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Title	Molecular, biochemical and behavioural evidence for a novel oxytocin receptor and serotonin 2C receptor heterocomplex
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Publication date	2021-02-01
Original Citation	Chruścicka, B., Cowan, C. S. M., Wallace Fitzsimons, S. E., Borroto-Escuela, D. O., Druelle, C. M., Stamou, P., Bergmann, C. A., Dinan, T. G., Slattery, D. A., Fuxe, K., Cryan, J. F. and Schellekens, H. (2021) 'Molecular, biochemical and behavioural evidence for a novel oxytocin receptor and serotonin 2C receptor heterocomplex', Neuropharmacology, 183, 108394, (17pp). doi: 10.1016/j.neuropharm.2020.108394
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.neuropharm.2020.108394
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Download date	2026-11-15 03:16:03
Item downloaded from	https://hdl.handle.net/10468/13173

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Neuropharmacology 183 (2021) 108394

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Molecular, biochemical and behavioural evidence for a novel oxytocin receptor and serotonin 2C receptor heterocomplex

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ARTICLE INFO

Keywords:
OTR
5-HTR$_{2C}$
Heteroreceptor complexes
GPCR crosstalk
Hypoactivity

ABSTRACT

The complexity of oxytocin-mediated functions is strongly associated with its modulatory effects on other neurotransmission systems, including the serotonin (5-hydroxytryptamine, 5-HT) system. Signalling between oxytocin (OT) and 5-HT has been demonstrated during neurodevelopment and in the regulation of specific emotion-based behaviours. It is suggested that crosstalk between neurotransmitters is driven by interaction between their specific receptors, particularly the oxytocin receptor (OTR) and the 5-hydroxytryptamine 2C receptor (5-HTR$_{2C}$), but evidence for this and the downstream signalling consequences that follow are lacking. Considering the overlapping central expression profiles and shared involvement of OTR and 5-HTR$_{2C}$ in certain endocrine functions and behaviours, including eating behaviour, social interaction and locomotor activity, we investigated the existence of functionally active OTR/5-HTR$_{2C}$ heterocomplexes. Here, we demonstrate evidence for a potential physical interaction between OTR and 5-HTR$_{2C}$ *in vitro* in a cellular expression system using flow cytometry-based FRET (fcFRET). We could recapitulate this finding under endogenous expression levels of both receptors via *in silico* analysis of single cell transcriptomic data and *ex vivo* proximity ligation assay (PLA). Next, we show that co-expression of the OTR/5-HTR$_{2C}$ pair resulted in a significant depletion of OTR-mediated Gαq-signalling and significant changes in receptor trafficking. Of note, attenuation of OTR-mediated downstream signalling was restored following pharmacological blockade of the 5-HTR$_{2C}$. Finally, we demonstrated a functional relevance of this novel heterocomplex, *in vivo*, as 5-HTR$_{2C}$ antagonism increased OT-mediated hypoactivity in mice. Overall, we provide compelling evidence for the formation of functionally active OTR/5-HTR$_{2C}$ heterocomplexes, adding another level of complexity to OTR and 5-HTR$_{2C}$ signalling functionality.

This article is part of the special issue on Neuropeptides.

1. Introduction

Oxytocin (OT) is a neurotransmitter produced, predominantly, in the paraventricular (PVN) and supraoptic nuclei of the hypothalamus (Du Vigneaud et al., 1953). The multiple established and proposed actions of OT are mediated by the OT receptor (OTR), which belongs to the rhodopsin (class A) G-protein coupled receptor (GPCR) family (Gimpl et al., 2008; Gimpl and Fahrenholz, 2001; Jurek and Neumann, 2018). The OTR is expressed peripherally in the uterus, kidney, thymus, bones, and heart as well as throughout the central nervous system, with differential expression in many brain areas such as the hypothalamus, hippocampus, striatum, pallidum, and some cortical areas (Mitre et al., 2016; Newmaster et al., 2020; Yoshida et al., 2009). The oxytocinergic system is well known to regulate lactation and parturition, circadian

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https://doi.org/10.1016/j.neuropharm.2020.108394
Received 22 May 2020; Received in revised form 11 October 2020; Accepted 5 November 2020
Available online 12 November 2020
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rhythm, heart rate, bone and muscle formation. OTR-mediated signalling within the CNS is mainly involved in modulation of complex social and cognitive related behaviours including bonding, attachment and trust, reward and motivation, as well as fear, anxiety and stress-related responses (Jurek and Neumann, 2018; Lee et al., 2009; Meyer-Lindenberg et al., 2011; Neumann and Slattery, 2016; Sobota et al., 2015; van den Burg and Hegoburu, 2020). OT by regulation of endocrine, physiological, and behavioural functions also promotes sedation and hypoactivity (Teng et al., 2013; Tunstall et al., 2019; Uvnäs-moberg, 1994; Uvnäs-Moberg et al., 1994). Over the past few decades, much effort has been put into understanding the complex behavioural effects of the OT neuropeptide (Keech et al., 2018; Meyer-Lindenberg et al., 2011; Song et al., 2016). However, the molecular mechanisms involved in mediating these functions, including neural targets and differences in OTR-mediated intracellular signalling pathways at various neuronal locations, are still not fully understood (Grinevich et al., 2016; Martinetz et al., 2019).

Interestingly, the complexity and diversity of OT-dependent functions are strongly associated with its modulatory effects on other neurotransmission systems, including the serotonin (5-HT) system amongst others (Jurek and Neumann, 2018). Growing evidence suggests an intriguing interaction between OT and 5-HT neurotransmitter signalling in the development of neural circuits and certain emotion-based behaviours (Eaton et al., 2012; Lefevre et al., 2018; Nagano et al., 2018). Administration of OT has been shown to increase the length and density of 5-HT axons in the amygdala and hypothalamus during development, demonstrating OT-mediated modulation of 5-HT innervation in early life in mice (Eaton et al., 2012). Several studies have also shown dysregulation of the OT system caused by elevated plasma 5-HT in the developmental hyperserotonemic model of Autism (Edwards et al., 2018; Madden and Zup, 2014; McNamara et al., 2008). In addition, coordinated OT and 5-HT activity in the nucleus accumbens of adult mice has been demonstrated to be crucial for the rewarding properties of social interactions (Dölen et al., 2013). The specific interaction between both neurotransmitter systems was also confirmed in nonhuman primates and in humans in the amygdala, insula, dorsal raphe nucleus, orbitofrontal cortex, and the hippocampus, key limbic regions implicated in the control of stress, mood, and social cognition (Lefevre et al., 2017; Mottolese et al., 2014).

The interaction of OT and 5-HT neurotransmitters is mediated by their specific receptors. Recent findings from our group demonstrated the formation of functional OTR/5-HTR$_{2A}$ heteroreceptor complexes *in vitro* in cells and *ex vivo* in the dorsal hippocampus and nucleus accumbens, indicating a potential role of OTR and 5-HTR heterocomplexes in the signalling crosstalk between their endogenous ligands, OT and 5-HT (Chruścicka et al., 2019). It has been suggested that the 5-HTR$_{2C}$ also participates in the specific OT/5-HT interaction. Similar to the 5-HTR$_{2A}$, the 5-HTR$_{2C}$ is well known to mediate OT secretion from the PVN of the hypothalamus (Jørgensen et al., 2003; Van de Kar et al., 2001; Zhang et al., 2002). Interestingly, the OT has also been shown to regulate 5-HT synthesis and release from 5-HT neurons in the midbrain raphe nuclei. This OT/5-HT interaction is driven by both OTR and 5-HTR$_{2A/2C}$ co-expressed in 5-HT neurons, leading to reduced anxiety-like behaviour in mice (Yoshida et al., 2009). The 5-HTR$_{2C}$ is the most widely expressed in the CNS among all serotonin receptors and mediates many central actions of 5-HT. Similar to the OTR, 5-HTR$_{2C}$-mediated signalling is particularly interesting in the regulation of mood, social affiliation, anxiety, aggression, food intake, and locomotor activity (Dekeyne et al., 2000; Nebuka et al., 2020; Palacios et al., 2017; Séjourné et al., 2015). The great complexity and diversity of OTR and 5-HTR$_{2C}$-mediated endocrine, physiological, and behavioural functions is regulated at many levels and includes the formation of homodimers, as well as functionally active heterooligomers, with other neurotransmitter receptors (Kamal et al., 2015; Maroteaux et al., 2019; Moutkine et al., 2017; Schellekens et al., 2015). Co-expression of the 5-HTR$_{2C}$ and OTR at a single cell level across multiple cortical and hippocampal regions was observed by analysis of the RNA-sequencing data in the Allen Brain Atlas (https://celltypes.brain-map.org/rnaseq/mouse_ctx-hip_smart-seq) (Fig. S1). Based on the overlapping distribution of 5-HTR$_{2C}$ and OTR in distinct brain regions at the cellular level together with their shared involvement in specific endocrine and behavioural outcomes, the formation of functional OTR/5-HTR$_{2C}$ heteroreceptor complexes must be considered when analysing the physiological or pathophysiological roles of OT and 5-HT signalling crosstalk in the brain (Dölen et al., 2013; Emiliano et al., 2007; Kohli et al., 2019; Yoshida et al., 2009).

In this study, we investigate the interaction between the OTR and 5-HTR$_{2C}$ in the context of their potential role in the specific crosstalk of OT and 5-HT neurotransmitters. We demonstrate colocalized expression using confocal microscopy and evaluate the possible formation of OTR/5-HTR$_{2C}$ heterocomplexes using a flow cytometry-based FRET (fcFRET) approach *in vitro* in a heterologous cell expression system. The formation of OTR/5-HTR$_{2C}$ heterocomplexes is further investigated *ex vivo*, in rat brain sections with the use of the Proximity Ligation Assay (PLA) with both receptors expressed at their endogenous levels. Functional cellular-based assays, including intracellular calcium mobilisation, IP-One (inositol monophosphate) accumulation, and receptors trafficking are used to demonstrate changes in Gαq-dependent signalling and trafficking of both receptors upon their co-expression in cells. The signalling crosstalk of both receptors is further confirmed *in vivo*, as observed by a significant potentiation of OT-induced hypolocomotor activity following coadministration of a 5-HTR$_{2C}$ antagonist (despite the hyperlocomotive effect of the antagonist alone). Together, these data provide compelling evidence for the formation of functionally active OTR/5-HTR$_{2C}$ heteroreceptor complexes adding another level of complexity to the 5-HTR$_{2C}$ and OTR signalling.

2. Materials and methods

2.1. Receptor ligands

Oxytocin (#O3251) and 5-hydroxytryptamine (#H9523) were purchased from Sigma-Aldrich (Wicklow, Ireland). Serotonin receptor antagonists, SB242084 (#2901) and RS102221, were purchased from Tocris Bioscience (Ellisville, MO) and Calbiochem (San Diego, CA), respectively. 3 mM stock of compounds were prepared in H_2O (Oxytocin, 5-HT, RS102221) or in DMSO (SB242084). Stock solutions were further diluted to the required concentrations in the proper assay buffer.

2.2. DNA constructs

DNA fragment encoding human unedited 5-HTR$_{2C}$–INI (NM_000868) C-terminally tagged with EGFP was purchased from Genecopeia (#H3309). Canonical sequence (transcript variant 1) of human OTR (NM_000916.3) C-terminally tagged with tGFP was supplied from OriGene (#RG211797, Rockville, MD). Both plasmids were used for stable transfection of Human embryonic kidney (HEK293A) cells. The coding sequences of 5-HTR$_{2C}$–INI and OTR were subcloned into the multicloning site of the HIV-based, replication deficient, lentiviral expression vector, pHR–SIN–BX-tRFP. Constructs containing 5-HTR$_{2C}$ sequence C-terminally tagged with tRFP or EGFP (pHR–SIN–BX-5-HTR$_{2C}$-tRFP and pHR–SIN–BX-5-HTR$_{2C}$-EGFP) were generated in house and described previously (Schellekens et al., 2013). Construct containing OTR sequence C-terminally tagged with tRFP (pHR–SIN–BX-OTR-tRFP) was generated by inserting the receptor coding sequence lacking its stop codon into the target vector with the use of *Bam*HI and *Xho*I restriction enzymes (Chruścicka et al., 2019). Lentiviral expression vector with the sequence of OTR C-terminally tagged with tGFP (pHR–SIN–B-X-OTR-tGFP) was obtained from pHR–SIN–BX-tRFP plasmid by replacing tRFP sequence with the sequence of OTR C-terminally tagged with tGFP using designed primers containing *Bam*HI and *Sda*I (*Sbf*I) sites

(5'_GTCGACTGGATCCGGTACCGAGG_3′ 5'_CGCCTACCTGCAGGCCG TTTAAACTCTTTCTTC_3'). All generated constructs were validated by restriction analysis followed by DNA sequencing.

2.3. Cell culture and stable transfection

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 Foetal Bovine Serum (FBS, #F7524, Sigma-Aldrich), 1% Non-Essential Amino Acids (NEAA, #11140035, Gibco Life Technologies, Gaithersburg, MD), and maintained at 37 °C in a humidified atmosphere with 5% CO_2. For stable transfection, HEK293A cells were transfected with the plasmid containing human OTR sequence fused with tGFP in the presence of Lipofectamine LTX Plus reagent according to the manufacturer's instructions (#15338100, Invitrogen). After 48 h, the cells were grown in DMEM supplemented with 500 ng/μl G-418 (#345812, Calbiochem), allowing for the selection of cells with stably integrated pCMV-OTR-tGFP plasmid. The cells with the highest CMV promoter-mediated expression of the receptor gene tagged with tGFP were selected using flow-assisted cell sorting (FACSAriaII, BD Biosciences) followed by clonal expansion. The cell line with the stable expression of 5-HTR_{2C}-EGFP was established in house and described previously (Schellekens et al, 2013, 2015). Expression level of the OTR and 5-HTR_{2C} in generated cell lines was routinely monitored and quantified using an epifluorescence microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences).

2.4. Lentiviral transfection and transduction

HEK293A cells stably expressing receptor C-terminally tagged with GFP were transduced with generated lentiviral expression plasmids to co-express receptor C-terminally tagged with red fluorescent protein (tRFP). Transduction was performed using a second generation packaging, gene delivery, and viral vector production system, previously described by our group (Schellekens et al., 2013). HIV-based lentiviral particles containing the OTR or 5-HTR_{2C} sequence were produced using HEK293T-17 cells, by transient co-transfection of the expression construct; pHR–SIN–BX-OTR-tRFP or pHR–SIN–BX-5-HTR_{2C}-tRFP, the packaging construct; pCMV ΔR8.91, and the envelope construct; pMD. G-VSV-G. Next, HEK293A cells were transiently transduced with the OTR or the 5-HTR_{2C} expressing lentiviral vector diluted in transduction media, consisting of DMEM with 2% heat-inactivated FBS, 1% NEAA and an additional 8 μg/ml polybrene (#H9268, Sigma). The transduction efficiency was monitored with the use of an epifluorescence microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences) before each experiment.

2.5. Flow cytometry fluorescence resonance energy transfer (fcFRET)

HEK293A cells stably expressing 5-HTR_{2C}-EGFP were transiently transduced with lentiviral OTR-tRFP. Following transduction, cells were washed twice and mechanically removed from the wells using Phosphate Buffer Saline (PBS). Cell suspension was then centrifuged for 4 min at 200×*g*, at room temperature. The cell pellet was resuspended in 400 μl of 2 nM EDTA (#E5134; Sigma) in PBS. Prior to analysis, cells were passed through a 100 μm nylon mesh cell strainer (#10199–658; VWR) and collected in a 5 ml round bottom polystyrene tube (#352054; Corning). FcFRET analysis was performed on a FACSAriaII cytometer (BD Biosciences) according to the protocol published by our group (Chruścicka et al., 2018). Briefly, GFP was excited at 488 nm from blue laser and detected with a 525/50 nm bandpass filter, whereas tRFP was excited at 561 nm from yellow/green laser and detected with a 610/20 nm bandpass filter. The fcFRET signal between GFP and tRFP was measured by excitation at 488 nm from blue laser and detection with a 610/20 nm bandpass filter located on the same laser. For the proper separation of GFP fluorescence and fcFRET emission from blue laser, a 505 Long Pass (LP) dichroic mirror (DM) was used. Parent HEK293A cells were used for initial instrument setup and to differentiate cells based on their size and granulation, according to the forward and side scatter plot (FSC/SSC). In the next step, cells expressing donor or acceptor construct were used to fine tune PMT settings and to perform the proper compensation for spectral bleed through and cross-excitation. The same number of cell (10^4) was recorded for each sample. Data were analysed using BD FACSDiva (BD Biosciences).

2.6. Colocalisation with the use of confocal microscope

HEK293A stably expressing 5-HTR_{2C}-EGFP were transiently transduced with lentiviral OTR-tRFP. Following transduction, cells were seeded on poly-L-lysine-coated (#P4707; Sigma) borosilicate glass slides (#631–0150; VWR International) at a density of 5×10^5 cells per well of a 24-well plate, followed by 24 h incubation in standard culture conditions. Colocalisation of the receptors was assessed in living cells using a laser scanning confocal fluorescent microscope (FV 1000 Confocal System; Olympus). Fluorescent images were acquired with a 63× objective lens (Plan-Apochromat, 1.4 Oil DIC) using Olympus fluoview FV3000 software. Colocalisation between the 5-HTR_{2C}-EGFP and OTR-tRFP was analysed by overlay with the use of ImageJ software (U.S. National Institutes of Health).

2.7. In situ proximity ligation assay (in situ PLA)

Experiments were performed using male Sprague-Dawley rats (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. Studies were approved by the Stockholm North Committee on Ethics of Animal Experimentation, in accordance with the Swedish National Board for Laboratory Animal and European Communities Council Directive (Cons 123/2006/3) guidelines for accommodation and care of Laboratory Animals.

To study OTR/5-HTR_{2C} heteroreceptor complexes, the *in situ* proximity ligation assay (*in situ* PLA) was performed as described previously (Borroto-Escuela et al., 2018; Chruścicka et al., 2019). Adult age-matched male Sprague–Dawley rats (n = 5) were anaesthetised and perfused intracardially with 4% (wt/vol) formalin in PBS. Brains were removed and post-fixed by immersion overnight in 4% formalin in PBS. Coronal sections (30 μm) were cut on a cryostat and processed for free-floating *in situ* PLA. Free-floating formalin fixed brain sections (storage at −20 °C in Hoffman solution) at Bregma level (−3.6 mm and 1.2 mm) were washed four times with PBS and quenched with 10 mM glycine buffer for 20 min at room temperature. After three washes in PBS, slices were permeabilised with a permeabilisation buffer (10% FBS and 0.5% Triton X-100 or Tween 20 in Tris buffer saline (TBS), pH 7.4) for 30 min at room temperature. Again, the sections were washed twice, 5 min each, with PBS at room temperature and incubated with the blocking buffer (0.2% BSA in PBS) for 30 min at room temperature. The brain sections were then incubated with the primary antibodies diluted in a suitable concentration in the blocking solution for 1–2 h at 37 °C or at 4 °C overnight. The day after, the sections were washed twice, and the proximity probe mixture (Duolink PLA probe anti-mouse MINUS and Duolink PLA probe anti-rabbit PLUS, Sigma-Aldrich, Stockholm, Sweden) was applied to the sample and incubated for 1 h at 37 °C in a humidity chamber. The unbound proximity probes were removed by washing the slices twice, 5 min each time, with blocking solution at room temperature under gentle agitation. The sections were then incubated in the hybridisation-ligation solution (BSA, 250 g/ml), T4 DNA ligase (final concentration of 0.05 U/μl), 0.05% Tween-20, 250 mM NaCl, 1 mM ATP and the circularisation or connector oligonucleotides (125–250 nM) in a humidity chamber at 37 °C for 30 min. Excess connector oligonucleotides were removed by washing twice, for 5 min

each, with the washing buffer A (Sigma-Aldrich, Duolink Buffer A [8.8 g NaCl, 1.2 g Tris Base, 0.5 ml Tween 20 dissolved in 800 ml high purity water, pH to 7.4]) at room temperature under gentle agitation. The rolling circle amplification mixture was added to the slices and incubated in a humidity chamber at 37 °C for 100 min. Next, the sections were incubated with the detection solution in a humidity chamber at 37 °C for 30 min. In a last step, the sections were washed twice in the dark, for 10 min each, with washing buffer B (Sigma-Aldrich, Duolink Buffer B [5.84 g NaCl, 4.24 g Tris Base, 26.0 g Tris-HCl, dissolved in 500 ml high purity water, pH 7.5]) at room temperature under gentle agitation. The free-floating sections were mounted on a microscope slide and a drop of appropriate mounting medium (e.g., Duolink Mounting Medium, Sigma-Aldrich) was applied. The cover slip was placed on the section and sealed with nail polish. The sections were protected against light and stored for several days at −20 °C before confocal microscope analysis. The *in situ* PLA experiments were performed using the following primary antibodies: rabbit polyclonal anti-5-HTR_{2C} (SAB4501477, 1 μg/ml; Sigma-Aldrich, Stockholm, Sweden) and goat polyclonal anti-OTR (ab87312, 5 μg/ml; Abcam, Stockholm, Sweden). As a neuronal marker the Neuro-ChromTM Pan neuronal marker antibody-Alexa488 conjugated (ABN2300A4, Merck/Sigma-Aldrich) was used. The PLA signal was visualised and quantified using a Leica TCS-SL confocal microscope (Leica, USA) and Duolink Image Tool software. A range of positive and negative controls have been used to guarantee the specificity of the PLA signal. The negative control consists in the suppression of the species-specific primary antibody corresponding to the 5-HTR_{2C} in the presence of the two PLA probes. As a positive control of the PLA assay, a parallel analysis of the Dopamine Receptor 2 – Oxytocin Receptor (D_2R-OTR) heteroreceptor complexes was performed. Detailed quality control analysis for the OTR antibodies have been reported previously (Borroto-Escuela et al., 2017; Romero-Fernandez et al., 2012). Furthermore, both anti-5-HTR_{2C} and anti-OTR antibodies were previously validated in our team in terms of their quality (in Western blot in collaboration with Human Atlas project and in HEK293 cells with and without expression of each receptor subtype using confocal analysis). Antibodies were used under optimal conditions, taking into consideration parameters such as concentration, targeted epitopes, fixation conditions, and antigen-retrieval (Borroto-Escuela et al., 2018).

2.8. Intracellular calcium mobilisation assay

Receptor-mediated changes in intracellular calcium (Ca^{2+}) were monitored with the use of an automatic fluorescent reader, FliprTetra (Molecular Devices, LLC Sunnyvale, CA). HEK293A cells expressing OTR, 5-HTR_{2C}, and cells co-expressing both receptors were seeded in black 96-well microtiter plates at a density of 3.0–4.0 × 10^4 cells/well and incubated overnight in standard culture conditions. 24 h before the experiment, growth media was replaced with serum-free DMEM containing 1% NEAA. On the day of the experiment, cells were incubated for 90 min with 80 μl of Ca5 dye diluted in assay buffer (1 x Hank's Balanced Salt Solution – HBSS #14025092; Gibco, containing 20 mM HEPES #15630080; Gibco) according to the manufacturer's protocol (#R8186; Molecular Devices). Fluorescent readings were taken for 120 s with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The addition of receptor ligands (40 μl/well) was performed using the automatic pipettor of the FLIPR Tetra High-Throughput Cellular Screening System from Molecular Devices. To investigate the effect of receptor antagonists, ligands were pre-incubated for 90 min with the Ca5 dye. The relative increase in intracellular calcium [Ca^{2+}] was calculated as the difference between the maximum and baseline fluorescence and depicted as percentage Relative Fluorescent Units (RFU) normalised to maximum response (100% signal) obtained with 3% FBS. Background fluorescence was recorded for non-stimulated cells and subtracted from RFU.

2.9. HTRF based IP_1 accumulation assay

The detection of IP_1 (inositol monophosphate) in HEK293A cells expressing OTR, 5-HTR_{2C}, and cells co-expressing both receptors was performed with the use of a homogeneous time-resolved fluorescence (HTRF) IP_1 assay, according to the manufacturer's instructions with minor modifications (#62IPAPEB; Cisbio Codolet, France). HEK293A cells were grown in standard culture conditions. 24 h before the 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×*g*. The cell pellet was then suspended in assay buffer (146 mM NaCl, 1 mM $CaCl_2$, 10 mM 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 μL of cell suspension was pipetted at the density of 3 × 10^5 per well to a flat bottom 96-well plate (#655075; Greiner Bio-One International) containing the appropriate concentration of compounds and incubated for 30 min at 37 °C. Following this step, 15 μL of IP1-d2 conjugate and 15 μL of anti-IP1 cryptate conjugate in lysis buffer were added. After 1 h of incubation at room temperature, the fluorescence at 620 nm and 665 nm was read on a FlexStation (Molecular Devices, LLC Sunnyvale, CA) using the readout setup recommended by the company. The results were calculated as the 665-nm/620-nm ratio multiplied by 10^4 and depicted as the percentage of RFU normalised to the maximum response (100% signal) obtained for non-stimulated OTR-expressing cells. Since the specific signal is inversely proportional to the concentration of endogenous IP_1 in the sample, fluorescence values were converted to demonstrate the directly proportional dependence of the compound concentration to the level of endogenous IP_1 in the sample.

2.10. Receptor trafficking assay

Receptor trafficking was analysed by monitoring fluorescent protein translocation away from the cellular membrane into vesicles within the cytosol. HEK293A cells stably expressing 5-HTR_{2C}-EGFP and transduced with lentiviral OTR-tRFP were seeded on 24-well plates at a density of 5 × 10^4 cells/well and incubated for 48 h in standard culture conditions. 24 h before the experiment, media was replaced with serum-free DMEM containing 1% NEAA. To investigate ligand-mediated changes in receptor trafficking, cells were incubated with different concentrations of 5-HTR_{2C} or OTR ligands for 5 min at 37 °C. To investigate the effect of 5-HTR_{2C} antagonism on ligand-mediated receptor trafficking, cells were pre-incubated for 60 min with the antagonist before addition of receptor agonists. After treatment, cells were fixed with 4% paraformaldehyde in PBS for 20 min and washed two times in PBS. Receptor trafficking was assessed using an inverted fluorescence microscope (IX71; Olympus). Fluorescent images were acquired with a 20X objective lens using Olympus cell R software. Results represent an average from three independent experiments each performed in duplicate (two wells for each condition in each experiment). Within each well, three images were captured. For each image, six cells were quantitatively analysed. Quantification of receptor trafficking was assessed by calculating the ratio between subcellular and membrane fluorescent intensity after excluding background fluorescence, with the use of a Java image processing program (ImageJ, US National Institutes of Health).

2.11. Behavioural tests

All *in vivo* experiments were performed using 12 to 13-week-old male NIH Swiss mice (Envigo, UK). Mice were group-housed 3 per cage in an environment controlled for light-dark cycle (12-h light; lights on at 7:00 a.m.), temperature (21 ± 1 °C), and humidity (55 ± 10%). Water and standard lab chow (2018S Teklad Global 18% Protein Rodent Diet, Envigo, Huntingdon, UK) were available ad libitum. All experiments were performed in accordance with the European Community Council directive (86/609/EEC) and approved by the Animal Experimentation

Ethics Committee of University College Cork (B100/3774). Behavioural testing occurred during the light phase, between 09:30 and 12:00. Animals were habituated to the experimental room (low light levels of 30 lux) for 1 h prior to the first injection.

The selective 5-HTR$_{2C}$ antagonist, SB242084 (SB), or vehicle (DMSO) was administered by intraperitoneal (i.p.) injection 15 min prior to test. Dose of SB (0.3 mg/kg in 1% DMSO) was chosen based on the literature and our own pilot experiments (Graf et al., 2003; Séjourné et al., 2015). OT or vehicle (saline) was administered by i. p. injection immediately before the test. Dose of OT (0.3 mg/kg in saline) was chosen based on our own prior pilot studies (data not shown). Sub-threshold doses of both SB and OT were selected to see a synergistic effect following co-administration of both ligands.

Following the second injection, animals were immediately placed in a regular home cage (20 × 37.4 cm) with opaque walls, limited bedding, and no play material. A transparent Perspex lid was used to allow recording of behaviour by a video camera positioned above the cages. Behaviour was analysed for 30 min. Locomotor activity (distance travelled and stationary time) was assessed using EthoVision XT 11.5 (Noldus). For stationary time, the "not moving" threshold was set at 1.75 cm/s. One animal in the SB-Saline group exhibited high levels of repetitive circling behaviour during the test and was excluded as an outlier (distance travelled: 4.49 SD from group mean, 5.71 SD from overall mean), although the results were the same with this outlier included.

2.12. Statistical analysis

All *in vitro* data were analysed using GraphPad Prism software (Prism 8; GraphPad Software Inc., San Diego, CA). The concentration-response curves of receptor ligands were generated using nonlinear regression. The curves were fitted to a three-parametric logistic equation, allowing for the determination of EC_{50} values.

Statistical comparison of the concentration−response curve parameters between cells co-expressing both receptors and cells solely expressing the corresponding receptor were performed using Student's t-test. Statistical comparisons of each compound concentration (each treatment) between cells expressing OTR, 5-HTR$_{2C}$ and cells co-expressing both receptors in all cell-based assays were performed using two-way analysis of variance ANOVA with follow-up Tukey's multiple comparison test. Statistical analysis of fcFRET signal was performed using Student's t-test.

Statistical analysis of *in vivo* data was performed using IBM SPSS Statistics Subscription. A repeated-measures ANOVA was used to evaluate potential time-dependent effects of drug administration, with follow-up one-way ANOVA and t-tests to investigate significant interactions. Where Mauchly's Test of Sphericity was violated, the Greenhouse-Geisser correction was applied. Where Levene's Test for Equality of Variances was violated, corrected values are reported.

All data are presented as mean ± SEM. The differences between groups were considered significant for $p < 0.05$, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. The number of independent experiments performed is provided in figure legends.

3. Results

3.1. Evidence for the formation of OTR/5-HTR$_{2C}$ heteroreceptor complexes in vitro

The interaction between the OTR and 5-HTR$_{2C}$ was assessed in human embryonic kidney (HEK293A) cells using flow cytometry-based FRET (fcFRET) (Fig. 1). FcFRET is a non-invasive, sensitive, and quantitative method that has been successfully used by our group and others to assess interactions between GPCRs (Chruścicka et al, 2018, 2019). Analysis of the fcFRET signal was performed on the gated population of single, live, and successfully transduced cells (Fig. 1). Two-dimensional dot-plots of GFP fluorescent signal against RFP fluorescent signal showed an equal and high transduction efficiency between HEK293A cells solely expressing OTR-tRFP, cells co-expressing 5-HTR$_{2C}$-EGFP with control construct (control-tRFP), and cells co-expressing 5-HTR$_{2C}$-EGFP with OTR-tRFP (Fig. 1D, see 3rd, 4th, and 5th panel from top). In addition, no significant differences were found between the percentage of GFP/RFP double fluorescence in 5-HTR$_{2C}$-EGFP cells co-expressing control-tRFP or OTR-tRFP (Fig. 1D, see 4th and 5th panel from top). Average values of GFP/RFP expression in 5-HTR$_{2C}$-EGFP cells across all experiments were 61 ± 6.9% and 74 ± 9.3% for control-tRFP and OTR-tRFP, respectively (Fig. 1A). Subsequent analysis of the fcFRET signal between the OTR and 5-HTR$_{2C}$ is presented as a two-dimensional dot-plot of the fcFRET signal against GFP fluorescence depicting the percentage of fcFRET positive cells (Fig. 1E), and as a histogram depicting the fcFRET median fluorescence (Fig. 1F). The average fcFRET signal between 5-HTR$_{2C}$-EGFP and OTR-tRFP (18.2 ± 2.9%) highlighted the constitutive and specific association between both receptors (Fig. 1B). The interaction was confirmed to be specific, as only weak and non-significant fcFRET signal (2.5 ± 0.2%) was observed in cells co-expressing 5-HTR$_{2C}$-EGFP with control-tRFP (Fig. 1B). The average fcFRET signal analysed as median fluorescence was also significantly higher in cells co-expressing both receptors (94.5 ± 20.5) compared to cells with the expression of donor construct 5-HTR$_{2C}$-EGFP (5.3 ± 5.3), acceptor construct OTR-tRFP (−17.5 ± 15.9), and cells co-expressing 5-HTR$_{2C}$-EGFP with the control-tRFP construct (28.3 ± 14.3) (Fig. 1C). Moreover, we have previously confirmed that the fcFRET signal detected between receptors under investigation is not due to an overexpression, random collision, or dimerisation of fluorescent proteins (Chruścicka et al, 2018, 2019; Schellekens et al., 2015). Together, the results obtained indicate a physical interaction between 5-HTR$_{2C}$ and OTR when co-expressed in HEK293A cells.

Subcellular localisation of the receptors was investigated using confocal microscopy in intact, living HEK293A cells co-expressing OTR and 5-HTR$_{2C}$. Both receptors were mainly found within the cell membrane, which was shown by the green fluorescence signal coming from 5-HTR$_{2C}$ fused with EGFP and the red fluorescence signal coming from OTR fused with tRFP (Fig. 2). In agreement with relatively high constitutive activity and internalisation of the OTR and 5-HTR$_{2C}$, both receptors are also found in the intracellular space (Berrada et al., 2000; Di Benedetto et al., 2014; Kinsey et al., 2007). Overlap between green and red fluorescence is indicated as yellow signal (Fig. 2, merged picture) indicating colocalized expression of both receptors on the cell membrane and within the cytoplasm of HEK293A cells. The apparent colocalisation of the OTR/5-HTR$_{2C}$ pair may indicate that the formation of OTR/5-HTR$_{2C}$ heteroreceptor complexes demonstrated by fcFRET may occur both on the plasma membrane as well as intracellularly.

3.2. Formation of OTR/5-HTR$_{2C}$ heteroreceptor complexes ex vivo, in rat brain regions

Our previous work demonstrated that the OTR/5-HTR$_{2A}$ heteroreceptor complexes are present in limbic regions, including the dorsal hippocampus, the cingulate and the ventral striatum of rat brain (Chruścicka et al., 2019). These regions are linked to social memory and recognition leading to social behaviour development (Mitre et al., 2016; Tirko et al., 2018) as well as social discrimination (Raam et al., 2017). Interestingly, in the current study, OTR/5-HTR$_{2C}$ heteroreceptor complexes were also found in the dorsal hippocampus. The positive PLA signal was observed in the pyramidal cell layer of the CA1, CA2, and CA3 regions (Fig. 3). The highest density of PLA clusters (red blobs) can be seen in the CA3 region of the hippocampus (Fig. 3A). The OTR/5-HTR$_{2C}$ complexes were mainly located in the pyramidal neurons building up this cell layer and operating through release of glutamate. A positive PLA signal was also identified in GABA interneurons scattered in the striatum radiatum and oriens of CA1 to CA3 regions (Fig. 3A, B and Fig. S2A). In the polymorphic layer of the dentate gyrus and in the

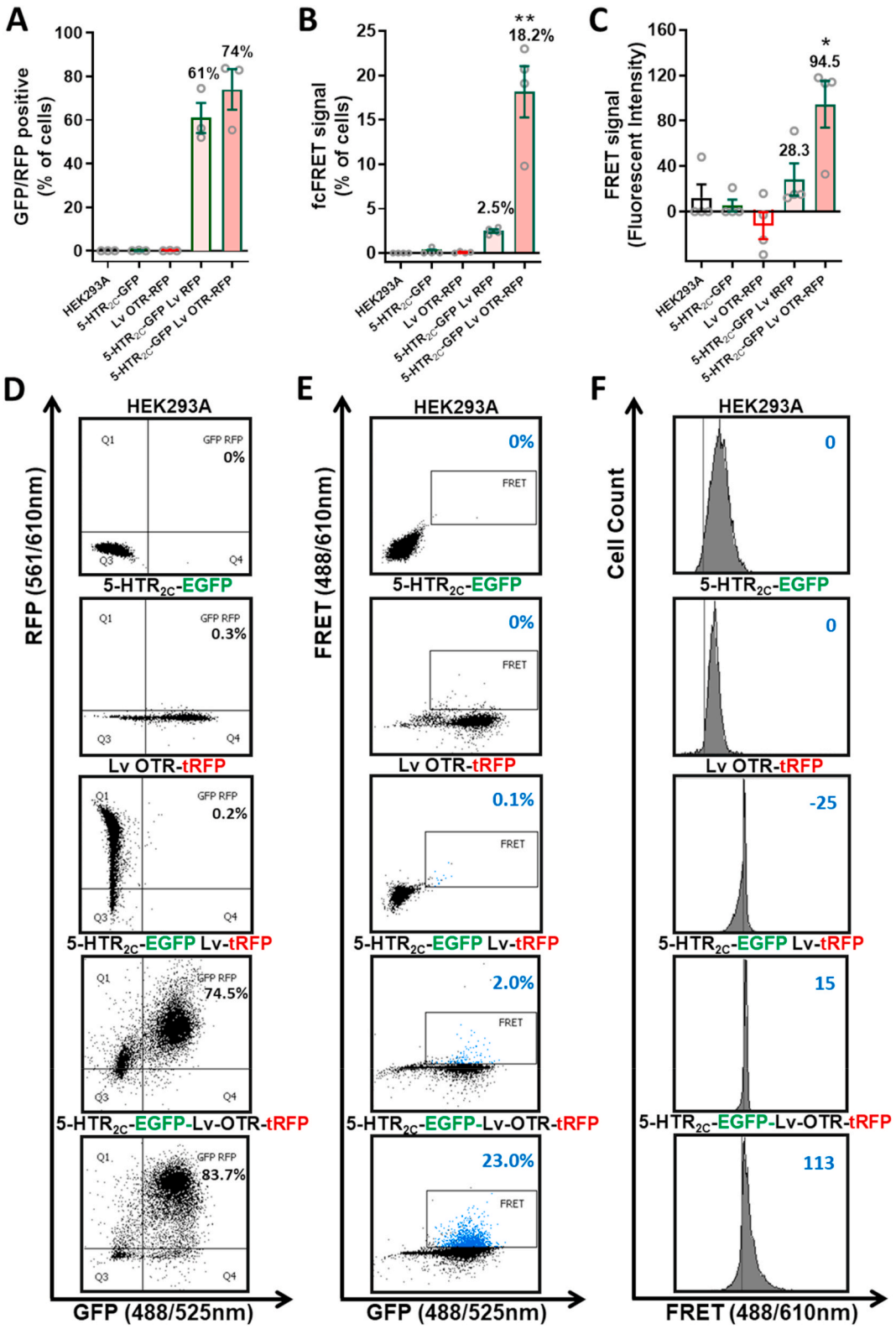


Fig. 1. Positive fcFRET signal between the 5-HTR$_{2C}$ and OTR. Wild-type HEK293A cells and cells stably expressing 5-HTR$_{2C}$-EGFP were transiently transduced with Lentiviral (Lv) vectors Lv-tRFP (control) or Lv-OTR-tRFP **(A,D)**. FcFRET signal is presented as a percentage of cells **(B,E)** and as median fluorescence **(C,F)** in wild-type HEK293A cells, cells expressing 5-HTR$_{2C}$-EGFP (donor), cells transiently transduced with Lv-OTR-tRFP (acceptor), cells co-expressing 5HTR$_{2C}$-EGFP and Lv-tRFP, and cells co-expressing 5-HTR$_{2C}$-EGFP and Lv-OTR-tRFP. Graphs represent mean ± SEM from four independent experiments. Statistical significance compared to cells expressing a donor with the control acceptor construct denoted by * for $p < 0.05$ and ** for $p < 0.01$. Representative dot plots show percentages of cells with EGFP and tRFP expression **(D)** and percentage of cells with fcFRET signal (fcFRET % depicted in blue) **(E)**. Representative histograms show median fluorescence of fcFRET signal (fluorescence values depicted in blue) **(F)**.

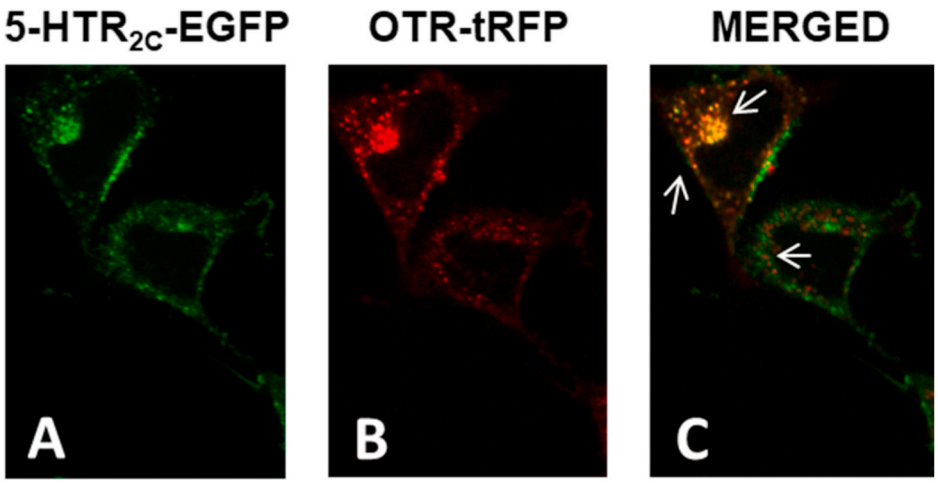


Fig. 2. Colocalisation between the 5-HTR$_{2C}$ and OTR. HEK293A cells stably expressing 5-HTR$_{2C}$-EGFP (green) (**A**) were transiently transduced with Lv-OTR-tRFP (red) (**B**). Merged picture (yellow) shows colocalisation of the two receptors within the cell in a confocal plane (**C**).

retrosplenial granular and agranular cortex moderate density of the OTR/5-HTR$_{2C}$ heteroreceptor complexes was observed (Fig. S2 B and C). The expression patterns of the OTR and 5-HTR$_{2C}$ in the dorsal hippocampus (García-Alcocer et al., 2006; Grinevich et al., 2016; Mitre et al., 2016; Yoshida et al., 2009) are compatible with the distribution of the OTR/5-HTR$_{2C}$ heteroreceptor complexes observed in the current study. The OTR is mainly restricted to the pyramidal neurons of the CA2 and CA3 regions of the hippocampus (Yoshida et al., 2009). The highest density of the OTR/5-HTR$_{2C}$ heteroreceptor complexes was in fact found in the CA3 pyramidal neurons (Fig. 3A).

Noteworthy, the PLA results are in agreement with the OTR and 5-HTR$_{2C}$ co-expression observed in mouse whole cortex and hippocampus (Fig. S1) and in human cortex (Fig. S1) following *in silico* analysis of the single cell RNA-sequencing data available via the transcriptomic explorer in the Allen Brain Atlas (https://celltypes.brain-map.org/rnaseq/mouse_ctx-hip_smart-seq). Co-expression of the 5-HTR$_{2C}$/OTR pair was observed across multiple cortical and hippocampal neurons at a single cell level in mouse as well as in human brain and reinforces the potential of these receptors to form heterocomplexes.

Taking together the formation of OTR/5-HTR$_{2C}$ heterocomplexes has been identified *ex vivo* in rat brain sections under endogenous expression level of both receptor protomers. More importantly, a specific distribution pattern of OTR/5-HTR$_{2C}$ heteroreceptor complexes indicates their potential functional outputs in the central nervous system.

3.3. Pharmacological assessment of signalling consequences of the OTR/5-HTR$_{2C}$ heteroreceptor complexes formation in vitro

Downstream signalling consequences following co-expression of the OTR and 5-HTR$_{2C}$ were investigated. The OTR and 5-HTR$_{2C}$ are known to mainly signal through the Gαq-mediated pathway, where activation of the Gαq protein leads to generation of the second messenger, D-myoinositol 1,4,5-triphosphate (IP3). Next, the second messenger, IP3 causes calcium release from the endoplasmic reticulum into the cytoplasm through the activation of endoplasmic-gated calcium channels (Berg et al., 1998; Berrada et al., 2000; Gimpl and Fahrenholz, 2001). Therefore, ligand-mediated changes in intracellular calcium mobilisation in HEK293A cells solely expressing the OTR or 5-HTR$_{2C}$ and cells co-expressing both receptors were assessed (Fig. 4).

As expected, a significant intracellular calcium release was detected following the addition of endogenous receptor ligands, OT and 5-HT (Fig. 4A and B). The potency of OT (EC_{50} = 0.22 ± 0.06 nM) in cells solely expressing OTR-tRFP (HEK293A Lv OTR-tRFP) and potency of 5-HT (EC_{50} = 0.24 ± 0.09 nM) in cells solely expressing 5-HTR$_{2C}$-EGFP (HEK293A-5-HTR$_{2C}$-EGFP) confirmed the functionality of receptors expressed in the heterologous expression system in coherence with the literature (Albizu et al., 2007; Bonhaus et al., 1995). Interestingly, the intracellular calcium release following an increasing concentration of OT was almost completely depleted (EC_{50} = NC) in cells co-expressing 5-HTR$_{2C}$ and OTR (HEK293A-5-HTR$_{2C}$-EGFP Lv OTR-tRFP) compared to cells solely expressing OTR (Fig. 4A). In addition, the intracellular calcium release following increasing concentrations of 5-HT was significantly reduced in cells co-expressing both receptors compared to cells expressing solely 5-HTR$_{2C}$ (Fig. 4B). The concentration-response curve of 5-HT was characterised by a lower potency (EC_{50} = 1.7 ± 0.9 nM) in cells co-expressing both receptors compared to cells solely expressing 5-HTR$_{2C}$ with no change in efficacy.

Similar results were obtained for cells co-expressing 5-HTR$_{2C}$ and OTR with reversed fluorescent tags (Fig. 4C and D), indicating this effect is independent of fluorescent proteins. Specifically, the intracellular calcium release following increasing concentration of OT was depleted in cells co-expressing OTR tagged with EGFP and 5-HTR$_{2C}$ tagged with tRFP (HEK293A-OTR-EGFP Lv 5-HTR$_{2C}$-tRFP) compared to cells solely expressing OTR-EGFP (EC_{50} = 0.23 ± 0.05 nM) (Fig. 4C). The potency of 5-HT to induce release of calcium was also significantly decreased in cells co-expressing OTR tagged with EGFP and 5-HTR$_{2C}$ tagged with tRFP (EC_{50} = 4.7 ± 2.7 nM) compared to cells solely expressing 5-HTR$_{2C}$-tRFP (EC_{50} = 0.6 ± 0.1 nM) (Fig. 4D). These results clearly show no effect of fluorescent protein tags (EGFP versus tRFP) or gene delivery mode (stable transfection versus transient lentiviral transduction) on observed changes in Gαq-dependent downstream signalling. It's well known that edited 5-HTR$_{2C}$ variants are characterised by significantly lower constitutive signalling and decreased level of Gq protein coupling (Herrick-Davis et al., 1999; Niswender et al., 1999; Price and Sanders-Bush, 2000). We therefore preformed additional experiments using cells co-expressing the OTR with the edited form of 5-HTR$_{2C}$ (5-HTR$_{2C}$-VSV). We observed a similar depletion of intracellular calcium release following increasing concentrations of OT in cells co-expressing both receptors (EC50 = NS) compared to cells solely expressing the OTR (EC50 = 0,2 nM). These experiments demonstrate that attenuation of OTR-mediated Gαq signalling is not dependent on high basal activity of the 5-HTR$_{2C}$ and sequestration of Gq proteins in cells (Fig. S4).

Furthermore, for each experiment performed, the level of OTR and 5-HTR$_{2C}$ expression in HEK293A cells was assessed. Flow cytometry analysis of EGFP and tRFP showed no changes in the level of receptor expression in cells solely expressing the respective receptor compared to cells co-expressing both receptors (Fig. S3). Thus, the observed effects are not caused by changes in receptor expression levels but appear to be driven by the specific interaction of both receptors co-expressed in a heterologous expression system.

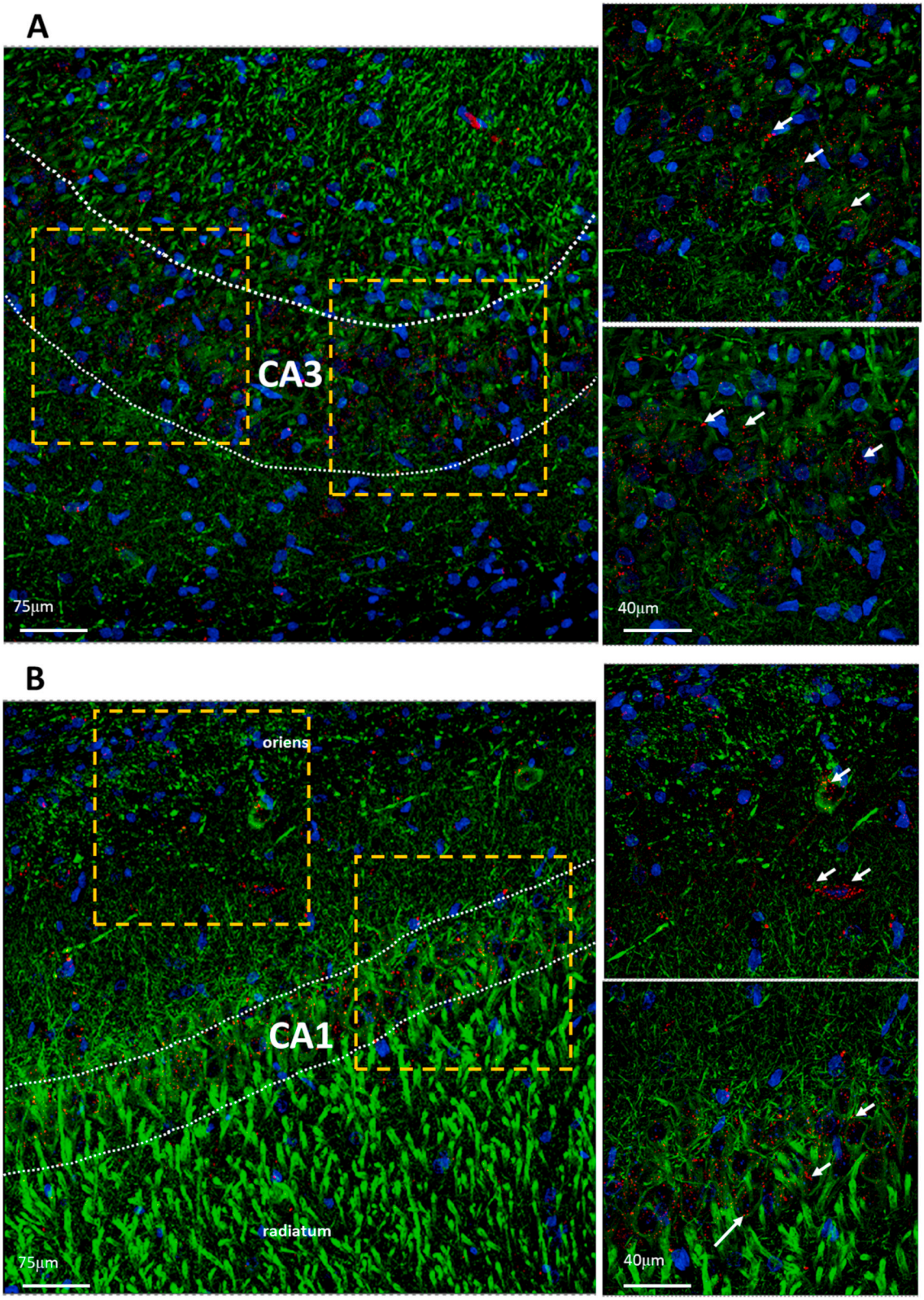


Fig. 3. PLA signal between the 5-HTR$_{2C}$ and OTR in the dorsal hippocampus of rat brain. Photomicrographs from transverse sections of the rat dorsal hippocampus show the distribution of OTR/5-HTR$_{2C}$ heteroreceptor complexes in the pyramidal cell layer as well as in GABA interneurons of CA3 (**A**) and CA1 (**B**) regions of hippocampus using the in-situ proximity ligation assay (PLA) technique and confocal laser microscopy. The positive PLA signal is demonstrated as red blobs (clusters, with examples indicated by white arrows), nuclei are shown in blue (DAPI), and neuronal cells in green (Alexa488 conjugated Neuro-Chrom Pan, neuronal marker).

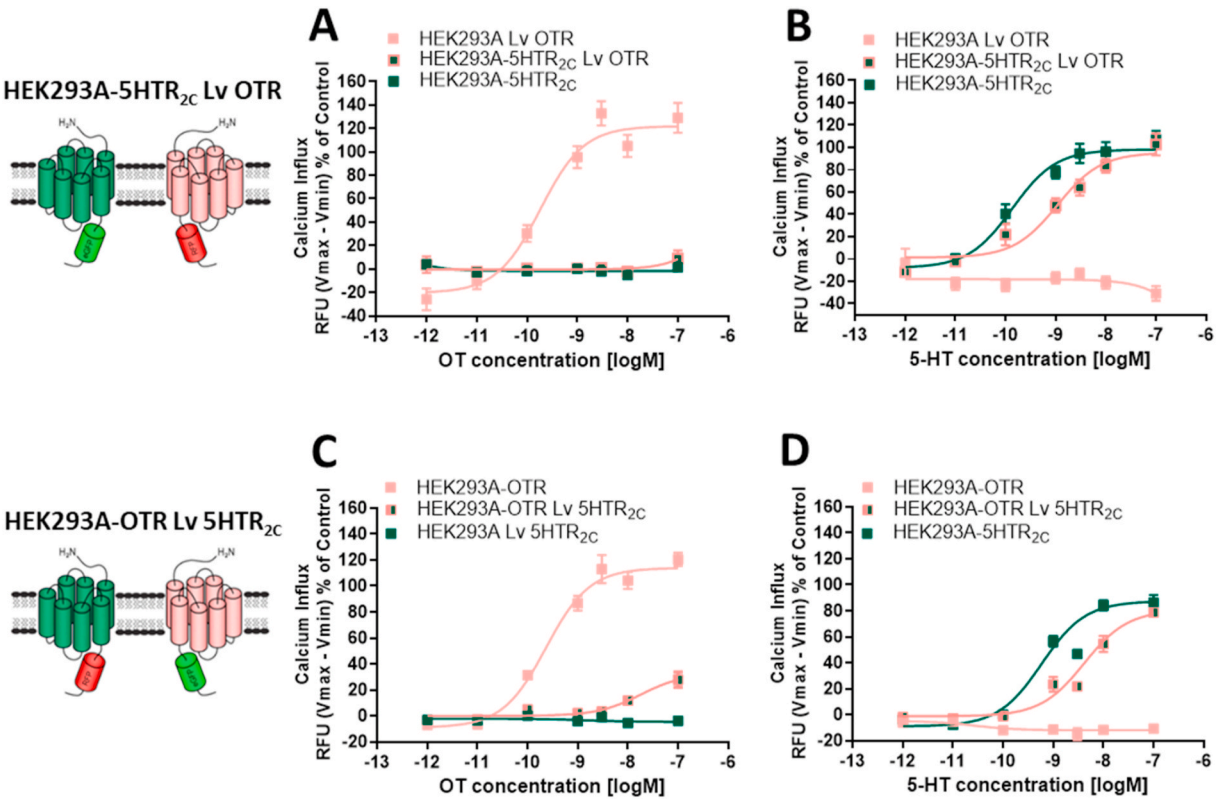


Fig. 4. Co-expression of the OTR and 5-HTR$_{2C}$ depletes OTR-mediated Gαq-dependent signalling. A, B - Intracellular calcium release induced by increasing concentration of OT (**A**) and 5-HT (**B**) in HEK293A cells stably expressing the 5-HTR$_{2C}$ tagged with EGFP, in cells transiently expressing the OTR tagged with tRFP, and in cells co-expressing both receptors. **C, D** - Intracellular calcium release induced by increasing concentration of OT (**C**) and 5-HT (**D**) in HEK293A cells stably expressing the OTR tagged with tGFP, in cells transiently expressing the 5-HTR$_{2C}$ tagged with tRFP, and in cells co-expressing both receptors. Intracellular calcium mobilisation is presented as a percentage of maximal calcium response elicited by the control (3% FBS). Graphs represent means ± SEM from six (A, B) or three (C, D) independent experiments run in duplicates.

Further experiments were conducted to investigate whether attenuation of OTR-mediated signalling depends on 5-HTR$_{2C}$ activation in cells co-expressing the 5-HTR$_{2C}$ and OTR (Fig. 5). For this purpose, we measured the OT-induced calcium response after 5-HTR$_{2C}$ blockade with its selective antagonists. As expected, pre-treatment with the 5-HTR$_{2C}$ selective antagonists, RS102221 (RS) and SB242084 (SB), at a 1 μM concentration, was able to inhibit 5-HT-induced calcium mobilisation in cells solely expressing the 5-HTR$_{2C}$ tagged with EGFP (Fig. 5D). On the other hand, neither of the 5-HTR$_{2C}$ antagonists had an effect on OTR-dependent calcium release in cells solely expressing OTR tagged with tRFP (Fig. 5C). These results demonstrate proper activity and specificity of the 5-HTR$_{2C}$ antagonists used.

Interestingly, pharmacological inhibition of 5-HTR$_{2C}$ signalling using both 5-HTR$_{2C}$ antagonists almost fully restored the depleted OTR-mediated calcium influx in cells co-expressing the OTR/5-HTR$_{2C}$ complex (Fig. 5A). The concentration-response curve of OT in the presence of SB and RS in cells co-expressing both receptors was characterised by a reduced potency (EC_{50} = 3.6 ± 1.3 nM and EC_{50} = 4.8 ± 2.2 nM, respectively), by one order of magnitude, compared to the OT potency in cells solely expressing OTR-tRFP (Fig. 5C). Similar effects were demonstrated in cells co-expressing both receptors with reversed tags (Fig. 5B). That is, OT-induced response in the presence of SB and RS was almost completely restored in cells co-expressing the 5-HTR$_{2C}$ tagged with tRFP and the OTR tagged with tGFP (EC_{50} = 5.1 ± 1.5 nM and EC_{50} = 4.9 ± 0.7 nM). None of the 5-HTR$_{2C}$ antagonists alone affected calcium release in any of the cell lines tested (Fig. S5). No significant difference in 5-HT-induced calcium release was observed in cells co-expressing both receptors, regardless of whether 5-HT was administered alone or in combination with OT (Figs. S6A and B). The lack of an OT effect on 5-HT-induced Gαq signalling further supports the specificity of the blockade of the OTR-dependent calcium response when co-expressed with the 5-HTR$_{2C}$.

Together, the above results highlight the attenuation of OTR-mediated Gαq-dependent signalling by the 5-HTR$_{2C}$, which likely occurs via OTR/5-HTR$_{2C}$ heteroreceptor complex formation.

Transient release of calcium from intracellular stores into the cytosol is driven by the second messenger, IP_3, which is subsequently converted to IP_2 and IP_1. To confirm the modulation of Gαq-dependent signalling when the OTR and 5-HTR$_{2C}$ are co-expressed, changes in IP_1 production were measured using an HTRF-based IP-One production assay (Fig. 6). The cellular response (increase in IP_1 production) was detected under basal and ligand-mediated conditions, following OT (1 nM, 10 nM, and 100 nM) and 5-HT (20 nM and 100 nM) treatment. As expected, an OT concentration-dependent increase in IP_1 production was observed in cells solely expressing OTR tagged with tRFP (Fig. 6A). However, in cells co-expressing both receptors there was no such relationship between OT concentration and IP_1 production (Fig. 6A). Consequently, IP_1 production following 10 nM and 100 nM OT was significantly reduced in cells co-expressing OTR and 5-HTR$_{2C}$ compared to cells solely expressing OTR (Fig. 6A). These results validate the ability of 5-HTR$_{2C}$ to attenuate OTR-mediated Gαq signalling as observed in the calcium mobilisation assay.

Similar to OT, there was a significant and expected 5-HT-mediated increase in IP_1 production compared to the control condition (untreated cells) in both cell lines (cells solely expressing 5-HTR$_{2C}$ and cells co-expressing both receptors) (Fig. 6B). A slight but non-significant decrease in IP_1 production induced by 20 nM and 100 nM 5-HT was observed in cells co-expressing both receptors compared to cells solely expressing 5-HTR$_{2C}$ (Fig. 6B).

Interestingly, a significant difference in ligand independent IP_1

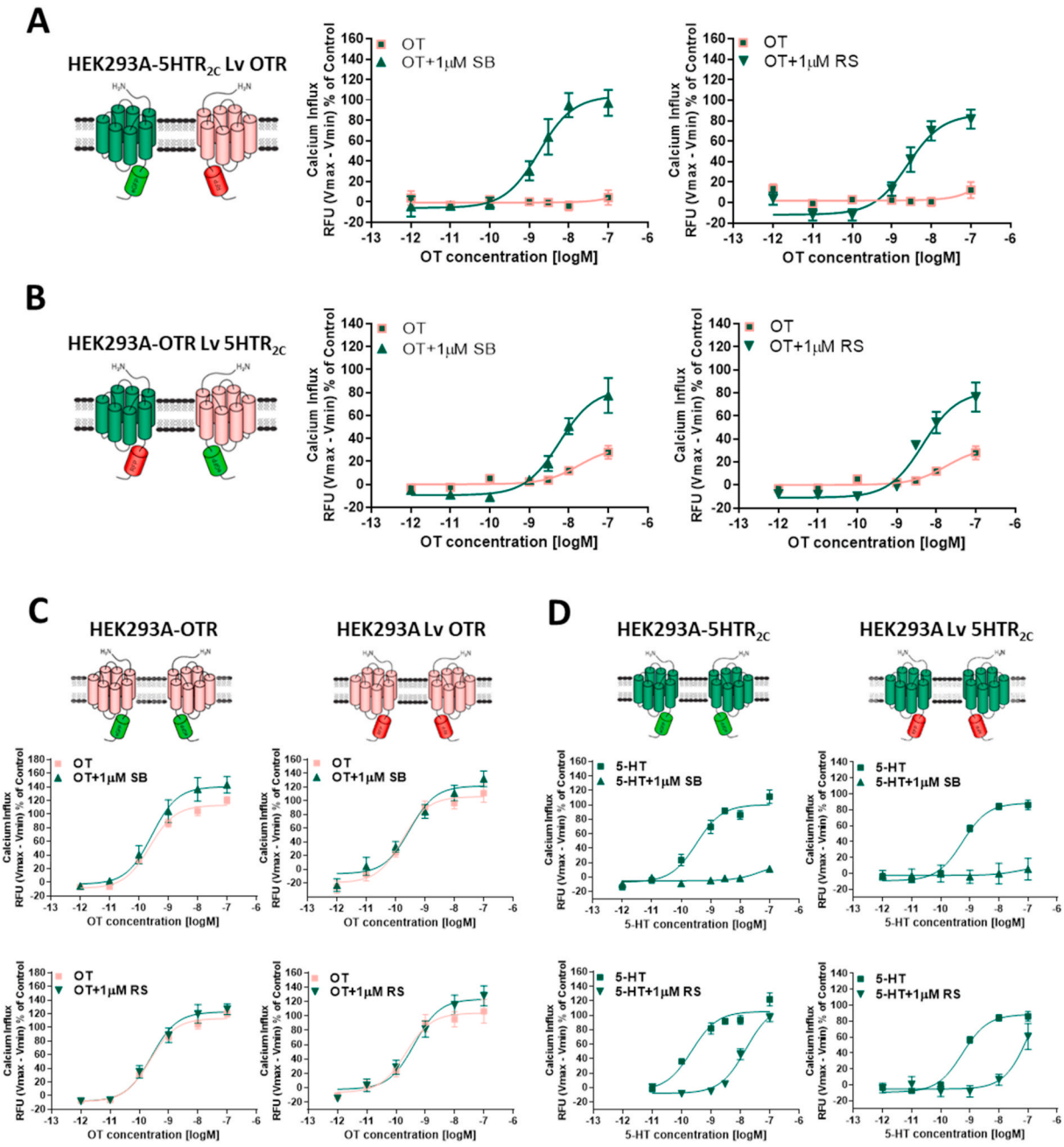


Fig. 5. OTR-mediated Gαq-dependent signalling restored when 5-HTR$_{2C}$ activity is blocked. Intracellular calcium release induced by increasing concentrations of OT alone and in the presence of 5-HTR$_{2C}$ antagonists; SB242084 (left graphs) and RS102221 (right graphs) in cells co-expressing the OTR-tRFP and 5-HTR$_{2C}$-EGFP (**A**) and cells co-expressing both receptors with switched tags (**B**). Intracellular calcium release induced by increasing concentrations of OT alone and in the presence of 5-HTR$_{2C}$ antagonists in cells solely expressing the OTR-tGFP or OTR-tRFP (**C**). Intracellular calcium release induced by increasing concentration of 5-HT alone and in the presence of 5-HTR$_{2C}$ antagonists in cells solely expressing the 5-HTR$_{2C}$-EGFP or 5-HTR$_{2C}$-tRFP (**D**). Graphs represent means ± SEM from at least three independent experiments run in duplicates.

production was observed between the three cell lines (Fig. 6C), with the highest level seen in cells solely expressing 5-HTR$_{2C}$. These results are in line with the relatively high constitutive activity reported for the 5-HTR$_{2C}$ (Martin et al., 2013). Cells solely expressing the OTR also showed ligand-independent PI$_1$ production, albeit at lower levels (Fig. S7). Of note, ligand-independent IP$_1$ production in cells co-expressing the OTR/5-HTR$_{2C}$ pair is significantly reduced compared to that in cells solely expressing 5-HTR$_{2C}$, suggesting a decrease in constitutive activity driven by the 5-HTR$_{2C}$ when in a heteroreceptor complex with the OTR.

Most GPCRs, including the OTR and 5-HTR$_{2C,}$ are internalised following agonist treatment for degradation or recycling back to the cell membrane (Berg et al., 1998; Conti et al., 2009; Hasbi et al., 2004). Desensitisation and subsequent internalisation of GPCRs provides an important physiological mechanism that protects cells against over-stimulation. In addition, β-arrestin-dependent internalisation inhibits G-dependent downstream signalling of GPCRs (Borroto-Escuela et al., 2011; Luttrell et al., 2018). Therefore, we investigated OTR and 5-HTR$_{2C}$ cellular trafficking when co-expressed in HEK293A cells under basal conditions and following 5 min treatment with their respective endogenous ligands, OT (100 nM) and 5-HT (1 μM) (Fig. 7). As expected, significant OT-mediated internalisation of the OTR was observed in cells solely expressing OTR (HEK293A-Lv-OTR-tRFP) (Fig. 7A). Similarly,

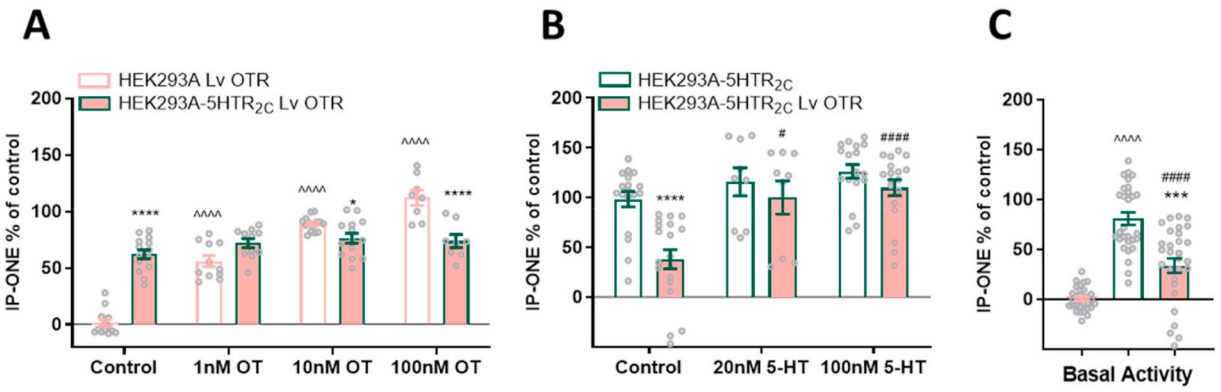


Fig. 6. Co-expression of the OTR and 5-HTR$_{2C}$ attenuates basal and ligand-mediated IP$_1$ production IP$_1$ production induced by increasing concentrations of OT (**A**) and 5-HT (**B**) in HEK293A cells expressing 5-HTR$_{2C}$-EGFP, OTR-tRFP, and co-expressing both receptors. Graphs represent means ± SEM from at least three independent experiments run in triplicates. The results were depicted as the percentage of RFU normalised to the maximum response (100% signal) obtained for non-stimulated OTR expressing cells. Statistical significance compared to cells expressing a single receptor denoted by *. Statistical significance compared to untreated group in cells expressing a single receptor denoted by ^, and in cells co-expressing both receptors denoted by #. Ligand-independent IP$_1$ production in cells expressing 5-HTR$_{2C}$-EGFP, OTR-tRFP, and co-expressing both receptors (**C**). Graph represents means ± SEM from eight independent experiments run in quadruplicates. Statistical significance compared to cells solely expressing OTR denoted by #, and compared to cells solely expressing 5-HTR$_{2C}$ denoted by *.

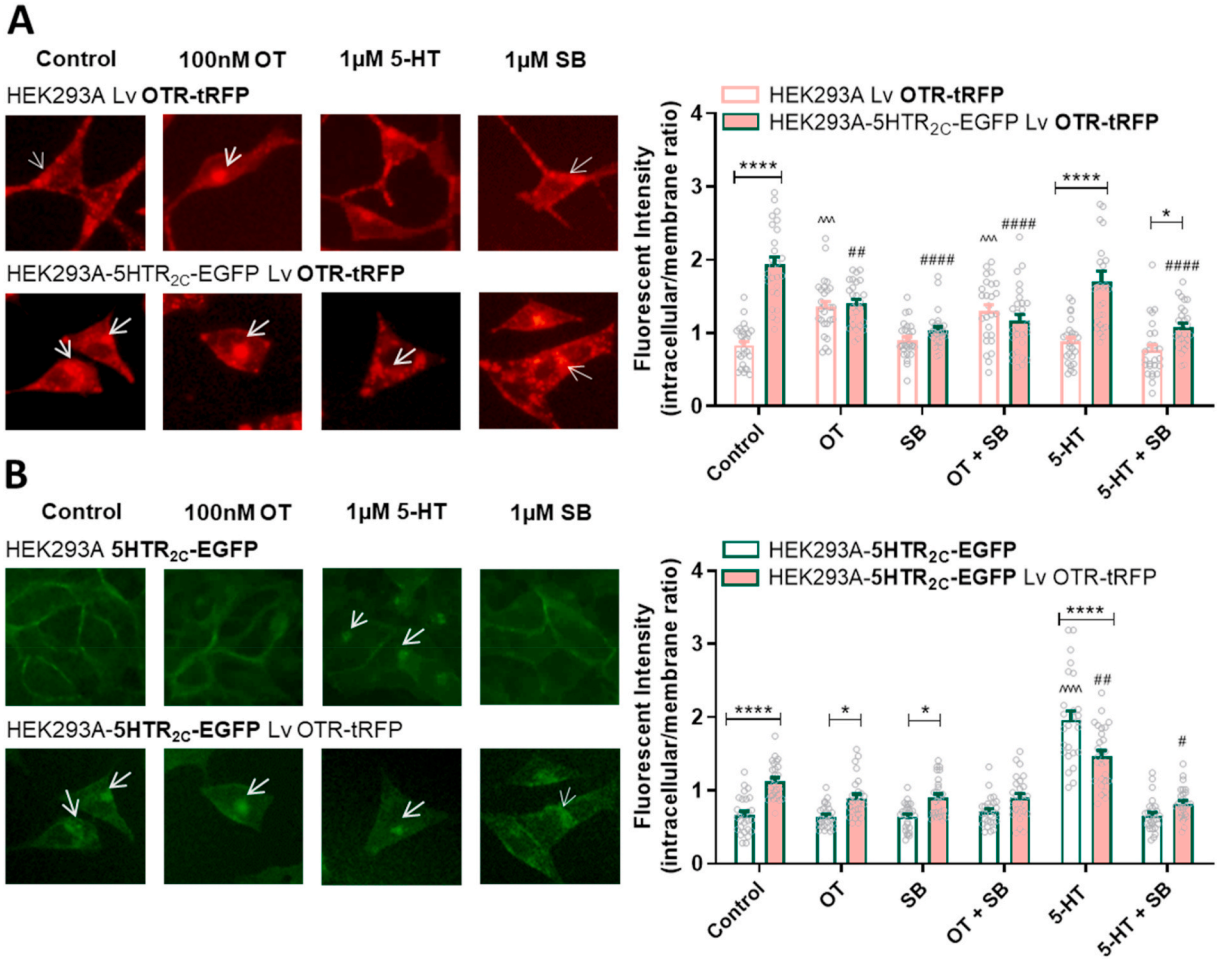


Fig. 7. Cellular trafficking of the OTR and 5-HTR$_{2C}$. Representative images and quantitative analysis of basal and ligand-mediated internalisation of the OTR tagged with tRFP (**A**) and 5-HTR$_{2C}$ tagged with EGFP (**B**) in cells expressing a single receptor versus cells co-expressing both receptors after 5 min incubation with ligands (100 nM OT or 1 μM 5-HT). Graphs represent mean ± SEM from three independent experiments. Statistical significance compared to cells solely expressing a single receptor denoted by *. Statistical significance compared to untreated control condition in cells solely expressing OTR or 5-HTR$_{2C}$ denoted by ^, and in cells co-expressing both receptors denoted by #.

there was significant internalisation of the 5-HTR$_{2C}$ following 5-HT treatment in cells solely expressing the 5-HTR$_{2C}$ (HEK293A-5HTR$_{2C}$-EGFP) (Fig. 7B). However, in cells co-expressing both receptors (HEK293A-5HTR$_{2C}$-EGFP-Lv-OTR-tRFP) a significant increase in basal internalisation of the OTR was noted (Fig. 7A). This increase in OTR trafficking was significantly attenuated after treating the cells with the 5-HTR$_{2C}$ antagonist, SB242084 (SB). Under these conditions, the rate of OTR internalisation in cells co-expressing both receptors was normalised to the basal level observed in cells solely expressing the OTR. In addition, pre-incubation with SB resulted in a normalisation of OT-induced OTR internalisation of cells co-expressing both receptors to the level observed in cells expressing only the OTR. This effect was no longer observed when cells were treated with OT for 30 min (Fig. S8). Similar to the OTR, basal internalisation of the 5-HTR$_{2C}$ in cells co-expressing both receptors was consistently increased compared to cells expressing only the 5-HTR$_{2C}$ (Fig. 7B). However, in contrast to the OTR, administration of 5-HT resulted in a further increase in 5-HTR$_{2C}$ trafficking in cells co-expressing both receptors, albeit to a lesser extent as in cells expressing 5-HTR$_{2C}$ alone. Pre-treatment of cells co-expressing both receptors with SB was again able to normalise the increased internalisation of the receptor complex. This effect was no longer observed following 30 min treatment of cells with OT (Fig. S8).

Together, the above results demonstrate that 5-HTR$_{2C}$-mediated downstream signalling is not affected to the same extent as OTR-mediated signalling is affected in cells expressing the OTR/5-HTR$_{2C}$ pair, which is in line with the calcium mobilisation and IP$_1$ data. In addition, changes in receptor trafficking appear to be mediated mainly by the 5-HTR$_{2C}$, as was the case for OTR-mediated Gαq signalling.

3.4. Signalling consequences of the OTR and 5-HTR$_{2C}$ crosstalk in vivo

Our *in vitro* data demonstrate that the 5-HTR$_{2C}$ attenuates OTR-mediated signalling, which is almost fully restored following 5-HTR$_{2C}$ antagonism. Therefore, we investigated whether pharmacological blockade of the 5-HTR$_{2C}$ is also able to enhance OTR-mediated behaviour *in vivo* in mice. To this end, OT-mediated locomotor activity was analysed following intraperitoneal administration of the specific and brain-penetrant 5-HTR$_{2C}$ antagonist, SB242084 (SB), followed by intraperitoneal injection of OT. The results were very similar for both measures of locomotor activity (i.e., time spent stationary and distance travelled) (Fig. 8). Repeated-measures ANOVA revealed a significant effect of OT (stationary time: $F_{1,\ 42} = 33.92$, $p < 0.001$; distance travelled: $F_{1,\ 42} = 30.22$, $p < 0.001$) but no significant effect of SB (stationary time: $F_{1,\ 42} = 0.12$, $p = 0.73$; distance travelled: $F_{1,\ 42} = 1.33$, $p = 0.25$). Importantly, there was a significant interaction between OT and SB for both measures (stationary time: $F_{1,\ 42} = 12.95$, $p = 0.001$; distance travelled: $F_{1,\ 42} = 11.20$, $p = 0.002$) (Fig. 8A and C). Follow-up t-tests indicated that the hypolocomotor effect of OT alone was relatively small but statistically significant or close to significant (stationary time: $t_{21} = 2.07$, $p = 0.051$; distance travelled: $t_{21} = 2.46$, $p = 0.022$). Most notably, co-treatment with SB enhanced the hypoactivity effect of OT with a significantly increased stationary time ($t_{21} = 5.58$, $p < 0.001$), and a significantly decreased distance travelled ($t_{21} = 4.92$, $p < 0.001$), in mice treated with subthreshold doses of both OT and SB in comparison to mice treated with SB alone. It is worth noting that this SB-mediated augmentation of the OT effect was not simply an additive effect of the ligands. In fact, when compared to the vehicle group, SB alone had the opposite effect, increasing locomotor activity, indicative of

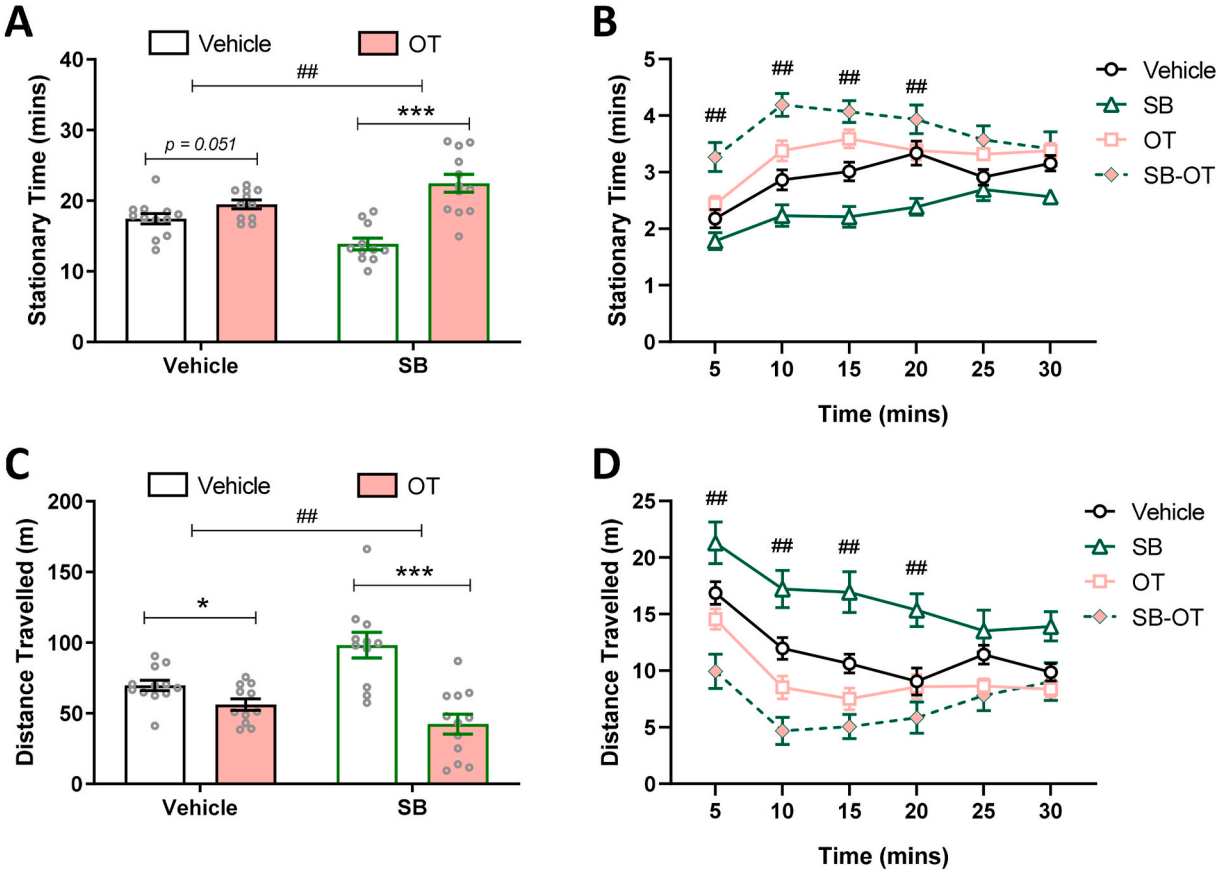


Fig. 8. Specific 5-HTR$_{2C}$ blockade potentiates OTR-mediated behavioural effects in mice. Following i. p. administration of SB and OT, or their respective vehicle, locomotor activity was measured as time spent stationary (**A, B**) and distance travelled (**C, D**). Data is presented as totals for the full 30-min observation period (**A, C**) and broken down into 5-min periods after OT injection (**B, D**). Graphs represent mean ± SEM. Statistical significance of *t*-test comparing OT-treated animals with control group denoted by *. Statistical significance of interaction between OT and SB treatment denoted by #.

hyperlocomotion (significant decrease in stationary time, $t_{21} = 3.29$, $p = 0.004$; significant increase in distance travelled, $t_{21} = 3.29$, $p = 0.007$).

In addition, significant effects of time were observed (stationary time: $F_{3.80,\ 159.44} = 27.30$, $p < 0.001$; distance travelled: $F_{3.79,\ 159.24} = 27.30$, $p < 0.001$) and significant interactions between time and OT (stationary time: $F_{3.80,\ 159.44} = 5.80$, $p = 0.006$; distance travelled: $F_{3.79,\ 159.24} = 6.82$, $p < 0.001$), and between time, OT, and SB (stationary time: $F_{3.80,\ 159.44} = 3.24$, $p = 0.015$; distance travelled: $F_{3.79,\ 159.24} = 4.14$, $p = 0.004$) (Fig. 8B and D).

Overall, the behavioural analysis revealed an OT-mediated decrease in locomotor activity, similar to what has been reported previously (Carson et al., 2010; Uvnäs-moberg et al., 1992; Uvnäs-Moberg et al., 1994). Due to the subthreshold dose of OT selected, these effects of OT alone were relatively small, allowing us to observe a synergistic effect of the combined drug treatment. Specifically, OT-induced hypoactivity was significantly augmented when OT was co-administered with the 5-HTR_{2C} antagonist, SB, and this was not simply an additive effect of the two drugs, as SB alone had a hyperlocomotive effect. These results are in keeping with our *in vitro* and *ex vivo* findings, providing further support for the functional relevance of the OTR/5-HTR_{2C} interaction.

4. Discussion

Here, we demonstrate, to our knowledge for the first time, a constitutive, specific and functional association between the OTR and 5-HTR_{2C} using a variety of approaches. The 5-HTR_{2C} and OTR are two GPCRs representing promising targets in the development of pharmaceutical drugs for the treatment of many neuropsychiatric disorders (Di Giovanni and De Deurwaerdère, 2016; Gauthier et al., 2016; Leppanen et al., 2017; Meyer-Lindenberg et al., 2011; Palacios et al., 2017). Interestingly, both the OTR and 5-HTR_{2C} are known to function as constitutive homodimers (Herrick-Davis, 2013; Herrick-Davis et al., 2005; Terrillon et al., 2003) and to form functionally active heterocomplexes with other GPCRs that must be considered when analysing the physiological or pathophysiological roles of OT and 5-HT signalling crosstalk in the brain (Maroteaux et al., 2019; Moutkine et al., 2017; Romero-Fernandez et al., 2012; Schellekens et al., 2015).

We initially demonstrated colocalized expression of the OTR and 5-HTR_{2C} and revealed an association between both receptors using a fcFRET approach, which indicates the potential of OTR/5-HTR_{2C} heteroreceptor complex formation. Further, we demonstrated a two-fold increase in ligand-independent intracellular localisation of the OTR and 5-HTR_{2C}, when co-expressed in cells. This is in line with the colocalized expression of both receptors observed not only on the cell membrane but also intracellularly. The significant intracellular presence of the OTR/5-HTR_{2C} pair may suggest that both receptors assemble as constitutive heterocomplexes at an early stage, during maturation and/or trafficking to the cell membrane. This hypothesis is supported by similar observations in the case of the OTR/5-HTR_{2A} and 5-HTR_{2A}-mGluR2 heteroreceptor complexes (Chruścicka et al., 2019; Wischhof and Koch, 2016). Alternatively, the above results may also indicate an increase in basal activity of both receptors and a subsequent higher internalisation rate as shown for cannabinoid CB1 and orexin OX1 receptor complexes (Ward et al., 2011). Importantly, both hypotheses indicate co-trafficking of both receptors within the cell and reinforce the formation of stable and constitutive OTR/5-HTR_{2C} heterocomplexes. Interestingly, specific pharmacological 5-HTR_{2C} antagonism restored elevated OTR trafficking in cells co-expressing both receptors to the level observed in cells solely expressing the OTR. This effect was no longer observed following 30 min treatment with OT. Moreover, after 30 min treatment with OT and 5-HT it becomes clear that OT can increase intracellular trafficking of the OTR when expressed alone, but there is no effect when co-expressed with the 5-HTR_{2C}. 5-HT treatment resulted in a very small but further increase in OTR trafficking compared to the control conditions in cells co-expressing both receptors. These results are in line with recent studies showing that the formation of heteroreceptor complexes can change the receptors mobility over time (Vasudevan et al., 2019), suggesting that changes in receptor co-trafficking over time may be regulated by the 5-HTR_{2C} in OTR/5-HTR_{2C} heterocomplexes.

Desensitisation and subsequent internalisation of GPCRs provides an important physiological mechanism that protects cells against overstimulation (Berg et al., 1998; Conti et al., 2009; Hasbi et al., 2004). However, the changes in β-arrestin dependent internalisation of receptors may also directly affect G protein-dependent downstream signalling of GPCRs (Borroto-Escuela et al., 2011; Luttrell et al., 2018). Indeed, oligomerisation of the OTR and 5-HTR_{2C} with other GPCRs has been previously shown to modulate G protein-dependent signalling pathways and thus exert a significant impact on receptor physiology and function (Chruścicka et al., 2019; de la Mora et al., 2016; Romero-Fernandez et al., 2012; Schellekens et al., 2015). The current study demonstrates a significant depletion of OTR-mediated Gαq-dependent signalling in cells co-expressing the OTR/5-HTR_{2C} pair, observed in both calcium mobilisation and IP_1 accumulation assays. In addition, the lack of OT synergistic effect on 5-HT-stimulated Gαq signalling further supports the specific depletion of OTR-mediated Gαq signalling upon co-expression with 5-HTR_{2C}. Finally, OTR-mediated Gαq-signalling was almost fully restored following pharmacological 5-HTR_{2C} antagonism. Interestingly, restoration of the OTR-mediated Gαq-signalling following 5-HTR_{2C} blockade is consistent with its effect on OTR trafficking. Although a small decrease in 5-HT-mediated downstream signalling was also observed, the effect of the 5-HTR_{2C} on OTR-dependent signalling is more pronounced in our *in vitro* cell expression system. Together, our findings strongly support the potential of the 5-HTR_{2C} to attenuate OTR-mediated downstream signalling via formation of functionally active OTR/5-HTR_{2C} heteroreceptor complexes.

These findings are similar to those previously demonstrated by our group for the 5-HTR_{2C} in heterocomplex with the GHSR-1a. In that case, a significant reduction in GHS-1a-mediated calcium release was restored following pharmacological 5-HTR_{2C} blockade (Schellekens et al., 2013). Such functional asymmetry has also been reported in the case of 5-HTR_{2C} heterodimers with the 5-HTR_{2A} and 5-HTR_{2B} (Moutkine et al., 2017). The 5-HTR_{2C} protomer again was able to blunt Gαq-dependent signalling of the partner protomer (5-HTR_{2A} or 5-HTR_{2B}) through conformational changes in the heterodimer. This phenomenon is explained by the loss of agonist binding in the 5-HTR_{2A} and 5-HTR_{2B} protomers in the presence of 5-HTR_{2C}. In the current experiments, OTR/5-HTR_{2C} heterocomplexes also behaved in an asymmetric manner with greater dominance of the 5-HTR_{2C} protomer. This may suggest that the 5-HTR_{2C} influences OTR binding properties via conformational changes caused by direct interaction of the two protomers within the complex, consequently affecting OTR-mediated downstream signalling. Moreover, restoration of OTR-mediated signalling following pharmacological 5-HTR_{2C} blockade is in line with previous findings in which homo- and heterocomplexes of the 5-HTR_{2C} can be regulated and even disrupted by its antagonists but not agonists (Millan et al., 2008; Schellekens et al., 2013; Ward et al., 2015).

We also confirmed the formation of functional OTR/5-HTR_{2C} heterocomplexes in rat brain sections under endogenous expression levels of both protomers. The hippocampus and cortex were chosen based on the *in silico* analysis of the co-expression of both receptors at the cellular level (Fig. S1). A PLA positive signal was observed particularly in the pyramidal cell layer of the CA3 and CA1 regions of the hippocampus, the polymorphic layer of the dentate gyrus, and the retrosplenial granular and agranular cortex. This specific distribution of OTR/5-HTR_{2C} heterocomplexes within differs from what was observed for 5-HTR_{2C} heteromers with GHSR-1a (hypothalamus and hippocampus) or MT2R (cerebral cortex and hippocampus) (Kamal et al., 2015; Schellekens et al., 2015) and for OTR heterodimers with 5-HTR_{2A} (nucleus accumbens, cingulate cortex, CA1 and CA3 regions of the hippocampus) or D2 (ventral and dorsal striatum and amygdala) (de la Mora et al., 2016;

Romero-Fernandez et al., 2012). The specific distribution pattern of OTR/5-HTR$_{2C}$ heterocomplexes in the brain is likely to correlate with their functional relevance in OTR-mediated behaviours.

Both the OTR and 5-HTR$_{2C}$ are known to be involved in modulation of locomotor activity (Demireva et al., 2018; Hicks et al, 2012, 2014; Nebuka et al., 2020). Here, we investigated the potential effect of OTR/5-HTR$_{2C}$ heterocomplex formation on OT-mediated hypoactivity. In line with previous data, we observed a small but significant decrease in locomotor activity in mice following peripheral administration of a subthreshold dose of OT (Carson et al., 2010; Uvnäs-moberg et al., 1992). OT has well established behavioural effects in rodents following peripheral administration, indicating that OT-mediated hypoactivity at least partially is centrally driven (Carson et al., 2010; Hicks et al., 2012; Maejima et al., 2015). We also showed that systemic administration of SB significantly increased 5-HTR$_{2C}$ driven motor activity in mice, in agreement with literature (Demireva et al., 2018; Nebuka et al., 2020). Most notably, specific antagonism of the 5-HTR$_{2C}$ significantly augmented OT-mediated hypoactivity in mice. This is not simply an additive effect of the two drugs, as SB alone had an opposite effect on locomotor activity, suggesting specific regulation of OTR signalling by the 5-HTR$_{2C}$. GPCR assemblies are known to possess distinct pharmacological profiles compared to their monomeric counterparts (Fuxe and Borroto-Escuela, 2016; Parmentier, 2015). Therefore, the effect of 5-HTR$_{2C}$ antagonism on OT-mediated hypoactivity may be driven by SB-mediated regulation of the OTR/5-HTR$_{2C}$ heterocomplex and independent of monomeric 5-HTR$_{2C}$ signalling. Although the involvement of other mechanisms in modulation of OT-mediated hypoactivity cannot be excluded, these findings are in line with our *in vitro* results and strongly support the potential of the 5-HTR$_{2C}$ to attenuate OTR-mediated functions by the formation of asymmetric heteroreceptor complexes. While we observed OTR/5-HTR$_{2C}$ heterocomplexes in the hippocampus and cortex (regions we selected based on our *in silico* analysis of the co-expression of both receptors at the cellular level), these regions are certainly not the only sites of heterodimerization, as both OTR and 5-HTR$_{2C}$ are widely distributed in the brain (Pompeiano et al., 1994; Warfvinge et al., 2020). Moreover, the site of action mediating the locomotor effects observed is not known and unlikely to be the hippocampus. Previous research has implicated the substantia nigra, a region with considerable OTR and 5-HTR$_{2C}$ expression, in the effects of oxytocin mediated locomotor activity (Angioni et al., 2016). Therefore, future research is now warranted to establish the broader distribution of OTR/5-HTR$_{2C}$ heterocomplexes throughout the brain and to identify specific regions implicated in the behavioural changes observed in our study. It is also worth noting that OT-induced hypoactivity reflects inhibition of the hypothalamus-pituitary-adrenal axis, causing a passive physiological and behavioural state that may facilitate social involvement (Hicks et al., 2014). This strongly suggests the potential functional involvement of these heteroreceptors in more complex OTR-mediated behaviours (Duque-Wilckens et al., 2020; Jurek and Neumann, 2018) and at least partially explain variation in the response to OT treatment (Evans et al., 2014; Gauthier et al., 2016). The opposing effect of SB also highlights the complex nature of the signalling crosstalk between multiple neurotransmitter systems and might partially explain the contradictory effects of 5-HTR$_{2C}$ agonists vs antagonists *in vivo*. Based on our *ex vivo* findings of OTR/5-HTR$_{2C}$ heteroreceptors in hippocampal and cortical areas, which are key brain regions associated with social discrimination, learning and memory (Lin and Hsu, 2018; Mitre et al., 2016; Raam et al., 2017; Teixeira et al., 2018), it would be worthwhile for future studies to explore the functional contribution of OTR/5-HTR$_{2C}$ complexes to these more sophisticated behaviours.

In conclusion, we demonstrate compelling evidence for the formation of functionally asymmetric OTR/5-HTR$_{2C}$ heteroreceptor complexes with significant inhibition of OTR-mediated downstream signalling. Furthermore, we provide evidence for the potential functional relevance of this OTR/5-HTR$_{2C}$ pair in the regulation of OT-mediated hypoactivity in mice. Together, these findings have uncovered a potential mechanism underlying the specific crosstalk between the OT and 5-HT neurotransmitter systems, paving the way for novel therapeutic approaches for the treatment of complex brain disorders. Future therapeutic strategies specifically targeting OTR/5-HTR$_{2C}$ heterocomplexes may benefit from a unique pharmacological profile, as have been seen for other GPCR assemblies (Fuxe and Borroto-Escuela, 2016; Parmentier, 2015), and further *in vivo* studies exploring their physiological and behavioural consequences are warranted.

CRediT authorship contribution statement

Barbara Chruścicka: designed all in vitro experiments, performed experiments that led to Figures 1, 2, 4, 5, and 6; analysed all in vitro results; and wrote the manuscript. **Caitlin S.M. Cowan:** performed and analysed in vivo data that led to figure 8. **Shauna E. Wallace Fitzsimons:** performed transduction of cells for in vitro experiments and analysed experiments that led to Figure 7. **Dasiel O. Borroto-Escuela:** performed and analysed ex vivo data. **Clémentine M. Druelle:** served as technician assistant in maintenance of cells, preparation of plasmid constructs, and calcium assay experiments. **Panagiota Stamou:** served as technician support in designing and performing of fcFRET experiments. **Cristian A. Bergmann:** performed additional experiments for revision manuscript and critically read the manuscript. **Timothy G. Dinan:** supervised the work and proofread the manuscript. **David A. Slattery:** advised on in vivo experimental design and critically proofread the manuscript. **Kjell Fuxe:** supervised the work and proofread the manuscript. **John F. Cryan:** supervised the work and proofread the manuscript. **Harriët Schellekens:** is the senior author responsible for the conception of the study, interpretation of the data and editing of the manuscript.

Acknowledgments

The authors declare no competing financial interest.

The study was funded by Science Foundation Ireland Research Centre Grant SFI/12/RC/2273 to the APC Microbiome Institute Ireland to Timothy G. Dinan, John F. Cryan and Harriët Schellekens. B. Ch. was supported by the National Science Centre (grant number: 2019/03/X/NZ1/00607), Poland. K.F. was supported by the Swedish Medical Research Council (62X-00715-50-3). D.O.B-E received support from Hjärnfonden (F02018-0286) and the Karolinska Institutet Forskningsstiftelser. K.F. and D.O.B-E also received support from Olle Engkvist Stiftelse.

The authors would like to thank Gerard Moloney for all technical assistance related to laboratory equipment and orders, Martin Codagnone for help with i. p. injections of mice, Nigel Theune for help with manual blind scoring of behaviour, Natasha Leeuwendaal for assistance with cell culture for additional experiments and Marcin Smaga for graphic work. The authors would like to acknowledge the Department of Anatomy & Neuroscience Imaging Centre, BioSciences Institute, University College Cork, and Suzanne Crotty for technical assistance in preparing, confocal microscopy imaging and analysing specimens for this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2020.108394.

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