situ PLA was performed using One-way ANOVA followed by
25 Tukey post-test. All data are presented as Mean \pm SEM. The differences between
26 groups were considered significant for $p < 0.05$. The number of independent
27 experiments performed is provided in figure legends.
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41 **ASSOCIATED CONTENT**

42 **Supporting information**

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44 Graphs presenting the results of additional control experiments: Figure S1. fcFRET
45 signal between the OTR-tGFP and the OTR-tRFP; Figure S2. fcFRET signal between
46 the tGFP and the tRFP; Figure S3. Confocal images presenting the expression pattern
47 of the OTR-tRFP in HEK293A. Figure S4. Flow cytometry analysis of EGFP and tRFP
48 in cells co-expressing receptors under investigation; Figure S5. The effect of OTR
49 ligands on intracellular calcium release in HEK293A and HEK293A-tGFP cells. Figure
50 S6. The effect of transduction procedure and fluorescent tags on G α_q -dependent
51 signalling; Figure S7. The effect of 5-HTR_{2A} antagonists on the OTR-mediated G α_q
52 signalling; Figure S8. The effect of OT and 5-HT co-treatment on G α_q -mediated
53 signalling in cells co-expressing 5-HTR_{2A} and OTR.
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Author Contributions

B.Ch. designed all in vitro experiments, performed experiments that lead to Figures 1, 2, 5, 6, 7, analysed all in vitro results and wrote the manuscript. S.W.F. performed transduction of cells for all in vitro experiments, performed and analysed experiments that lead to Figure 4. D.B.E. designed, performed and analysed all ex vivo data. C.D. served technician assistant in maintenance of cells, preparation of plasmid constructs and calcium assay experiments. S.P. served as technician support in designing and performing of fcFRET experiments. J.F.C., T.G.D., K.N. and K.F. supervised the work and critically read the manuscript. H.S. was involved in the conception of all experiments, supervised the work and edited the manuscript.

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Notes

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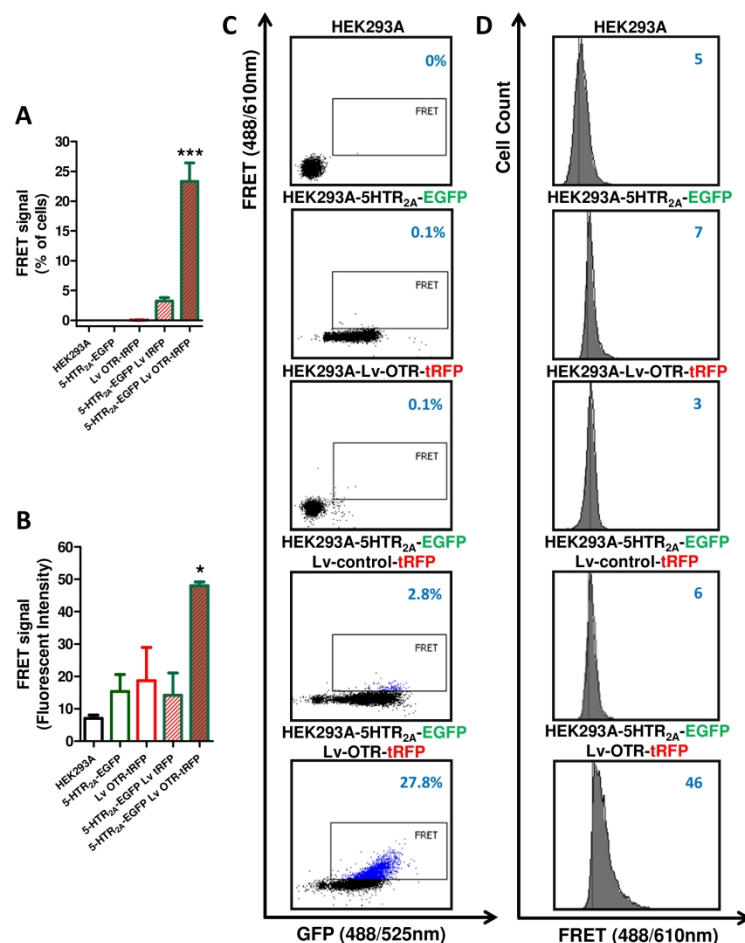
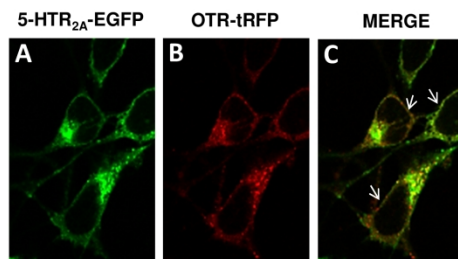


Figure 1. fcFRET between the OTR and 5-HTR_{2A}. The fcFRET signal is presented as a percentage of cells (A,C) and as median fluorescence (B,D) in wild-type HEK293A cells, cells stably expressing the 5-HTR_{2A} tagged with EGFP (donor), cells transiently transduced with lentiviral vector expressing OTR tagged with tRFP (acceptor), cells expressing 5-HTR_{2A} tagged with EGFP and the control-tRFP, and cells co-expressing 5-HTR_{2A} tagged with EGFP and OTR tagged with tRFP. Graphs represent mean \pm SEM from three independent experiments (A,B). Statistical significance of fcFRET signal in cells co-expressing both receptors compared to cells expressing donor with the control acceptor constructs is denoted as * for $p < 0.05$ and *** for $p < 0.001$. Dot plots (C) show percentage of cells with fcFRET signal (FRET vs EGFP plots), histograms (D) demonstrate median fluorescence of fcFRET signal (Cell count vs FRET signal). Dot plots and histograms are representative of three independent experiments.

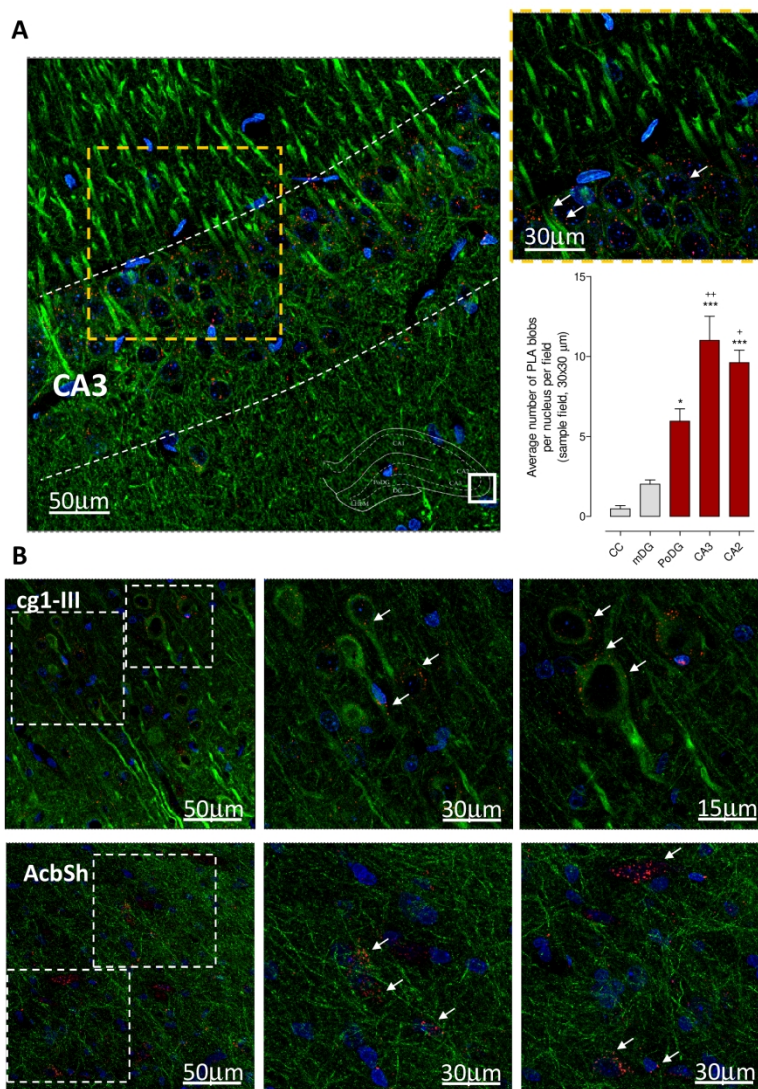
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45 **Figure 2. Cellular colocalization of the OTR/5-HTR_{2A} pair.** HEK293A cells stably expressing the 5-
46 HTR_{2A} tagged with EGFP (green) (A) were transiently transduced with lentiviral vector expressing OTR
47 tagged with tRFP (red) (B). Merged picture (yellow/orange) shows colocalization of the two receptors within
48 the cell (C).

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45 **Figure 3. Illustration of the OTR-5HT_{2A} heteroreceptor complexes in the dorsal hippocampus**
 46 **and nucleus accumbens of rat brain.** Microphotographs from transverse sections of the rat dorsal
 47 hippocampus (Bregma level: -3.6 mm) show the distribution of the OTR-5HT_{2A} heteroreceptor complexes
 48 in CA3 using the in situ proximity ligation assay (in situ PLA) technique. The square outlines the CA3 area
 49 from which the picture was taken. Receptor complexes are shown as red PLA blobs (clusters) found in high
 50 densities per cell in a large number of nerve cells in the pyramidal cell layer using confocal laser microscopy.
 51 No specific PLA blobs were found in the stratum moleculare and radiatum of the CA3-CA2 regions (cornu
 52 ammonis). The nuclei are shown in blue by DAPI staining and the neuronal marker in green. In the higher
 52 right panel the PLA blobs are presented in higher magnification in the pyramidal cell layer. In the lower right
 53 part of the figure the density (per nucleus per sampled field) of the PLA positive complexes in PoDG
 54 (polymorph layer of the dentate gyrus), CA3, and CA2 are highly significantly different (***) from the
 55 density found in crus cerebri (CC) and the granular cell layer of the dentate gyrus (gDG). The density is also
 56 significantly higher in the CA2 (+) and CA3 (++) versus PoDG (Mean ± SEM, 4 rats per group) (A). The
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3 upper panel of B show representative examples of these PLA receptor complexes from transverse sections of
4 the rat cingulate cortex, area 1 (Bregma level: 1.2 mm). They present the distribution of OTR-5HTR_{2A}
5 heteroreceptor complexes. They are shown as red PLA blobs (clusters) with high densities in layer III and
6 low to moderate densities in layer II. Layer III represents the external pyramidal cell layer where large PLA
7 positive clusters are found and appear to be located on the surface of many pyramidal cells. Higher
8 magnifications of the two squares outlined in left panel are shown in the two right panels. The nerve cell
9 bodies and apical dendrites are seen in green (neuronal marker). The lower panel in B is taken from nucleus
10 accumbens shell (AcbSh). The neuronal marker (Neuro-Chrom™ Pan neuronal marker antibody-Alexa488
11 conjugated, ABN2300A4) shows the neurite network. Discrete nerve cell bodies are associated with a high
12 density of PLA positive blobs representing OTR-5HTR_{2A} heteroreceptor complexes that may also have an
13 intracellular location through trafficking. The outlined squares in the left panel are shown in higher
14 magnifications in the two right panels (B).

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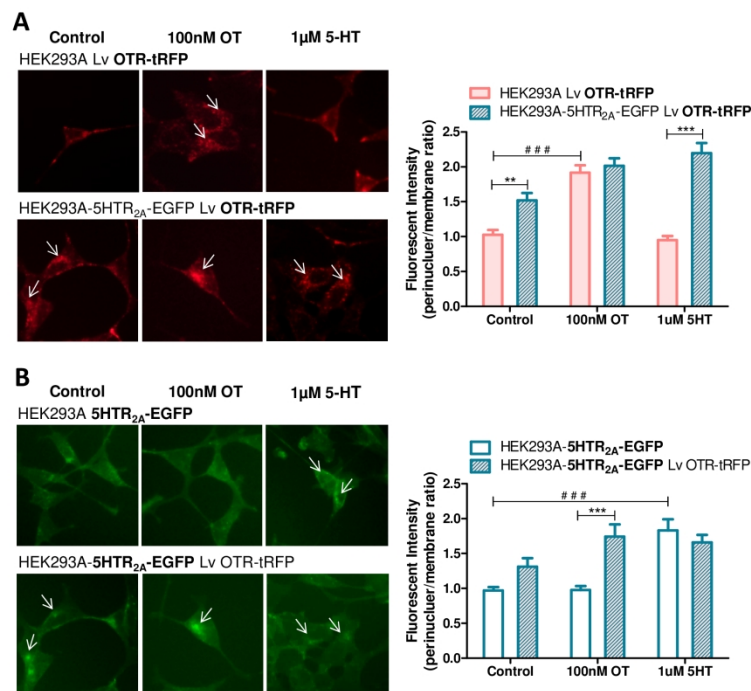


Figure 4. Cellular trafficking of the OTR and 5-HTR_{2A}. Representative images (left panel) and quantitative analysis (right panel) of ligand-mediated internalization of OTR tagged with tRFP (A) and 5-HTR_{2A} tagged with EGFP (B) versus cells co-expressing both receptors. Graphs represents mean \pm SEM from three independent experiments run in triplicate. Statistical significance of cells co-expressing both receptors compared to cells solely expressing the corresponding receptor is denoted as; ** indicating $p < 0.01$; or *** indicating $p < 0.001$. Statistical significance of cells following OTR or 5-HT treatment compared to the control condition is denoted as; # # # indicating $p < 0.001$.

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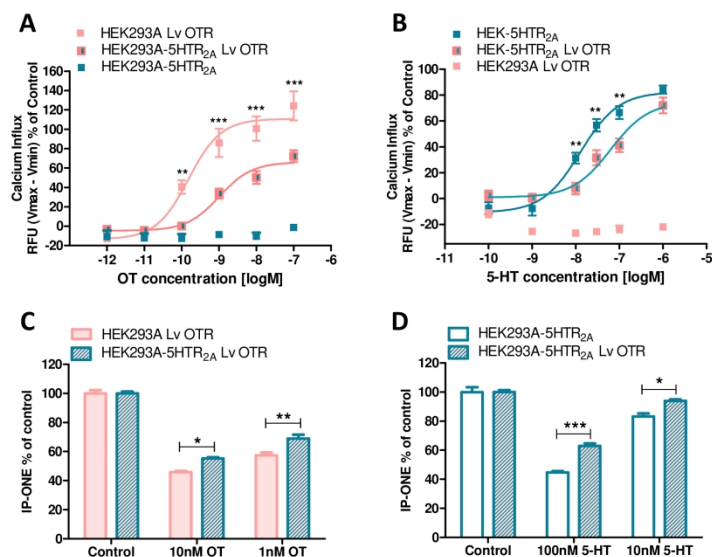


Figure 5. Co-expression of the OTR and 5-HTR_{2A} attenuates G α q-dependent signalling of both receptors. Intracellular calcium release induced by increasing concentration of OT (A) and 5-HT (B) in HEK293A cells stably expressing the 5-HTR_{2A} tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. Intracellular calcium mobilization is presented as a percentage of maximal calcium response elicited by the control (3% FBS). Graphs represent means \pm SEM from at least three independent experiments run in triplicates. IP-One production induced by 10 nM and 1 nM OT (C), and 100 nM and 10 nM 5-HT (D) in HEK293A cells stably expressing 5-HTR_{2A} tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. IP-One production is presented as a percentage of control (100% for non-stimulated cells). Graphs represent means \pm SEM from experiments run in triplicate. Statistical significance of cells co-expressing both receptors compared to cells solely expressing one receptor is denoted as * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.001$.

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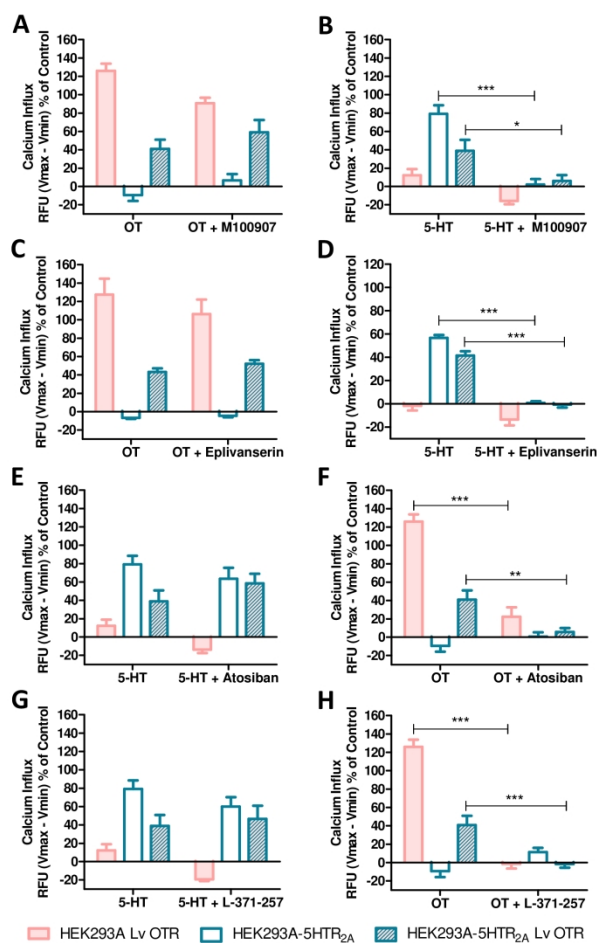


Figure 6. Pharmacological inhibition of the OTR or 5-HTR_{2A} does not affect the OTR-5-HTR_{2A} heterocomplex specific G_{αq}-dependent signalling. Intracellular calcium release in cells solely expressing OTR, cells solely expressing 5-HTR_{2A}, and cells co-expressing both receptors induced by 10 nM OT alone and in the presence of 1 μM 5-HTR_{2A} antagonists; M100907 (A) and Eplivanserin (C), as well as 1 μM OTR antagonists; Atosiban (F) and L-371-257 (H). Intracellular calcium release induced by 100 nM 5-HT alone and in the presence of 1 μM OTR antagonists; Atosiban (E) and L-371-257 (G), as well as 5-HTR_{2A} antagonists; M100907 (B) and Eplivanserin (D). All graphs represent means ± SEM from at least two independent experiments run in triplicates, demonstrated as percentage of maximum calcium response (3% FBS). Statistical significance of cells co-expressing both receptors compared to cells solely expressing corresponding receptor is denoted as * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.001$.

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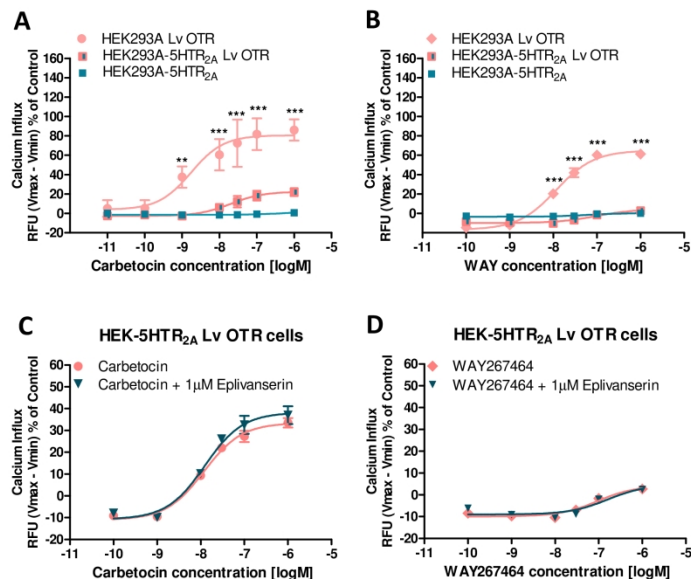
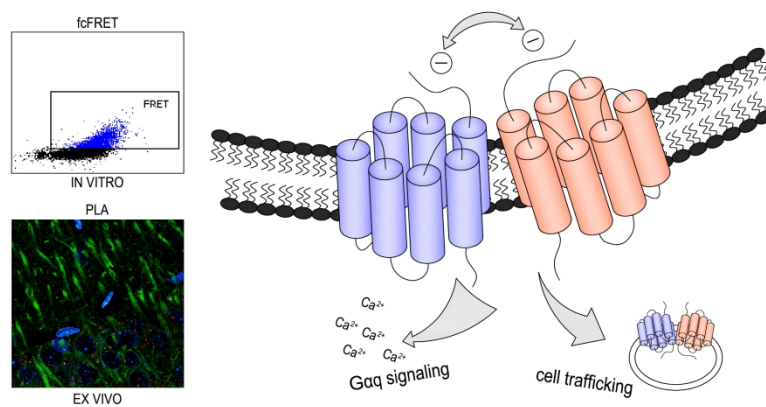


Figure 7. Synthetic OTR ligand-mediated attenuation of G α q-dependent signalling in cells co-expressing the OTR and 5-HTR_{2A}. Intracellular calcium release induced by increasing concentration of Carbetocin (A) and WAY265464 (B) in cells expressing OTR, in cells expressing 5-HTR_{2A}, and in cells co-expressing both receptors. Graphs present mean \pm SEM from at least two independent experiments run in triplicates. Statistical significance of cells co-expressing both receptors compared to cells solely expressing corresponding receptor is denoted as ** for $p < 0.001$, and *** for $p < 0.001$. Intracellular calcium release induced by increasing concentration of Carbetocin (C) and WAY (D) in the presence of 5-HTR_{2A} antagonist; Eplivanserin in cells co-expressing both receptors. Graphs present mean \pm SEM from an experiment run in triplicate. Results are demonstrated as a percentage of maximum calcium response (3% FBS).

209x297mm (300 x 300 DPI)



Graphical table of contents