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Oxytocin receptor heteromerization-a novel convergence of central molecular signalling and G-protein coupled receptor crosstalk

Thesis presented by

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for the degree of Doctor of Philosophy

2019

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Author contributions

All experiments were designed, performed and analysed by me, Shauna Wallace Fitzsimons, except for experiments described below. Both Clementine Druelle and Dr. Barbara Chruścicka aided in the development of the stable HEK-OTR-tGFP cell line and Lv-OTR-tRFP plasmid used throughout all chapters. Dr. Ken Nally for allowing us to use the flow cytometry platform. Dr. Stamou Panagiota and Dr. Barbara Chruścicka designed the program required to measure flow cytometry-based fluorescence resonance energy transfer. Experiments described in chapter four were designed by Dr. Barbara Chruścicka and performed equally (Chapter 4). Proximity ligation assays were performed and analysed by Dr. Dasial Borroto-Escuela (Chapter 4). Experiments in Chapter 5 were designed, and calcium mobilization and IP-One assays performed by by Dr. Barbara Chruścicka.

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“When you have seen one ant, one bird, one tree, you have not seen them all”

E.O. Wilson

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Summary

G-protein coupled receptors (GPCR) are the largest family of cell membrane receptors, eliciting a multitude of effects. Due to their wide array of function, GPCRs have become widely studied, specifically within the pharmaceutical market; where over 30% of currently approved drugs target such GPCRs. It is now over three decades since the concept of a GPCR-GPCR interaction emerged, leading to the discovery of GPCR heterodimers which is the interaction of two different GPCRS, homodimers the interaction of two of the same GPCRS and high-order oligomers, the interaction of 3 or more GPCRs. Intriguingly these heterocomplex formations led to differential signalling of the GPCRs within these complexes compared to their monomer state. Such studies ushered in an exciting new field in GPCR research. Yet despite the large therapeutic effect produced from targeting GPCRs, the full ability of GPCR heterocomplex signalling remains largely unexplored.

The oxytocin receptor (OTR), is one of the best studied central GPCRs located in multiple regions throughout the central nervous system and periphery. In addition to its ability to facilitate social behaviours it also known to impact multiple other interrelated behaviours such as food intake and mood. So, the question can be asked how such a well-studied receptor in its monomer state, with only one known endogenous ligand can elicit such a multitude of responses? Is it doing so in its monomer state or does it have the ability to form GPCR heterocomplexes subsequently altering its signalling ability?

Therefore, this thesis focuses on the ability of the OTR to form a multitude of heterocomplexes which may impact its multifaceted signalling abilities. With a focus on the interplay between social behaviour, appetite and mood multiple GPCRs associated with such functions were screened for co-localisation with the OTR in HEK293A cells. Due to previous studies linking their signalling pathways and known co-expression in different brain regions associated with social behaviour, appetite and mood, a cellular screening platform was established to aid in the identification of an OTR heterocomplex with receptors such as the ghrelin 1a receptor (GHSR) (Chapter 2), glucagon like peptide 1 receptor (GLP-1r) (Chapter 3), serotonin 2a receptor (5-HT_{2A}) (Chapter 4) and the serotonin 2c receptor (5-HT_{2C}) (Chapter 5),

receptors known too to signal in either social behaviour, food intake and mood. Chapter 1 will further expand on the current knowledge and impact of GPCR heterodimerization on GPCR signalling and where it stands as pharmacotherapeutic target. Moreover, introducing these receptors, there signalling abilities and central expression patterns, finally, finishing on the link between these receptors and how they may play a role in the intricate interplay between social behaviour, food intake and mood. An interplay possibly driven by such heterocomplex formations?

Chapters 2 through 5 focuses on these heterocomplex formations, using a multitude of assays, including heterocomplex identification (co-localization and FRET, PLA) and functional assays (calcium mobilization, inositol monophosphate and ligand mediated trafficking, cyclic adenosine monophosphate) the ability of the OTR to form functional heterocomplexes was studied. Excitingly, data revealed the OTR to be a hub for GPCR heterocomplex formations, each leading to unique signalling pathways associated with these heterocomplex formation. Lastly, chapter 6 focuses on the current scientific knowledge of OTR heterodimerization and the impact of these dimer formations on such knowledge, moreover the possible impact these heterocomplexes may have in future therapeutics.

In conclusion, this thesis highlights the ability of the OTR to heterodimerize with a number of other GPCRs, possibly implicating these heterodimer pairs in the ability of the OTR to regulate multiple behaviours. Thus, fully understanding the full extent of OTR heterocomplex signalling will aid in a better understanding of current therapies and may lead to the development of much needed novel, more potent and selective pharmacotherapies. An area which is already proving promising with the use of bivalent ligands.

Chapter 1

Introduction

1.1 G-protein coupled receptors

G-protein coupled receptors (GPCR) are the largest protein family of receptors involved in cell membrane transduction. GPCR genomic analysis has revealed 802 GPCRs within the human genome alone (Fredriksson et al., 2003). These GPCRs play a key role in normal physiology and produce a diverse array of functions including neurotransmission, cellular metabolism, differentiation and growth, and inflammatory and immune responses (Betke et al., 2012, Husted et al., 2017, Smith et al., 2018, Carmona-Rosas et al., 2018). Due to this GPCR's have become one of the leading therapeutic targets worldwide (Chakraborty and Chattopadhyay, 2015, Bouvier, 2001).

GPCRs are characterised by their seven transmembrane α-helical structure which alternate between intracellular and extracellular loops, the N-terminus being extracellular while the C-terminus is intracellular (Kobilka, 2007). The GPCR downstream signalling pathways are mediated via GPCR specific ligands, which attach to the binding pockets of the GPCR, this initiates a cascade of biochemical events which allow for GPCRs to elicit their effect (DeVree et al., 2016, Gurevich and Gurevich, 2017). These biochemical events are mediated via receptor trafficking and binding of G-proteins which bind intracellularly to GPCRs; activating such proteins as Gαq, Gαi, Gαs and $G\alpha_{12/13}$ which all lead to different downstream signalling as seen in Figure 1.2 (Hilger et al., 2018, Gurevich and Gurevich, 2017). Due to the range of available G-proteins, it has been shown that different receptor ligands can activate different G-proteins on a GPCR leading to a biased differential downstream signal, this results in the ability of ligands to activate specific signalling pathways of a receptor allowing for a more targeted response of the associated GPCR (Ramirez et al., 2018).

GPCRs can be classified depending on which of two systems is used, these systems include the "GRAFS" system which stands for the GPCRs (G) glutamate receptors, (R) rhodopsin receptors, (A) adhesion receptors, (F) frizzled receptors and (S) the secretin receptors (Fredriksson et al., 2003). The next system classifies GPCRs from class A-F, the Class A consists of rhodopsin-like receptors, Class B the secretin receptors, Class C the metabotropic glutamate receptors, Class D the fungal mating

pheromone receptors, Class E cyclic adenosine monophosphate receptors and finally the Class F frizzled receptors (Munk et al., 2016, Kolakowski, 1994). Both these systems differentiate these classes based on receptor functionality and genetic homology (Attwood and Findlay, 1994). This thesis will mainly focus on class A rhodopsin-like receptors, which is the largest studied class of GPCRs as they compose 80% of known GPCRs, which include hormones and neurotransmitters (Hu et al., 2017), and also include the oxytocin receptor (OTR), ghrelin 1a receptor (GHSR), 5-HT 2a receptor (5-HT_{2A}) and the 5-HT 2c receptor (5-HT_{2C}). The class B GPCR family, which contain receptors associated with hormonal regulation, metabolic functioning and are associated with disorders of the nervous system (Hollenstein et al., 2014) includes the glucagon like peptide 1 receptor (GLP-1R).

The GPCRs, OTR, GHSR, 5-HT_{2A}, 5-HT_{2C} and GLP-1r, are known to play critical roles in variety of physiological process such as metabolism, and central appetite regulation, mood and social behaviour (Wei et al., 2017, Grammatopoulos, 2017, Wardman et al., 2016, Im, 2017). Moreover, alterations to the downstream signalling of GPCRs is shown to affect their role, leading to the onset of disorders associated with these areas such as obesity (Skrzypski et al., 2018, Funato et al., 2009), depression (Borroto-Escuela et al., 2018a) and autism (Brigida et al., 2017). In addition, to the complex pharmacology of GPCR signalling, it is now well accepted that GPCRs function not only as monomers but can also crosstalk with other GPCRs and even form dimers or higher order oligomers (Gomes et al., 2016). Such dimerization can result in alterations to GPCR downstream signalling, often effecting the physiological function of such GPCRs (Schellekens et al., 2015b, Borroto-Escuela et al., 2017a). However, the scale of GPCR dimerization and its role in the centrally-regulated mechanisms of food intake, mood, social interactions and other behaviours is still underappreciated and only beginning to emerge.

1.2 G-protein coupled receptors crosstalk and heterodimerization

The concept of G-protein coupled receptor (GPCR) homo-, hetero- and oligomerization, where single GPCR monomers form dimers or even higher order complexes (Figure 1.1), was first conceptualised over three decades ago, revealing a direct physical interactions between these seven transmembrane spanning receptors

on the cell membrane (Agnati et al., 1980, Fuxe et al., 1981). But the first accepted demonstration of functional GPCR heterodimerization came from the GABA B receptors, where it was shown heterodimerization of the GABA B receptor R1 and R2 subtypes was required for receptor functionality (Marshall et al., 1999). Since then the importance of GPCR heterodimerization and the putative downstream signalling consequences in the periphery and the central nervous system have become evident and it has been hypothesized that GPCR interactions play a significant role in the pathophysiology and treatment of many centrally-regulated disorders such as depression (Borroto-Escuela et al., 2017a). These GPCRs consist of homodimers, where the physical interaction involves two of the same receptors, for example an OTR-OTR homodimer; shown to stably exist on the cell membrane of COS-7 cells and human embryonic kidney cells independent of ligand treatment (Devost and Zingg, 2004b, Terrillon et al., 2003). Alternatively, heterodimerization can occur, which consist of a physical interaction between two different GPCRs. Previously, a novel heterodimer between the GHSR- and the 5-HT_{2C} was described by Schellekens et. al., who showed that interaction with 5-HT_{2C} was able to decrease GHSR signalling in vitro, furthermore, attenuating its ability to increase food intake in vivo (Schellekens et al., 2015b). Finally, evidence exists for higher order oligomers, where multiple GPCRs interact in multidomain complexes. For example, the dopamine D2 receptor is seen to form a physical interaction with four D2 receptors at a time on the membrane of a cell showing the existence of higher order homodimers (Guo et al., 2008).

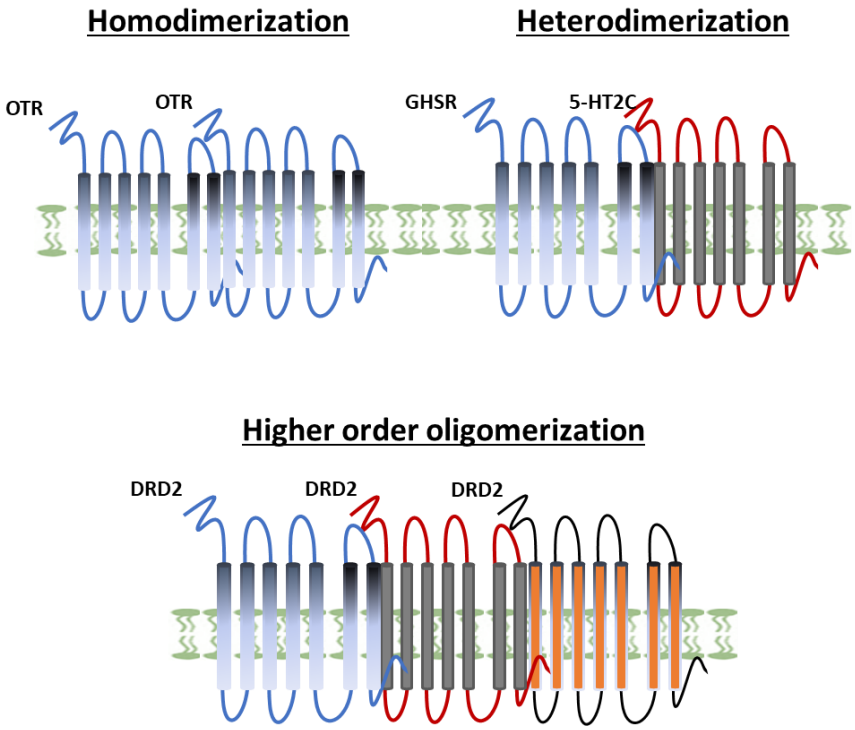

Figure 1.1: *Simplified schematic highlighting the different organisational structures of GPCR dimerization. Including homodimerization binding of two of the same receptors, heterodimerization the physical interaction between two different GPCRs and higher order oligomerization the binding of 3 or more GPCRs.*

Tools have now been developed that allow for the examination of GPCR topology and networking, using information from databases such as STRING and SCOPUS (Borroto-Escuela et al., 2014a). Websites such as G protein-coupled receptor heterodimer network (gpcr-hetnet.com) use connectivity analysis to reveal dominance between intra- and interfamily connections allowing for an easy view of possible strong and weak GPCR heterodimers. This allows for easy access to currently known dimer pairs (Borroto-Escuela et al., 2014a).

Such studies show the exciting potential of GPCR dimerization and its downstream signalling consequences, which will ultimately have an impact on functionality (Schellekens et al., 2013b, Borroto-Escuela et al., 2017a). Functionally, heterodimerization of specific GPCRs can result in the altered binding of receptor ligands (Siddiquee et al., 2013b). The binding of G-proteins to the intracellular domain of GPCRs can be altered, further resulting in changes to downstream signalling such as calcium (Ca2+) influx (Cudmore et al., 2012). Similarly pathways

such as β-arrestin recruitment, which control the trafficking of GPCRs from the membrane to the cytoplasm and vice versa can be altered, effecting the regulation of GPCR and ligand synthesis (Rocheville, 2000).

This aspect of GPCR signalling adds a novel dimension to GPCR pharmacology and paves the way for new GPCR-targeting therapeutics. In addition, GPCR species specific interaction may uncover pharmacological differences between animal and human therapeutic efficacies (Suratman et al., 2011). A wide range of these heterodimers and homodimers have now been identified, many centrally expressed GPCRs not only crosstalk but directly interact on the plasma membrane, with varying levels of interaction (Fuxe et al., 2010, Borroto-Escuela et al., 2013a, Borroto-Escuela et al., 2013b).

The identification of novel receptor-receptor interactions will to lead to new targets outside that of direct specificity for the receptor in question. Studies have shown that modulatory ligands of specific GPCRs can influence and activate receptors in which they have no specific affinity for due to the formation of a GPCR dimers. Thus, with 34% of current pharmacotherapies targeting unique GPCRs and 20% of current drugs in clinical trials targeting these receptors, it is vital that we understand the full extent of GPCR signalling, including these novel GPCR interactions (Schellekens et al., 2013c, Naumenko et al., 2014, Hauser et al., 2017, Hauser et al., 2018). Crosstalk and interactions between central GPCRs as described above are poised to have significant ramifications for drug response and treatment outcome (Goupil et al., 2013, Hübner et al., 2016). Indeed, GPCR interactions may allow for the use of combined drug treatments or bivalent drugs (Hübner et al., 2016, Berque-Bestel et al., 2008, Coke et al., 2016, Arnatt et al., 2016), which will allow a more specific and targeted drug treatment. Together, this highlights the pressing need to identify novel GPCR interactions within the central nervous system and to elucidate the downstream signalling consequences and functional relevance.

1.3 G-protein coupled receptors heterodimerization- A role in pharmacotherapy?

Over 50% of drugs are known to fail late stage clinical trials, with 57% of these drugs failing due to inadequate efficacy in stage 3 clinical trials (Hwang et al., 2016). Interestingly, such results are often unknown, but genetic variation of GPCRs are seen between individuals which can result in altered GPCR binding, explaining the reduced efficacy leading to failure of clinical trials or it can be hypothesised that genetic variations result in reduced or enhanced heteroreceptor complex formation (Hauser et al., 2018). Although a second hypothesis could be made, as 35% of available pharmacotherapies target GPCRs, the failures associated with GPCR targeted clinical trial drugs could be due to the formation of GPCR heterodimers (Sriram and Insel, 2018).

Both *in-vitro* and *in-vivo* studies have shown the physiological relevance of heterodimer pairs in this regard. Such that the analgesic effect of opioids is mediated by opioid receptor heterodimers, where *in-vitro* the highest effect of opioid receptor ligand 6'-guanidinonaltrindole was seen only when the opioid receptors were forming a heterodimer (Waldhoer et al., 2005). Moreover *in-vivo* studies revealed specific heterodimerization sites where the ligand 6'-guanidinonaltrindole was only active in the presence of this heterodimerization, this effect subsequently being blocked by antagonising at least one of the receptors within the heterodimer pair (Waldhoer et al., 2005).

Class F GPCRs such as the frizzled (Fz) Wnt receptors have also been implicated in the physiology of familial exudative vitreoretinopathy, specifically the heterodimerization of the Fz 4 and Fz 1 inactive receptor isoform with wild type FZ 4 and Fz1 respectively. Interestingly, this results in the trapping of the wild type functional receptors within the endoplasmic reticulum, resulting in reduced activity of the receptors which may explain the genetic component associated with familial exudative vitreoretinopathy (Kaykas et al., 2004).

Moreover, the anxiolytic effect of the OTR is altered by heterodimerization with the dopamine D2 receptor (de la Mora et al., 2016). The initial evidence of this

heterodimer pair was found by proximity ligation assays, heterodimerization being prominent in the nucleus accumbens and striatum (Romero-Fernandez et al., 2013). A follow-up study showed that blockade of the dopamine D2 receptor attenuated the oxytocin receptors anxiolytic effect within the amygdala (de la Mora et al., 2016). This effect can be linked the functional consequences of these heterocomplexes, the oxytocin receptor enhancing the MAPK signalling and reducing the PKA signalling of the dopamine D2 receptor, furthermore, the dopamine D2 enhancing the MAPK and IP-3 pathways of the oxytocin receptor (de la Mora et al., 2016).

The expression of GPCRs is likely also affected following dimerization and may vary between monomer expression and heterodimerization formation. Such that, ligand treatment has been shown to induce the formation of heterodimers. Indeed, heterodimerization between the cannabinoid 1 receptor and dopamine D2 receptors was increased with chronic cannabinoid treatment (Przybyla and Watts, 2010). This increase was attenuated following antagonism of either receptor, indicating a ligand-mediated bias in the formation of a cannabinoid 1 receptor and dopamine D2 receptor heterodimer (Przybyla and Watts, 2010). These findings are likely to have significant implications in antipsychotic treatment as many antipsychotics function as D2 receptor antagonists. Therefore, further understanding the differences in heterodimerization expression throughout the central nervous system and impact of this heterodimerization on receptor functionality may help with understanding the variation seen in drug treatments and further explain the large percentage of drug failures in clinical trials, as receptor expression differs across development, between sexes or physiological state such as hunger versus satiation, consequently effecting heteroreceptor formation, therefore altering treatment efficacy.

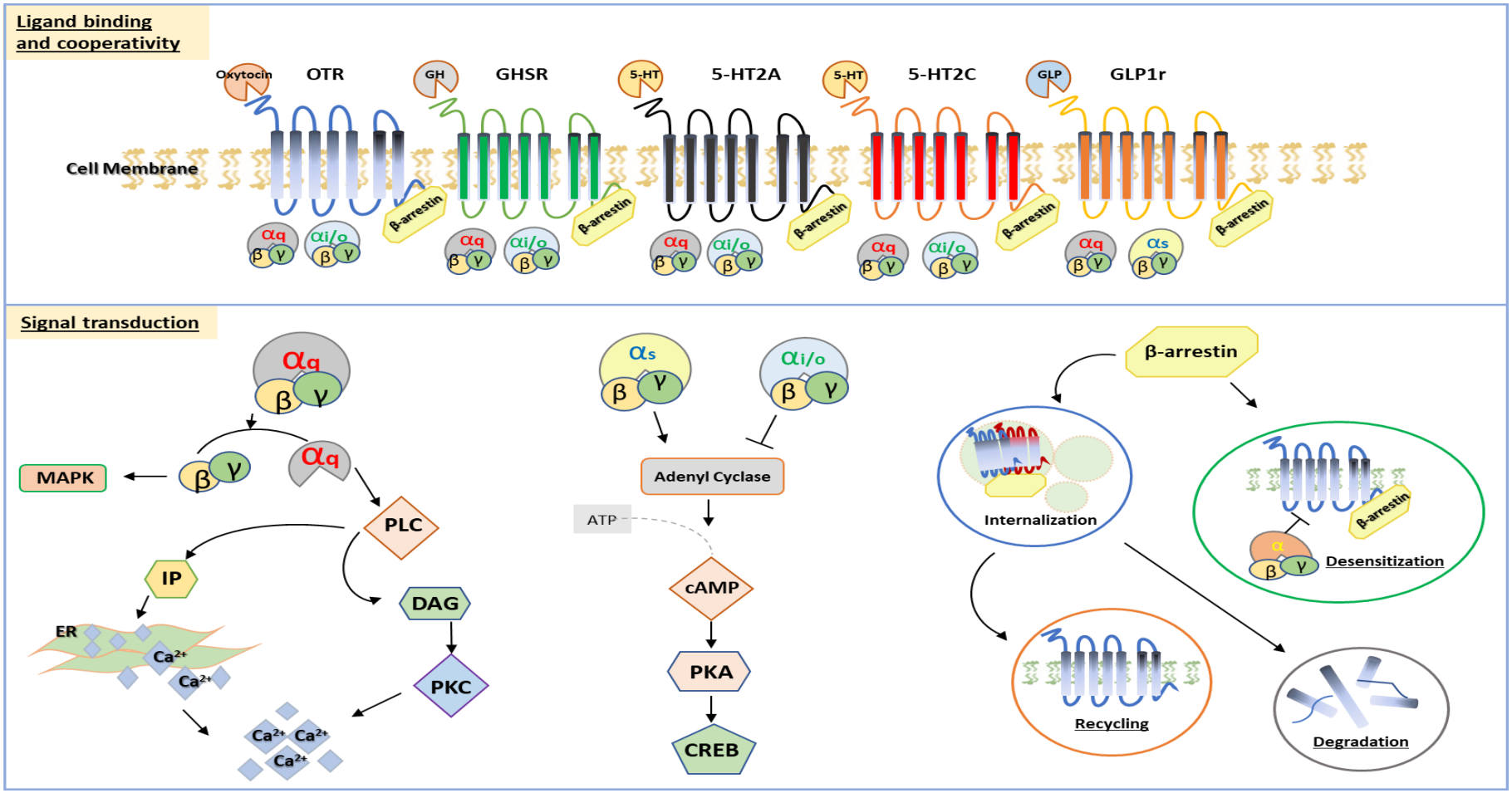

Figure 1.2: *Schematic illustrating the coupling of the OTR, GHSR, GLP-1R, 5-HT$_{2C}$ and 5-HT$_{2A}$ with their relative G-proteins and its subsequent downstream signalling pathway.*

1.4 Oxytocin and the oxytocin receptor

Oxytocin is an oligopeptide containing nine amino acyl residues, shown to be expressed in such sites as the paraventricular nucleus and the supraoptic nucleus of the hypothalamus and secreted through the posterior pituitary (Lam et al., 2006). The diverse functions of this hormone are regulated by the vast expression and subsequent activation of its GPCR known as the oxytocin receptor (OTR). The OTR is a 7-transmembrane receptor widely expressed throughout the central nervous system. Upon binding of oxytocin, the oxytocin/OTR mediated signalling occurs through Gαi/Gαq/Gαo proteins, which lead to further downstream signalling as described in Figure 1.2 (Chatterjee et al., 2016). The oxytocin system has classically been associated with labour and milk ejection during lactation but behavioural studies have now suggested a more central role in social bonds, maternal behaviour, separation response and social contact (Insel, 1992).

1.4.1 Structure and signalling

Oxytocin was first discovered in 1909 by Sir Henry Dale in extractions taken from posterior pituitary gland and linked to the onset of contractions in female cats (Dale, 1906). Following this in 1953 oxytocin became the first neuropeptide to be fully sequenced and synthesised (Du Vigneaud, 1956). The oxytocin peptide is transcribed from chromosome 20p13 from the oxytocin-Neurophysin I gene and synthesised in the paraventricular and supraoptic nucleus. It is initially synthesised as an inactive precursor, where co-synthesis carrier protein neurophysin I transports it to the posterior pituitary for subsequent maturation (Rao et al., 1992). The pre-oxytocin gene consists of three exon regions; the first exon spans the signalling region, oxytocin gene, GLL processing signal and into the neurophysin; the second exon encodes the middle section of the neurophysin; the third exon codes for the end of the neurophysin. The protein is synthesised as a precursor with neurophysin, which is cleaved by enzymatic digestion at the glycyl-lysyl-arginine (GLL) region. This results in the three exons and four intron active oxytocin gene which is used to synthesis the oxytocin protein, a nine-amino acid peptide connected by a disulphide bond at position 1&6 (cysteine-cysteine) (Gimpl and Fahrenholz, 2001b). Thus, secreted into the bloodstream and the brain via axon terminals and collaterals of the

magnocellular oxytocin neurons of the hypothalamus, once secreted being simultaneously projected to the forebrain and posterior pituitary, studies showing that oxytocin release from these axonal endings can lead to control in behaviour (Knobloch et al., 2012).

The OTR is a 388-amino acid polypeptide, with a 7-transmembrane structure as expected for a GPCR. The OTR responds to oxytocin with great specificity and binding affinity (Kimura et al., 1992b). The OTR gene consists of three introns and four exons across a region of 17 kilobases, a number of single nucleotide polymorphisms in this gene have been studied and are associated with many social disorders (Feldman et al., 2015).

The OTR is ubiquitously expressed throughout the body, including in tissues such as the kidney, heart, thymus, pancreas, and adipocytes and cells including cancer cells, endothelial and myoblasts and the brain, explaining its vast array of functionalities, including reproduction, social behaviour and appetite regulation (Ott et al., 2013a, Meziane et al., 2015, Gonzalez-Iglesias et al., 2015, Gimpl and Fahrenholz, 2001b). Oxytocin is released in both close and distant OTR expressed regions, where, depending on the location of the receptor, its exact physiological function is initiated. Thus, the specific OTR-mediated signalling cascades and effects are being determined by both the localization of the receptor (i.e hypothalamus and limbic system) as well as the concentrations of the neuropeptide in the ECF and receptor density (Landgraf and Neumann, 2004, Neumann and Landgraf, 2012).

Once bound by an agonist the OTR activates multiple possible pathways; such as the MAPK (Mitogen-activated protein kinase) signalling pathway, which is implicated in the transduction of cell signals to cell responses such as proliferation, development, transformation, and apoptosis (Zhang and Liu, 2002). Another signalling pathway that is activated following oxytocin binding to OTR is the modulation of neuronal excitability through the activation of the G-protein inwardly-rectifying potassium channels (GIRK) (Lüscher and Slesinger, 2010). The main pathway of the OTR is through the activation of the Gαq alpha subunit, which subsequently activates Phospholipase C (PLC), which cleaves Inositol trisphosphate (IP3) and diacylglycerol (DAG) (Billups et al., 2006). DAG phosphorylates protein kinase C (PKC) (Wang, 2006),

this reaction leads to a number of events either being implicated in MAPK signalling through the proto-oncogene serine/threonine-protein kinase (New and Wong, 2007), or protein synthesis and subsequent tropic effect via Eukaryotic elongation factor-2 kinase (CAMKIII) (Devost et al., 2008). The activation caused by both PKC-cyclic ADP ribose hydrolase (CD38) and IP3- Inositol trisphosphate receptor(IP3R) and Ryanodine receptor (RYR), lead to the release of calcium (Ca2+) from intracellular stores, this calcium induces a cascade of activity shown to produce contractions in labour, aid in cardiovascular control, endothelial cell migration, neuronal activation and feedback control of oxytocin release (Viero et al., 2010). These signalling pathways can be targeted using select ligands that activate specific pathways (Busnelli et al., 2012).

1.4.2 Central oxytocin receptor expression

OTR is located in multiple regions across the brain as shown in Figure 1.3 from (Grinevich et al., 2015). Using *in-situ* hybridisation OTR was shown in areas such as the prefrontal cortex, anterior olfactory nuclei, olfactory tubercle, and sections of the hypothalamus, the amygdala, hippocampus and the dorsal motor nucleus of the vagus (Yoshimura et al., 1993, Loup et al., 1991). Studies have implicated these OTR areas in specific functioning such as steroid sensitive reproductive behaviours (hypothalamic ventromedial nucleus), maternal behaviour (ventral tegmental area), learning and memory (hippocampus) and reinforcement (frontal cortex, amygdala) (Ostrowski, 1998).

Using blood oxygenation level dependent (BOLD) fMRI another study looked at areas of the brain that responded to oxytocin using centrally administered oxytocin, which demonstrated a reduction in anxiety like behaviour, enhanced by fear (Febo et al., 2009). In addition, an effect in both olfactory regions and the forebrain was found. An, increase in BOLD was found in the anterior cingulate, bed nucleus of stria terminalis and perirhinal area, while decreasing BOLD in areas such as motor cortex and prelimbic prefrontal cortex. This suggests, that oxytocin and its receptor increase emotion and cognition while lowering autonomic and skeletal motor responses, revealing that a myriad of brain region activation by oxytocin induces significant changes in emotion and cognitive state (Febo et al., 2009).

Another method employed by B.R.Gould and H.H.Zingg used a oxytocin receptor-LacZ reporter mouse. The use of this procedure allowed for novel areas of OTR expressing neurons to be discovered in accessory olfactory nucleus, the medial septal nucleus, and the facial nucleus (Gould and Zingg, 2003). Heon-Jin Lee et al used a conditional knockout mouse line to aid in better understanding the role of OTR in in specific brain regions. In this protocol there were two mouse lines used to eliminate the OTR from brain areas at specific times, either using Cre recombinase in all tissue (OTR (-/-)) or just in the forebrain (OTR (FB/FB)). As a result, OTR (-/-) had reduced social memory while OTR(FB/FB) could not recognise individuals but could differentiate between species. Such studies allowing for a greater more precise understanding of the OTR role in the brain (Lee et al., 2008).

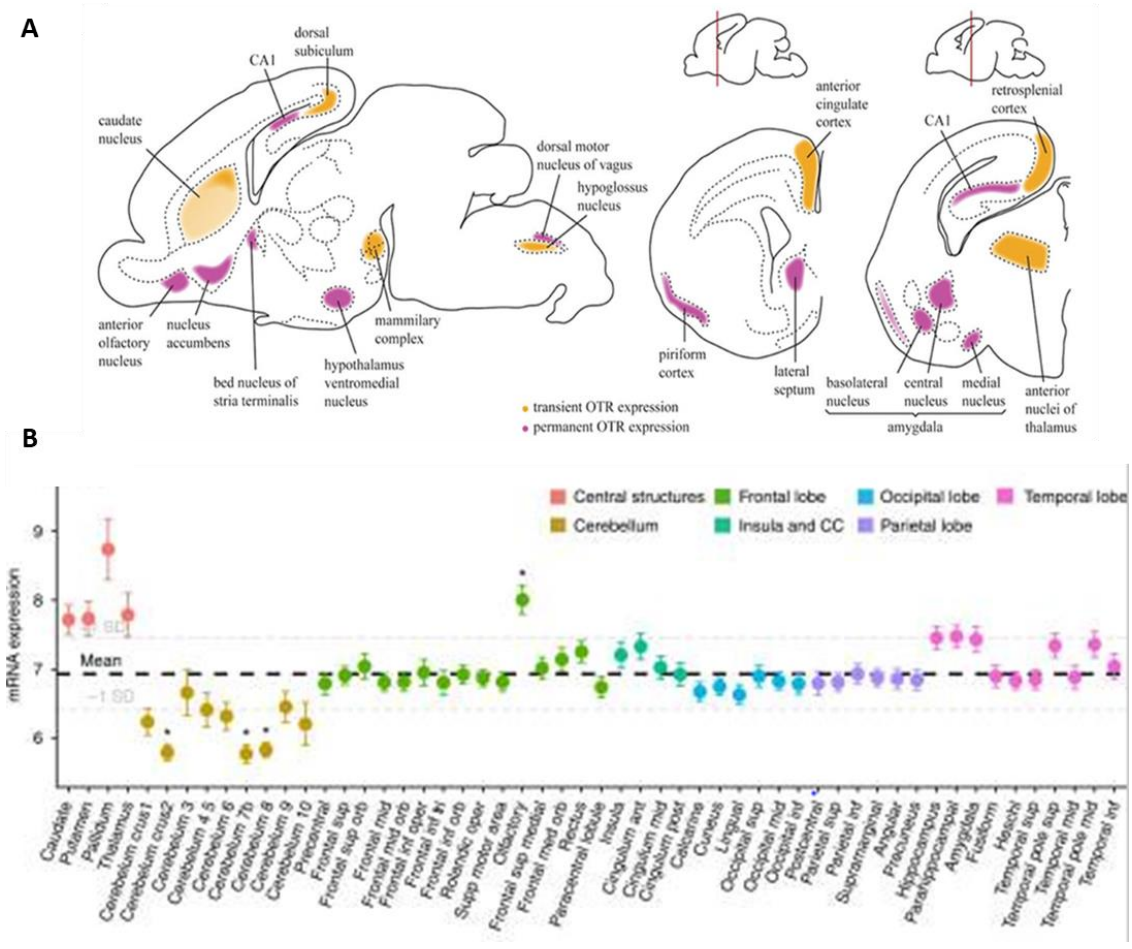

Figure 1.3: (A) *Illustration of OTR developmental expression in rats, revealing regions of both transient and permanent OTR expression (Grinevich et al., 2015).* ***(B)*** *OTR expression in the human adult brain (Quintana et al., 2019).*

A human OTR central expression study was recently conducted, analysing 1 female and 5 males of different ethnicities from known mRNA distribution data. Central structures, such as pallidum and thalamus and regions of the cerebellum such as 3 and 10 had the highest level of variability between subjects with expression in other areas remaining constant across the 6 subjects (Quintana et al., 2019). The highest levels of OTR expression was found in the olfactory region of the frontal lobe (Quintana et al., 2019), figure 1.3B highlights the varying expression across brain regions of these subjects. However, OTR expression can be altered in individuals with disorders such as schizophrenia, with the use of qRT-PCR OTR levels were shown to be reduced in areas such as the Brosmann 10&21, the caudate nucleus and the vermis, furthermore altered receptor binding is also observed in these regions (Uhrig et al., 2016). This reduction in OTR expression and binding is influenced by the level of cholesterol in the system, where cholesterol has been shown to stabilise the OTR against thermal and proteolytic degradation, while acting as a positive allosteric modulator to allow for a high affinity OTR state (Gimpl et al., 2008, Gimpl and Fahrenholz, 2002). Therefore, when examining OTR signalling *in-vivo* such changes to receptor expression and binding affinity must be taken into consideration.

1.4.3 Oxytocin interactions, crosstalk and heterodimerization

The oxytocin receptor plays a role across multiple centrally related behaviours such a food intake (Lawson, 2017), social behaviour (Churchland and Winkielman, 2012) and mood (Broadbear et al., 2014). Therefore, oxytocin is known to crosstalk with other systems to elicit its effect in such behaviours as shown in table 1.1. For example, oxytocin is known to interact with orexigenic hormone ghrelin, where intravenous oxytocin administration is known to reduce the circulating levels of ghrelin (Vila et al., 2009). Furthermore, ghrelin treatment on neurohypophyseal cells resulted in enhanced oxytocin secretion within the cells (Gálfi et al., 2016). Oxytocin is also known to have a modulatory effect on serotonin (5-HT), where oxytocin administration results in modulation of the serotonergic system, including modulation of 5-HT synthesis and 5-HT receptor binding potential (Mottolese et al., 2014). Such crosstalk can be implicated in the areas of appetite control, social

behaviour and mood disorders, therefore fully elucidating such crosstalk via heterodimerization studies will enhance the current knowledge of these behaviour. Table 1.1 summarises the current known oxytocin receptor heterodimer pairs and the main signalling and functional consequences of these heterodimer pairs within these studies.

Table 1.1: Currently known OTR heterodimers

Heterodimer receptor (OTR+)	Model	Main Findings	Reference
β2-adrenergic receptor	*In-vitro*- hTERT-C3 myometrial cells, HEK293 cells	A physical interaction was shown between OTR and the β2-adrenergic receptor indicating receptor heterodimerization, with consequent functional impacts listed below: - oxytocin-induced ERK1/2 signalling was significantly attenuated following β2-adrenergic receptor agonism - β2-adrenergic receptor antagonism increased oxytocin-induced ERK1/2 signalling - OTR antagonism decreased β2-adrenergic receptor-induced ERK1/2 signalling	(Wrzal et al., 2012)
Dopamine D2 receptor (DRD2)	*In-vitro* - HEK293 cells *Ex-vivo*- Rat	- Co-localization of OTR and DRD2 in the nucleus accumbens and striatum indicates formation of a GPCR heterodimer. - Oxytocin treatment enhanced the quinpirole induced inhibition of the PKA-pCREB signalling and enhanced signalling of the MAPK pathway. - DRD2 agonism enhanced the oxytocin-mediated increases in PLCbeta-IP3-calcineurin and MAPK signalling. - DRD2 antagonism inhibits oxytocin-mediated anxiolytic effect	(de la Mora et al., 2016, Romero-Fernandez et al., 2013)
Vasopressin 1a & 2 receptor & OTR (homodimer)	*In-vitro* - HEK293 cells	- Bioluminescence resonance energy transfer and co-immunoprecipitation reveal a physical interaction between OTR with OTR or vasopressin 1a & 2 receptor	(Terrillon et al., 2003, Devost and Zingg, 2004a)

1.5 Ghrelin and the ghrelin 1a receptor

Ghrelin was first discovered in 1999 in extracts from a rat stomach and is a 28-amino acid peptide, with a n-octanoyl serine-3 modification which is essential for its action (Kojima et al., 1999). Ghrelin in both rats and humans have similar homology, where in humans the ghrelin gene is located on the chromosome within the 3p25-26 region (Sato et al., 2012). To date it is the only known orexigenic hormone to be secreted in the periphery, eliciting its central effects by passing the brain blood barrier. Ghrelin is the endogenous ligand for the ghrelin 1a receptor also known as the growth hormone secretagogue receptor (GHSR), which was first discovered in 1996, found at the time to be expressed in both the pituitary and hypothalamus with properties that controlled growth hormone release (Howard et al., 1996).

1.5.1 Structure and signalling

Ghrelin is a peptide produced in the stomach (Ariyasu et al., 2001) following transcription and translation from the preproghrelin gene, located on chromosome 3 (3p25-26). Preproghrelin is a 117-amino acid residue, where 23 amino acids encode signalling peptides and 94 amino acids encode proghrelin (Delporte, 2013). Preproghrelin is post-translationally modified to produce ghrelin. Here, peptidase enzymes, the endoprotease prohormone convertase 1/3 and carboxypeptidase- B, are responsible for cleavage and subsequent conversion of preproghrelin to ghrelin (Zhu et al., 2006, Delporte, 2013). Interestingly, preproghrelin is distinguishable from other prohormones in that it does not only result in post-translational production of ghrelin but also other peptides. These peptides include des-acyl ghrelin hypothesised to act upon a different receptor to that of ghrelin, but the exact receptors are still unknown and identified to interact functionally to inhibit ghrelin (Delhanty et al., 2013, Satou et al., 2011). Obestatin, another derivative of preproghrelin is known to again to act upon a different receptor to that of ghrelin but also has an opposite effect of ghrelin, reducing food intake (Zhang et al., 2005). Intriguingly, ghrelin is conserved across all mammals, in that 10 of the N-terminal amino acids are identical with n-octanoylation of serine-3 occurring across most vertebrates. Ghrelin exerts its central physiological effect through its ability to pass the brain blood barrier, reaching multiple regions of the brain such as the hypothalamus and hippocampus, to where

it subsequently activates the GHSR (Rhea et al., 2018). The GHSR is a 366-amino acid 7 transmembrane receptor, belonging to the class A rhodopsin GPCRs (Howard et al., 1996). A truncated ghrelin receptor known as the ghrelin 1b receptor also exists, although the direct function of this isoform is unknown and is not activated by ghrelin (Navarro et al., 2016). Moreover, it is thought that the GHSR function is partially controlled by heterodimerization of the ghrelin 1b receptor as shown in table 2.2 (Leung et al., 2007, Navarro et al., 2016). The GHSR is known to primarily signal through the Gαq signalling pathway (Damian et al., 2015) as depicted in Figure 1.2. Similar to that of the OTR, activation of the Gαq subunit results a cascade of events ending in the release of calcium from intracellular stores (Billups et al., 2006). Moreover, GHSR is known to mediate multiple other signalling pathways, including the $G_{12/13}$ which activates the Ras homolog gene family, member A (RhoA), both the Gαq and $G_{12/13}$ are responsible for activation of the serum response element associated with GHSR activation (Sivertsen et al., 2011). Internalization of the GHSR and its high basal constitutive activity is mediated by C-terminal recruitment of β-arrestin (Evron et al., 2014). Interestingly, the activation and reduction of these signalling pathways is dependent on which ghrelin receptor ligand is bound, such that the synthetic GHSR ligand SP-analogue attenuates β-arrestin signalling, while acting as an inverse agonist for other G proteins (Ramirez et al., 2018).

Ghrelin mediates a multitude of centrally-regulated physiological responses, one of the main areas of study in this regard is in appetite regulation (De Vriese and Delporte, 2007). Overproduction of ghrelin in the hypothalamus through viral methods results in increased food intake, the effect of which is reversed after 3 weeks due to a compensatory mechanism in which the pro-opiomelanocortin mRNA levels are significantly up-regulated (Qi et al., 2015). Moreover, peripheral injection of ghrelin and GHSR agonists similarly result in increased food intake and substantial weight gain in both humans and animal models (Wren et al., 2001, Holubova et al., 2013). Ghrelin aids in the regulation of glucose metabolism; acute ghrelin administration results in hyperglycaemia, while chronic treatment regulates this and restores insulin levels again indicating a possible compensatory mechanism, possibly by interactions with the insulin receptor leading to altered receptor signalling

(Goshadrou et al., 2015). This may also explain the effect of hyperglycaemia in abolishing satiety signalling after food consumption, due to increases in ghrelin revealing a possible feedback loop between ghrelin and plasma glucose (Knudsen et al., 2014, Sim et al., 2014).

1.5.2 Central ghrelin receptor expression

The GHSR is shown throughout many regions of the brain, particularly those involved in food intake and reward, as depicted in Figure 1.4 (Schellekens et al., 2013a). *In-situ* hybridisation revealed the expression of GHSR mRNA in both mouse and rat brains. Here the expression between both mice and rats varied, within the hippocampus, strong expression was seen in the cornu ammonis (CA) regions, CA1, CA2 and CA3 and dentate gyrus in rats compared to inconsistent expression in mice. Similarly, this variation is seen throughout the hypothalamus, within the ventromedial nucleus, with expression in rats only seen in the central and dorsomedial ventromedial nucleus and ventrolateral and capsule of mice, with consistent expression between mice and rats in the arcuate nucleus and suprachiasmatic nucleus. All areas of the substantia nigra, ventral tegmental area, nucleus of the solitary tract, magnocellular nucleus remained consistent in both mice and rats. Many other areas throughout the midbrain, pons and medulla oblongata demonstrated differential expression, which may contribute to the varied behaviours associated with ghrelin receptor activity (Zigman et al., 2006a). Although few studies have investigated central GHSR expression in animal models even fewer have identified central expression in the human brain with only one study showing expression in the cerebrum and cerebellum (Ueberberg et al., 2009).

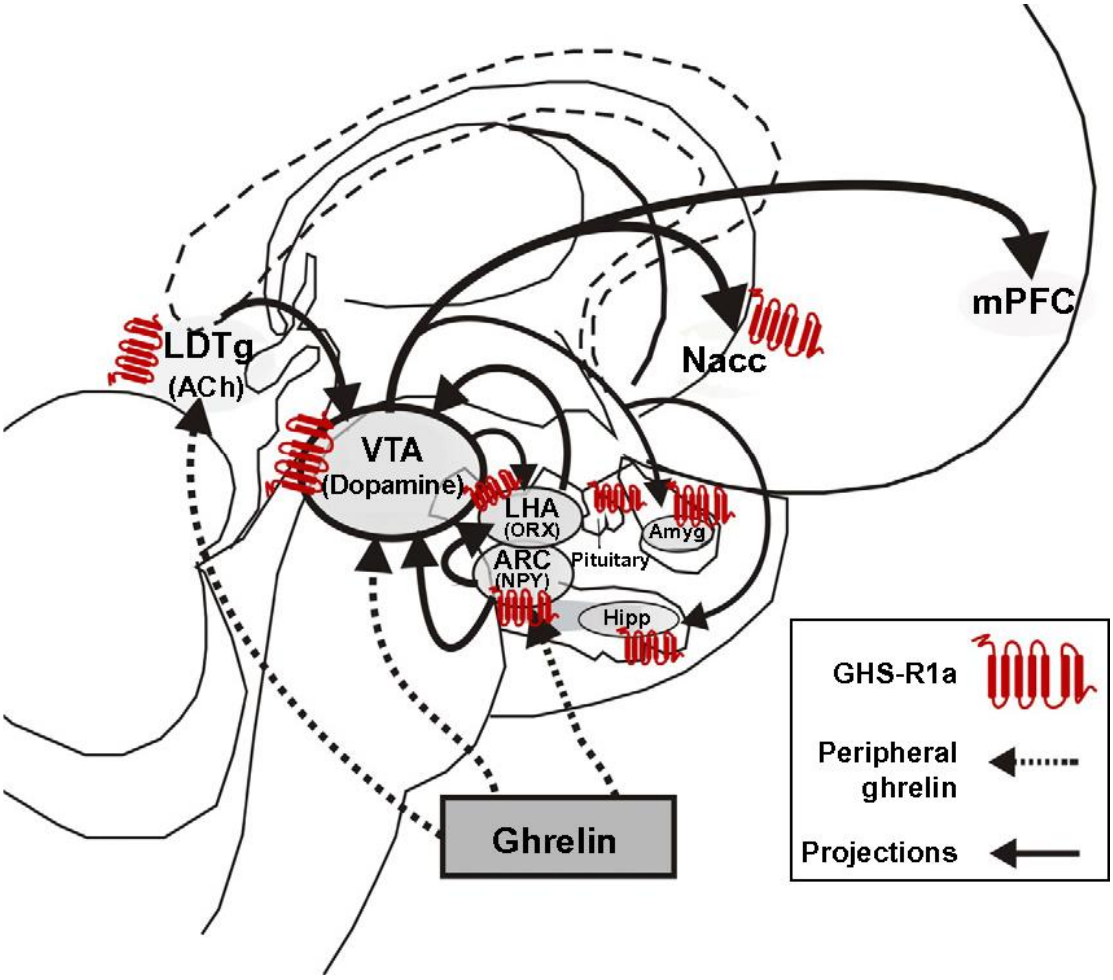

***Figure 1.4**: Schematic of GHSR expression in areas involved in obesity and reward, including projection lines and areas receiving peripheral ghrelin (Schellekens et al., 2013a).*

1.5.3 Ghrelinergic crosstalk and heterodimerization

The ghrelinergic system is long known to crosstalk with other pathways, influencing both signalling and secretion of other receptors and peptides as seen in table 1.2 (Blanco et al., 2017). Many of these are pathways that are also involved in appetite regulation, such that in neuropeptide Y (NPY) containing neurons of the arcuate nucleus, which are influenced by ghrelin in a dose dependent manner increase calcium signalling. The addition of orexin further increased ghrelins response and leptin attenuated ghrelin's response in these neurons, implicating ghrelin crosstalk with these systems in the regulation of appetite within the arcuate nucleus (Kohno et al., 2003). Knockdown of leptin expression in the lateral hypothalamus attenuates ghrelin's mediated effect on hypocretin/orexin expressing neurons, the neurons of the lateral hypothalamus involved in increasing food intake (Brown et al., 2017). The

cannabinoid receptor type one (CB1), like GHSR is also known to increase food intake, interestingly, antagonism of CB1 results in decreased food consumption and reduced plasma ghrelin levels in both fed and fasted animals (Cani et al., 2004). Such effects can be linked to the paraventricular nucleus of the hypothalamus where ghrelin's mediated increase in food intake is again attenuated by antagonism of CB1, again showing the functional crosstalk in ghrelinergic signalling required in appetite regulation (Tucci et al., 2004). Ghrelin crosstalk has also been implicated in the reward system (Abizaid et al., 2006). Ghrelin's effect in the reward system was shown to be facilitated through a glutamatergic-dopaminergic-ghrelinergic pathway within the ventral tegmental area, in which antagonism of the N-methyl-D-aspartic acid receptor attenuated ghrelin effect, although no change was seen when antagonists of the orexin or opioid receptors were present, highlighting the specificity of this crosstalk between behaviours and brain regions (Jerlhag et al., 2011). The ghrelinergic system is known to crosstalk with other pathways, but the GHSR itself is also known to form functional heterodimers with other receptors as described in table 1.2, where it can mediate the signalling of these other receptors, but also affects GHSR signalling. The table below highlights the main findings associated with all GHSR heterodimers currently known.

Table 1.2: Currently known ghrelin receptor heterodimers

Heterodimer receptor (GHSR+)	Model	Main Findings	Reference
5-HT_{2C} receptor	*In-vitro* - HEK293a *Ex-vivo* - rat primary cultures *In-vivo* - C57BL/6 mice	- 5-HT_{2C} co-expression significantly attenuated GHSR Gαq signalling which was rescued by 5-HT_{2C} antagonism - *In-vivo,* 5-HT_{2C} antagonism significantly increased GHSR-mediated food-intake.	(Schellekens et al., 2015a, Schellekens et al., 2013e)
Probable G-protein coupled receptor 83 (Gpr83)	*In-vitro* - HEK293a *In-vivo* - C57BL/6 mice	- Gpr83 co-expression attenuates GHSR-mediated IP3 signalling - *In-vivo,* Gpr83 knockout results in increased ghrelin-induced food intake.	(Muller et al., 2013)
Melanocortin receptor 3 (MCR3)	*In-vitro* - HEK293a	- GHSR and MCR3 dimerization results in function alteration to both receptors - GHSR increases MCR3-mediated Gαs signalling - MCR3 co-expression reduces GHSR Gαq signalling	(Schellekens et al., 2013e, Rediger et al., 2011)
Vasoactive prostanoid receptors	*In-vitro* - HEK293a	- Oligomerization of the prostaglandin E2 receptor subtype EP3-I, prostacyclin receptors and thromboxane A2 with	(Chow et al., 2008)

		GHSR results in reduced activation of GHSR-induced phospholipid C signalling	
Somatostatin receptor type 5 (SST5)	*In-vitro* – HEK293a and INS-1 832/13 cells	- SST5 receptor and GHSR form a functional heterodimer - Dimerization cause coupling of Gαi to GHSR to be increased which resulting in suppression of cAMP - GHSR-mediated glucose-stimulated insulin secretion is enhanced in the presence of the SST5 receptor	(Park et al., 2012)
Dopamine D1 receptor (D1DR)	*In-vitro* – HEK293a and SK-N-SH cells	- Co-expression of D1DR and GHSR increases D1DR cAMP accumulation - A synergistic effect during co-expression is shown upon ghrelin and dopamine co-administration	(Schellekens et al., 2013e, Jiang et al., 2006)
DRD2	*In-vitro* – HEK293a *In-vivo* - C57BL/6 mice	- GHSR and DRD2 dimerization increases DRD2 -mediated calcium mobilization - Antagonism of the GHSR *in-vivo* blocks DRD2-mediated anorexigenic effect	(Kern et al., 2012)
Ghrelin 1b receptor	*In-vitro* – HEK293a	- Upon heterodimerization of GHSR and the Ghrelin 1b receptor increased migration into the cytosol was seen	(Navarro et al., 2016, Leung et al., 2007)

		- GHSR-mediated Gαi signalling was enhanced upon co-expression of both receptors.	
Orexin 1 receptor	*In-vitro* - HEK293a and SH-SY5Y cells	- Heterodimerization enhances GHSR-mediated Gαs signalling - The orexin 1 receptor reduces the GHSR-enhanced Gαq signalling pathway, indicating a shift in GHSR G protein bias	(Xue et al., 2018)

1.6 Serotonin and the 5-HT_{2A} and 5-HT_{2C} receptor

The serotonergic system is one of the most ambivalent centrally-regulated systems due to it's different associated outcomes across behavioural studies. Serotonin (5-HT) plays a critical role in early development (Sundstrom et al., 1993, Gaspar et al., 2003), where variation in genetic make-up or expression patterns of 5-HT in early life can lead to individuals being more sensitive to environmental factors that effect and regulate (Brummelte et al., 2017). It is also involved in glucose metabolism and type 2 diabetes (Zhou et al., 2007a). The serotonergic system also play a role in anxiety (Akimova et al., 2009), depression (Blier and El Mansari, 2013), autism (Muller et al., 2016), anorexia (Haleem, 2012), obesity (Crane et al., 2015), schizophrenia (Bleich et al., 1988) and Parkinson's disease (Politis and Niccolini, 2015).

Circulating 5-HT elicits its effect across multiple receptors. The 5-HT receptors are classified into 7 families (5-HT 1-7) which are further split into subgroups; the 5-HT1 receptor family are GPCRs primarily known to signal through the Gαi alpha subunit, decreasing cAMP accumulation. 5-HT1 consists of the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors. The 5-HT2 receptors are also classified as GPCRs primarily signalling through the Gαq alpha subunit enhancing intracellular calcium release and are sub-grouped into 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (McCorvy and Roth, 2015). The 5-HT3 family are the only non-GPCR classified 5-HT receptors, belonging to the Cys-loop superfamily of ligand-gated sodium and potassium ion channels, sub grouped into 5-HT_{2A} and 5-HT_{3B} (Thompson and Lummis, 2006). The 5-HT4, 5-HT6 and 5-HT7 are also GPCRs with no subgrouping and signal through the Gαs alpha subunit, increasing intracellular cAMP release. The 5-HT5 family is sub-grouped into 5-HT_{5A} and 5-HT_{5B} which signal through the Gαi subunit (McCorvy and Roth, 2015). For the purpose of this thesis, the following section will focus on the 5-HT_{2A} and 5-HT_{2C} receptors.

1.6.1 Structure and signalling

5-HT was first discovered in 1948 (Rapport et al., 1948). Interestingly 5-HT was first isolated from the gastrointestinal tract in 1937, then named enteramine; was found to cause smooth muscle contraction and in 1952 it was discovered that enteramine was indeed 5-HT (Erspamer and Asero, 1952). In 1957 it was thought that 5-HT was only neurotransmitter (Brodie and Shore, 1957) with the central serotonergic system

first being classified in 1964 following identification of specific 5-HT containing nuclei throughout the brain (Dahlstrom and Fuxe, 1964). 5-HT is biosynthesised from the dietary precursor tryptophan. Tryptophan is oxidised by tryptophan hydroxylase to produce 5-hydroxytryptophan which is subsequently decarboxylated by L-aromatic amino acid decarboxylase to yield 5-hydroxytryptamine (5-HT) (Clark et al., 1954).

As previously described 5-HT acts upon multiple receptors one of these being the 5-HT_{2A} receptor (5-HT2A). 5-HT_{2A} was first cloned in 1990 (Julius et al., 1990), a 471 amino acid sequence located on the human chromosome 13 (13q14-q21) (Sparkes et al., 1991). Changes to 5-HT_{2A} and its signalling are linked to many disorders such as, depression (Celada et al., 2004), addiction (Underwood et al., 2008) and schizophrenia (Muguruza et al., 2013a). Interestingly, the psychosis of schizophrenia has been inked to 5-HT_{2A} due to the research into hallucinations associated with recreational drug usage, found to elicit their effect through activation of 5-HT_{2A} (Lopez-Gimenez and Gonzalez-Maeso, 2018).

5-HT_{2C} receptor is a 458-amino acid sequence located on the X chromosome, this gene spans across 20kb of four exons where its sequence is highly conserved between different mammals (Stam et al., 1994). Similar, to 5-HT_{2A}, 5-HT_{2C} plays a role in many centrally-regulated functions such as mood (Serretti et al., 2004), anxiety (Kimura et al., 2009), depression (Millan, 2005), appetite (Halford and Harrold, 2012) and sexual behaviour (Popova and Amstislavskaya, 2002).

The 5-HT2 receptor family signalling is depicted in Figure 1.2. This family of 5-HT receptors is known to primary signal through the Gαq alpha subunit, subsequently activating phospholipase C (PLC) resulting in the production of inositol triphosphate (IP3) and diacylglycerol (DAG), which aids in the release of calcium from intracellular stores (Roth et al., 1998). Moreover, 5-HT_{2A} and 5-HT_{2C} are both known to signal through the Gαi subunit; upon activation of this pathway, Gαi inhibits adenylate cyclase which in hand decreases the synthesis of cyclic adenosine monophosphate (cAMP), therefore reducing phosphorylation of protein kinase A (PKA) (Coward et al., 1998).

1.6.2 Central serotonin receptor expression

All 5-HT receptors subtypes are expressed widely throughout the brain as shown in Figure 1.5 (Compan et al., 2012). Using in-situ hybridisation 5-HT_{2A} was analysed in the brain of a marmoset, where high densities of 5-HT_{2A} were found to localise in the central lateral nucleus, thalamic reticular nucleus; the hippocampus had expression in sub-regions such as the CA1, CA2, CA3 and dentate gyrus, with the highest densities in the lateral amygdala and the medial mammillary nucleus of the hypothalamus, and throughout multiple regions of the striatum (Shukla et al., 2014). Using autoradiographic mapping and RT-PCR, post-mortem analysis saw similar distributions of 5-HT_{2A} in humans; the most prominent expression was seen in cortical brain regions, with intermediate expression in the amygdala, hypothalamus, hippocampus, thalamus and nucleus accumbens, with little to no expression in the cerebellum and putamen (Dwivedi and Pandey, 1998, Pazos et al., 1987). In situ hybridisation on brain slices from adult Wister rats revealed high expression of 5-HT_{2C} in the lateral nucleus of the hypothalamus, substantia nigra, CA1-3 regions of the hippocampus and throughout the brain stem and spinal cord (Pompeiano et al., 1994).

The expression of 5-HT_{2A} and 5-HT_{2C} has also been examined throughout development in the rat brain using western blotting and immunohistochemistry (Li et al., 2004). Expression between brain regions and expression throughout development changes between receptors, with high expression of 5-HT_{2C} seen in the epithalamus, thalamus, and subthalamus at later stages of development compared to low expression with 5-HT_{2A} with similar expression throughout the amygdala and basal ganglia (Li et al., 2004). Overall, as can be seen in Figure 1.5B the 5-HT_{2A} receptor tends to increase through development, while the 5-HT_{2C} receptor can increase and decrease across development as seen in the brain stem. The dynamic expression of the 5-HT_{2A} and 5-HT_{2C} receptors should therefore be taken into account when examining the physiological function of these receptors *in-vivo*, an example of this is the effect of 5-HT_{2C} on the hallucinogen 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), here reduction in 5-HT_{2A} results in a blunting of DOI induced

locomotor activity, therefore such trials in younger animals would not be as effective due to reduced expression of 5-HT_{2A} (Halberstadt et al., 2009).

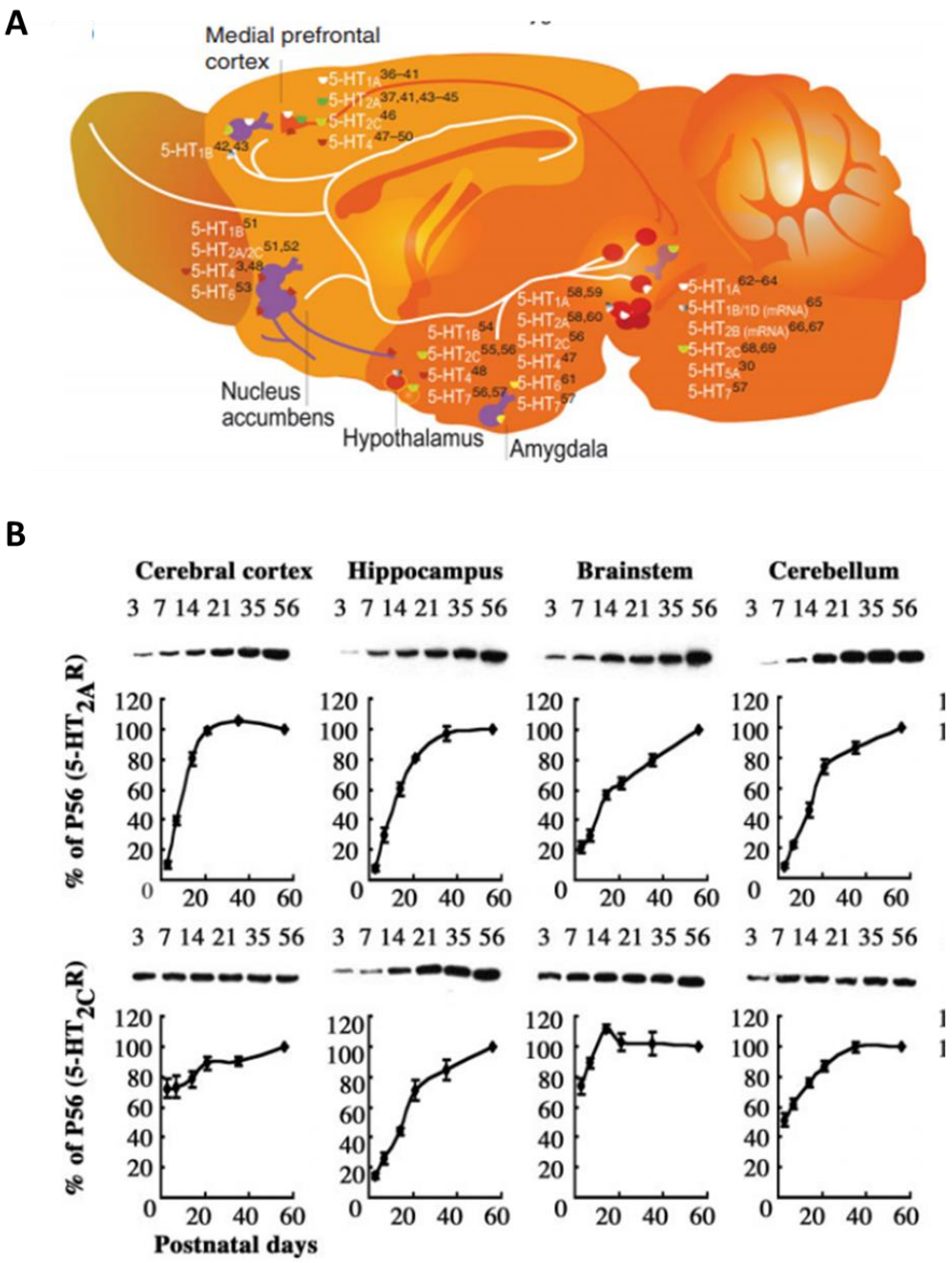

Figure 1.5: (A) *Schematic illustrating some of the many brain regions expressing 5-HT receptor signalling, specifically those involved in eating disorders such as the medial prefrontal cortex, nucleus accumbens, hypothalamus and amygdala (Compan et al., 2012)* ***(B)*** *Western blot of male wistar rat brain, showing the expression of 5-HT_{2A} and 5-HT_{2C} across multiple brain regions, ranging from postnatal day 3 to postnatal day 56 (Li et al., 2004).*

1.6.3 Serotonergic crosstalk and heterodimerization

Similarly to oxytocin and ghrelin, 5-HT is known to interact with other signalling pathways in the central nervous system whether through crosstalk or heterodimerization of the 5-HT receptors as seen in table 1.3. Within depression 5-HT is known to impact norepinephrine signalling. Specifically the activation of serotonergic neurons is known to have an inhibitory effect on norepinephrine signalling, therefore drugs targeting the 5-HT system have corresponding effects on the norepinephrine system (Blier, 2001). Oxytocin administration reduces 5-HT neurotransmission on areas associated with emotional regulation, again highlighting the importance of 5-HT crosstalk in psychiatric disorders (Mottolese et al., 2014). Heterodimerization of the 5-HT receptors in particular the 5-HT_{2A} and 5-HT_{2C} have been implicated in the dysregulated signalling associated with multiple disorders, table 1.3 highlights these currently known 5-HT_{2A} and 5-HT_{2C} heterodimer pairs.

Table 1.3: Currently known 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor heterodimers

Heterodimer	Model	Main Findings	Reference
5-HT$_{2C}$ and GHSR	*In-vitro* - HEK293a *Ex-vivo* - rat primary cultures *In-vivo* - C57BL/6 mice	- 5-HT$_{2C}$ co-expression significantly attenuated GHSR Gαq signalling which was rescued by 5-HT$_{2C}$ antagonism - *In-vivo,* 5-HT$_{2C}$ antagonism significantly increased GHSR-mediated food-intake.	(Schellekens et al., 2015a, Schellekens et al., 2013e)
5-HT$_{2C}$ and melatonin type 2 receptor (MT2)	*In vitro* – HEK293a	- Dimerization results in increased 5-HT$_{2C}$-mediated Gαq signalling - Unidirectional transactivation of the 5-HT$_{2C}$ protomer was induced by MT2 receptor agonist melatonin	(Kamal et al., 2015)
5-HT$_{2A}$ and metabotropic glutamate receptors (mGluR)	*In-vitro* – HEK293a *Ex-vivo* – human post-mortem brain slices	- Upon heterodimerization activation of the mGluR abolished the hallucinogenic properties of 5-HT$_{2A}$ - Dimer expression is reduced in schizophrenia; linked with associated psychosis	(Gonzalez-Maeso et al., 2008, Moreno et al., 2012)
5-HT$_{2A}$ and DRD2	*In-vitro* – HEK293a *In-vivo* - 129S6/SvEv mice	- DRD2- 5-HT$_{2A}$ dimerization enhances hallucinogenic binding of 5-HT$_{2A}$ - Co-administration of receptor agonists enhances both 5-HT$_{2A}$ and DRD2-mediated Gαq signalling	(Borroto-Escuela et al., 2014b, Albizu et al., 2011, Borroto-Escuela et al., 2010b)

		- Co-administration of receptor agonists reduces DRD2 -Gαi mediated signalling	

1.7 Glucagon-like peptide 1 and the glucagon like peptide 1 receptor

Glucagon-like peptide 1 (GLP-1) is best known for its role as an incretin, aiding in insulin secretion, and subsequently reducing blood glucose following the consumption of food (Nauck, 2004). GLP-1 is one of the first peptides released following food consumption, involved in the suppression of appetite, with active involvement of both the central and peripheral GLP-1-mediated pathways via the brain-gut axis (Barrera et al., 2011, Shah and Vella, 2014). In addition to its role in appetite, GLP-1 has been implicated in several centrally-regulated functions such as neurogenesis and neuroprotection, which has led to animal studies involving GLP-1 as a prophylactic against neurodegenerative disorders such as Parkinson's, Alzheimer's and multiple sclerosis (DellaValle et al., 2016, Lee et al., 2018, Harkavyi et al., 2008, Perry et al., 2003, Li et al., 2010).

GLP-1 binds and subsequently activates the glucagon-like peptide one receptor (GLP-1R) which is expressed throughout the peripheral and central nervous systems (Donnelly, 2012). GLP-1R is a Class B GPCR, also known as a family of secretin receptors, composed of 15 receptors, all sharing similar sequence identity with the most variation occurring at the N-terminal region in which the extracellular ligand binding occurs (Lagerstrom and Schioth, 2008, Hollenstein et al., 2014).

1.7.1 Glucagon-like peptide 1 signalling and structure

GLP-1 is a 30-amino acid long peptide synthesised from the posttranslational processing of the preproglucagon gene. Neurons throughout the brain in areas such as the nucleus tractus solitarius, the olfactory bulb, and the dorsal and ventral medulla, are known to induce GLP-1 secretion (Merchenthaler et al., 1999, Manandhar and Ahn, 2015). Here, prohormone convertase 1/3 cleaves preproglucagon to produce several peptides including GLP-1, GLP2, glicentin and oxyntomodulin (Larsen et al., 1997, Vrang and Larsen, 2010). Interestingly GLP-1 can be truncated to form two separate peptides, GLP-1 (7-37) and GLP-1 (7-36), both shown to be the active peptide forms of GLP-1 *in vivo* (Brubaker et al., 1997, Donnelly, 2012), although the half-life of these peptides is short (~2 mins) following degradation by dipeptidyl peptidase IV (Kieffer et al., 1995, Mentlein et al., 1993).

Therefore many synthetic compounds have been produced to replicate the actions of GLP-1 and target the GLP-1R for disorders such as diabetes (Lee and Lee, 2017).

GLP-1 elicits its effect by binding to the GLP-1R a 463-amino acid residue belonging to the class B family of GPCRs. GLP-1R first being cloned from humans in 1993, showing 90% homology with the rat GLP-1R (Dillon et al., 1993, Thorens et al., 1993). The GLP-1R signalling pathway as depicted in Figure 1.2, is known to primarily activate the Gαs signalling pathway which results in activation of adenylate cyclase and subsequent cAMP accumulation; from here PKA is activated, influencing such pathways as the cAMP response element-binding protein (CREB), and subsequently impacting GLP-1 transcription (MacDonald et al., 2002). β-arrestin recruitment influences this signalling, where knockout of β-arrestin-1 attenuated cAMP accumulation and CREB activation (Sonoda et al., 2008). The GLP-1R is also known to influence calcium mobilization from intracellular stores through both IP3 and the ryanodine receptor, which are connected to the activation of the Gαq signalling pathway (Harikumar et al., 2012, Zhang et al., 2015a, Tsuboi et al., 2003). Each of these signalling pathways leads to numerous cellular functions which can have major physiological effects (Salcedo et al., 2012, Yusta et al., 2015, Smith et al., 2014).

1.7.2 Central glucagon-like peptide 1 receptor expression

GLP-1R expression has been examined across multiple models. One such model shown in Figure 1.6 uses Cre-recombinase technology to identify GLP-1R positive cells (Cork et al., 2015). Immunohistochemical analysis of C57BL/6 mice revealed vast expression of GLP-1R in the mouse brain, in areas such as telencephalon, (nucleus accumbens, amygdala and dentate gyrus) the diencephalon (hypothalamus) the mesencephalon (dorsal raphe and substantia nigra) and many areas of the pons and medulla (Jensen et al., 2018).

Furthermore, GLP-1R mRNA has been examined using *in*-situ hybridization in Sprague Dawley rat, where unlike in mice, expression in the telencephalon was minimal, with positive signal seen in areas of the basal ganglia, septum and olfactory bulb (Merchenthaler et al., 1999). Wider expression was seen throughout the diencephalon with high expression observed in areas of the thalamus, hypothalamus such as the arcuate nucleus, paraventricular nucleus and supraoptic nucleus. Higher

expression in the area of the mesencephalon and medulla were also observed with lower expression in the in the pons (Merchenthaler et al., 1999).

In rhesus macaque primates, in situ hybridisation, in situ ligand binding and immunohistochemistry techniques were used to analyse primate GLP-1R expression. This analysis revealed the highest expression of GLP-1R in the hypothalamus, particularly the supraoptic nucleus, paraventricular nucleus and arcuate nucleus. This high expression was also seen in the dorsal tegmental nucleus of the midbrain, the area postrema and the of the hind brain. Mid to low expression of GLP-1R was observed in areas of the amygdala, striatum and nucleus accumbens (Heppner et al., 2015).

Finally, using human post-mortem tissue, immunohistochemistry was utilised to examine human GLP-1R mRNA expression. Here, GLP-1R was expressed on neurons within the hypothalamus, medulla and parietal cortex; consistent expression between subjects was observed in the arcuate nucleus and paraventricular nucleus, with more varied expression between subjects in the ventromedial hypothalamus, area postrema, large and small pyramidal cells of the parietal cortex (Farr et al., 2016). Consistent expression of GLP-1R across mice, rat, primates and human was witnessed in areas of the hypothalamus such as the arcuate nucleus highlights the homology of GLP-1R across different species, in areas such as appetite regulation (Adams et al., 2018, Abbott et al., 2005, van Bloemendaal et al., 2014).

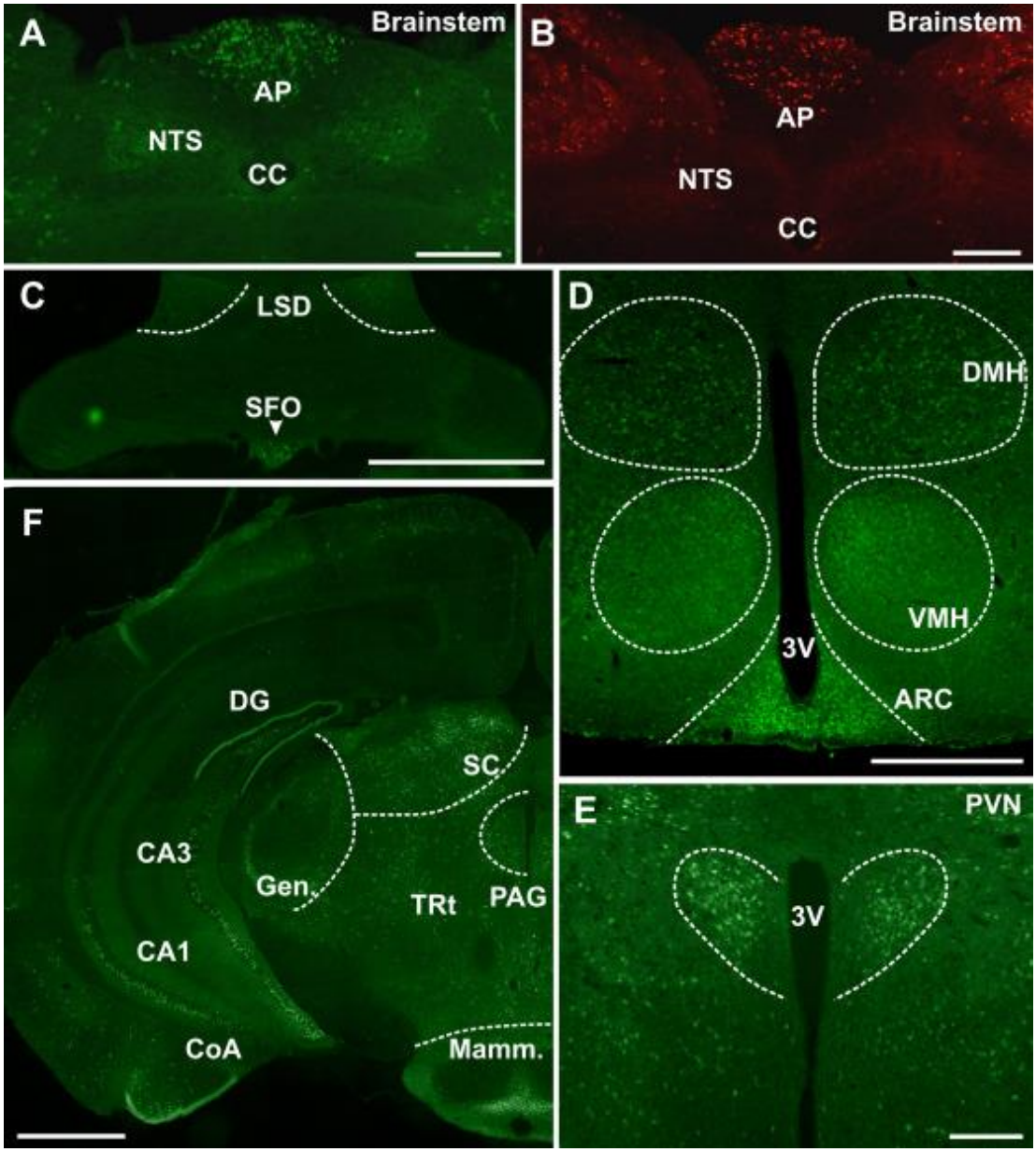

***Figure 1.6**: Cre-recombinase technology in mice revealing the expression of GLP-1R in sections of the **(A, B)** area postrema and nucleus tractus solitarius **(C)** dorsal lateral septum organ **(D)** ventrolateral hypothalamus, arcuate and dorsomedial hypothalamus **(E),** hypothalamic paraventricular nucleus **(F)** and hippocampus (Cork et al., 2015)*

1.7.3 Glucagonergic crosstalk and heterodimerization

In the context of appetite regulation, the GLP-1R and GHSR are known to crosstalk aiding in the control of food intake, here GLP-1R agonism significantly reduced circulating ghrelin levels in fasted rats (Perez-Tilve et al., 2007). Moreover, intraperitoneal injection of ghrelin in mice significantly increases GLP-1 stimulated by glucose, whereas antagonism of GHSR reduced circulating GLP-1 levels (Gagnon et

al., 2015). Similarly, GLP-1 release is altered by 5-HT; the serotonergic pathway influences GLP-1 induced energy expenditure. Here, reduction of 5-HT in rats impairs the activity of GLP-1R agonist exendin 4; interestingly agonism of the GLP-1R reduced expression of 5-HT_{2A} and 5-HT_{2C} in the hypothalamus (Anderberg et al., 2017, Ripken et al., 2016). Crosstalk between oxytocin and GLP-1 influences the OXTR mediated anorexigenic effect, where antagonism of the GLP-1R followed by addition of oxytocin impairs the anorexigenic effect of oxytocin when compared to oxytocin added alone (Rinaman and Rothe, 2002). It appears evident that the GLP-1 pathway mediated its effects by cross-talking with multiple other pathways (Roed et al., 2015); although little research has been done in regards to GLP-1R heterodimerization, table 1.4 highlights the currently known GLP-1R heterodimer pairs.

Table 1.4: Currently known GLP-1R heterodimers

Heterodimer (GLP-1R +)	Model	Main Findings	Reference
Gastric inhibitory peptide receptor (GIPr)	*In-vitro*: HEK 293T	- GLP-1R and GIPr heterodimerization reduces GLP-1R-mediated calcium influx - GLP-1R arrestin binding is reduced when co-expressed with GIPr	(Schelshorn et al., 2012)
Secretin receptor (SecR)	*In-vitro*: Chinese hamster ovary cells *Ex-vivo:* Pancreatic islets	- Dimerization of SecR and GLP-1R reduced SecR-mediated calcium signalling - *Ex-vivo* studies revealed GLP-1R to modulate the SecR-induced secretin response.	(Harikumar et al., 2017)
Glucose-dependent insulinotropic polypeptide receptor (GIP)	*In-vitro*: Chinese hamster ovary cells and INS-1 pancreatic cells and HEK293a	- GLP-1R co-expression rescues GIP receptor mutant lacking N-glycosylation, increasing receptor half-life on the membrane. - Dimerization reduces GLP-1-mediated calcium signalling via impaired recruitment of arrestins to GLP-1R.	(Whitaker et al., 2012, Roed et al., 2015)

1.8 Methods used in heterodimerization research

There is currently a wide array of techniques used to study the formation of GPCR heterodimers; due to the ability of heterodimers to alter the pharmacology and function of GPCRs. These techniques look at the physical interaction of receptors such a co-immunoprecipitation (Matsubara et al., 2017) and fluorescent resonance energy transfer (Boyer and Slesinger, 2010) as well as functional alterations such as changes to G-protein signalling (Schellekens et al., 2013e), ligand binding (Goupil et al., 2013) and receptor trafficking (Whitaker et al., 2012). However, most of these assays are performed *in-vitro*, more specifically in HEK293 (Human embryonic kidney) cells. The use of HEK293 cells is well established, being the second most popular cell line when it comes to cell biology and biotechnology (Stepanenko and Dmitrenko, 2015). This popularity is due to its capability to post-translationally fold and process functional, mature proteins, furthermore its quick growth rates, easy maintenance and its tractable nature towards transfection and transduction (Thomas and Smart, 2005). Within the neuroscience field HEK293 cells have also gained traction due to its neuronal properties allowing for benefits from both embryonic and neuronal cultures (Shaw et al., 2002, Stepanenko and Dmitrenko, 2015). Confirmed neuronal cell lines such as SH-SY5Y, SH-EP, BE(2)-M17, SK-N-AS and IMR-32 have also been used in to study of GPCR function (Tummler et al., 2017, Xue et al., 2018, Ferlemann et al., 2017). These neuronal cell lines have enhanced neuronal properties compared to HEK293 as they do not share these properties with embryonic cells. Although advantageous, neuronal cell lines can have a slower growth rate and require more nourishment (Chen et al., 2014), they also need to be approached with care as a change in physiology can be observed between cell lines and the phenotype of the cell itself can be altered due to culture conditions i.e. growth factors (Gordon et al., 2013). Alternatively, neuronal primary cultures can be utilised but are more difficult to establish and cannot be used for long term studies as cell division slows down and eventually stops in mature neurons (Giordano and Costa, 2011, Gordon et al., 2013). Thus, primary cultures are more advantageous when used in combination with immortalised cell lines so as to back up a possible result or theory. Though each method and choice of expression system alone has

limitations, in combination these techniques provide strong evidence of GPCR heterodimerization (Milligan and Bouvier, 2005).

1.8.1 Functional assays

One of the earliest GPCR heterodimer studies began by looking at the functional consequence of a heterodimer formation on receptor signalling as opposed to the physical interaction of receptors themselves (Marshall et al., 1999, Maggio et al., 1993). An interaction of the M3 muscarinic acetylcholine receptor (mAChR) and the α2c-adrenergic receptor was discovered using co-expression of receptors in a COS-7 cell line, where radioligand binding and inositol monophosphate (IP1) assays revealed co-expression to increase the binding capacity of the M3 mAChR and the α2c-adrenergic receptor antagonists, reducing IP1 accumulation compared to M3 mAChR expressed alone (Maggio et al., 1993).

1.8.1.1 Gαq signalling pathway

During Gαq signalling analysis, different studies have used different points along the Gαq signalling pathway as illustrated in Figure 1.2; the first of these is phospholipase C (PLC). The role of PLC in the Gαq signalling pathway is often measured by blockade of PLC using antagonists. Investigations into the dopamine D1 (DRD1) and D2 (DRD2) receptors utilised this method. In this study, calcium mobilization was measured using the automated FlexStation reader from *Molecular Devices LLC. California,* where DRD1 and DRD2 heterodimers increased Gαq calcium mobilization, pre-treatment with PLC antagonist U73122 abolished the increase in calcium mobilization, implicating the role of PLC and therefore Gαq signalling in DRD1 and DRD2 heterodimerization (Lee et al., 2004, Hasbi et al., 2009).

Secondly, inositol phosphate (IP) can be measured via IP1 and inositol triphosphate (IP3) accumulation analysis. Commercial assays such as homogeneous time-resolved fluorescence (HTRF) from *Cisbio*, *France,* are available; these high throughput assays require only HTRF reagents and a fluorescent plate reader, setup with HTRF specific settings (Moutkine et al., 2017). Another method utilises [3H]myoinositol labelled IP cells, isolated via chromatography and measured using scintillation counters (Wilson et al., 2004, Quitterer et al., 1999, Sherrill and Miller, 2006).

The main secondary messenger, as a result of Gαq activation, is intracellular calcium influx. Therefore, this is one of the most common markers for measuring Gαq functionality. Similar to the IP assay, commercial assays such as the Ca5 kit from *Molecular Devices LLC. California* can be used to measure calcium influx from intracellular stores; they are ratiometric dyes which measure the shift in emission or excitation between free and bound calcium within the endoplasmic reticulum (Schellekens et al., 2015a). In such kits, cells are pre-incubated with a calcium dye, followed by ligand stimulation of GPCRs coupled with subsequent ratiometric fluorescence readings that correlate with calcium influx (Matsubara et al., 2017). Other studies have used calcium dyes such as Fura-2; here, a change in calcium mobilization is measured via luminescence alterations with a spectrophotometer, although this method can be more cumbersome then commercial assay kits (Uberti et al., 2005, Wilson et al., 2004). Non-ratiometric calcium dyes such as fluo-4 and x-rhod-1 are also used to examine changes in calcium; these dyes are analysed using laser scanning microscopy, which has a lower throughput than the analyses previously described. While non-ratiometric dyes correlate directly to calcium concentration, a major disadvantage of this method lies with the fact that florescence intensity can easily be impacted by factors other than calcium concentration, such as acquisition settings within the microscope (Evans and Walker, 2008).

Gαi/Gαs signalling

The Gαs and Gαi subunits play opposing roles in the accumulation of cAMP. Gαi activation results in inhibition of cAMP while Gαs activation enhances cAMP production as seen in Figure 1.2 (Wang et al., 2004). Time-resolved flowcytometry fluorescent resonance energy transfer (TR-FRET) was used to measure the effect of Gαi and Gαs signalling on adenosine receptors 1 and 2a, detecting cAMP accumulation with the LANCE Ultra cAMP kit from Perkin Elmer (Navarro et al., 2018). Similarly, enzyme immunoassays have been used to identify cAMP accumulation following activation of the beta 2 adrenergic receptor and bradykinin receptor heterodimer. This assay uses a fixed concentration of peroxidase labelled cAMP to measure native cAMP levels (Haack et al., 2010). Most cAMP assays, although using

different measurement techniques and kits, work from the same idea, where a company labelled cAMP molecule competes with native cAMP for an anti-cAMP antibody; therefore the signal is inversely proportional to the concentration of cAMP (Koole et al., 2010, Harikumar et al., 2012).

β-arrestin recruitment

β-arrestin binding to GPCR mediates receptor trafficking (Figure 1.2), controlling the localization of the receptor within the cell and degradation and this subsequently effects the binding of ligands to GPCRs due to varied expression of the GPCR on the cell membrane (Takenouchi et al., 2018). Binding and dissociation of β-arrestin influences rate of endocytosis and subsequent recycling of GPCRs back to the membrane, where quick dissociation of β-arrestin results in enhanced membrane recycling (Terrillon et al., 2004). Therefore, it is important to analyse such pathways as this can have consequences not only on receptor trafficking but receptor signalling due to receptor availability. β-arrestin recruitment can be measured both directly and indirectly. Some studies incorporate the use of a β-arrestin fluorescent tag to monitor the movement of β-arrestin attached to the GPCR of interest, which also allows for kinetic analysis of receptor trafficking (Angers et al., 2000, Terrillon et al., 2004). Similarly, it can be measured using bioluminescence resonance energy transfer (BRET) experiments in which the β-arrestin is cloned into a plasmid expressing such BRET detector tags as renilla-luciferin 2-monooxygenase, or mutant yellow fluorescent protein (Liu et al., 2016). In such studies the GPCR of interest will be cloned to express the opposing BRET tag for β-arrestin, the proximity of both tags results in a bioluminescent reading. Therefore, an amplified bioluminescent signal correlates to increased β-arrestin recruitment. Many studies use internalization assays as an indirect measure of β-arrestin recruitment. Such assays tend to be quicker with a higher throughput. A frequent method used for this requires ligand treatment, followed by staining with an ELISA based antibody, resulting in an absorbance reading which inversely correlates with receptor endocytosis (Pfeiffer et al., 2002, Jordan, 2001). GPCR internalisation can be analysed through stable fluorescent tagging of the GPCRs expressed within an *in-vitro* model; from this,

fluorescent microscopy can be used to quantify internalisation of receptors within the cell (Ellis et al., 2006).

Binding assays

The novel signalling pathways associated with heterodimer formation can often be influenced by the ability of ligands to form a complex with their corresponding receptor (Siddiquee et al., 2013a). This ligand binding can also influence the formation of the heterodimer itself (Schellekens et al., 2015a). Radioligand binding is currently the most common method of identifying changes in ligand binding within a heterodimer pair (Zhang and Xie, 2012). This assay incorporates the use of radioactively tagged ligands (radioligands), which can be incubated alone, or with increasing concentrations of a second untagged ligand to determine radioligand affinity. The cells are subsequently lysed, and the sample analysed to determine radioactivity (i.e. level of bound ligand) (Herrick-Davis et al., 2005, Levoye et al., 2006, Ayoub et al., 2002, Harikumar et al., 2006).

1.8.2 Co-localization assays

Bioluminescence resonance energy transfer (BRET)

BRET transfer is a method often employed for the detection of heterodimer formations (Achour et al., 2011). This detects the close proximity (50–100 Å) of receptors within an *in-vitro* system using GPCR constructs with opposing bioluminescent donor or acceptor proteins. Close proximity of these proteins results in a shift in fluorescent wavelength measurable through automated fluorescent plate readers (Kufareva et al., 2013). Many combinations of bioluminescent donor and acceptor proteins are used in heterodimerization studies, such as the (1) renilla luciferase donor and yellow fluorescent protein (Percherancier et al., 2005, Siddiquee et al., 2013b), (2) the μ luciferase and yellow fluorescent protein (Gomes et al., 2004), and (3) renilla luciferase and the green fluorescent protein (Mercier et al., 2002).

Fluorescence resonance energy transfer (FRET)

FRET uses a similar concept to BRET, but in the case of FRET both the donor and acceptor molecules are fluorescent proteins which overlap on the fluorescent

spectra, therefore spatial proximity of these proteins results in the transfer of energy from the donor to the acceptor, resulting in emission from the acceptor protein as opposed to the donor (Cottet et al., 2012). FRET can be measured in multiple ways, such that FRET intensity can be calculated using fluorescent microscopy (Bai et al., 2014, Vilardaga et al., 2008). As previously shown in our lab flow-cytometry based FRET can be employed to calculate the percentage of positive FRET expression within an *in-vitro* system, allowing for a higher throughput system (Chruścicka et al., 2018). Automated systems can be used incorporating the use of time resolved FRET and automated fluorescent readers, advantageously reducing the amount of background fluorescence observed in traditional FRET measurement techniques (Wilson et al., 2005, Watts et al., 2013).

Co-immunoprecipitation

The concept of Co-immunoprecipitation (Co-IP) differs from both BRET and FRET; in this assay, both GPCRs of interest are again co-expressed in an *in-vitro* system. However, at this point cells are lysed, and an antibody specific for the first GPCR allows for immunoprecipitation of the GPCRs out of the sample, which is then further analysed by detection of the second antibody (Khare et al., 2016, DeFazio-Eli et al., 2011). The most common methods for detection of the second GPCR is western blotting. Following immunoprecipitation, GPCRs are isolated using gel electrophoresis. The sample is then transferred to a western blot for detection of the second GPCR using its specific antibody (Bai et al., 2014, Hwang et al., 2014, War and Kumar, 2012). Therefore, the presence of the second antibody from the western blot indicates co-immunoprecipitation of both receptors, and consequently heterodimerization.

1.9 An intricate interplay between sociability, mood and appetite

Oxytocin has a multitude of centrally-regulated functions especially at the level of behaviour (McCarthy and Altemus, 1997, Lawson, 2017, Shamay-Tsoory and Young, 2016). The best studied of these is the well described role of oxytocin signalling in social behaviour including social bonding (Bosch and Young, 2018), social cognition (Ebert and Brune, 2018) and affiliative behaviour (Ross et al., 2009) and within disorders such as autism (Yamasue and Domes, 2018) and schizophrenia (Shilling and Feifel, 2016). Oxytocin has been linked to the control of appetite (Leng et al., 2008, Sabatier et al., 2013) and associated with disorders such as obesity (Olszewski et al., 2017). The oxytocin pathway has been associated with mood (Broadbear et al., 2014), particularly disorders such as depression and anxiety (Stuebe et al., 2013, Cyranowski et al., 2008). All these behaviours are intertwined, and often dysfunction of one lead to an alteration in the others, leading to multifaceted disorders.

An individual's social functioning defines how they interact and the role they play in environments such as social activities, employment and relationships, with many social behaviours feeding in to this, such as social cognition, bonding and social interaction (Hari et al., 2015, Lieberwirth and Wang, 2014, Frith, 2008). As a result, many areas of the brain play key roles in regulating these behaviours as depicted in Figure 1.7 by Billeke and Aboitiz. Sub-regions of the hypothalamus, hippocampus, amygdala, cortex, striatum and ventral tegmental area are known to function in the regulation of social behaviours (Chen and Hong, 2018). Moreover, such pathways which act within and between these areas are known to further play a role in the regulation of the social brain, such as the dopaminergic, serotonergic and oxytocinergic pathways (Skuse and Gallagher, 2009, Lesch, 2007). A link in the regulation of social function is seen when compared to other areas such as mood and appetite, where alterations to social functioning can often lead to the development and onset of mood-related disorders such as depression (Matthews et al., 2016, Choi et al., 2015).

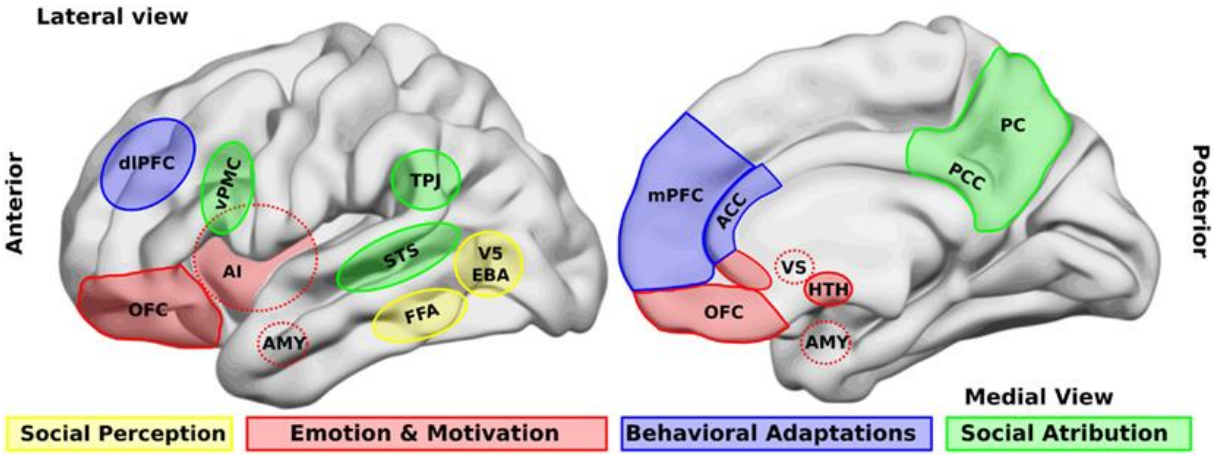

***Figure 1.7**: Illustration depicting the numerous areas involved in the signalling of the "social brain", including social perception, emotion, adaption and social attribution (Billeke and Aboitiz, 2013).*

Mood disorders are defined by an individual's emotional state, impacted by long-term factors such as positive and negative life experiences; these disorders can represent a major cause of disability and mortality worldwide (Nettle and Bateson, 2012). Major depressive disorders and bi-polar disorder are common psychiatric illnesses defined under mood disorders (Marvel and Paradiso, 2004). When compared to the social circuitry similar sub-regions of the hypothalamus such as the paraventricular nucleus have been implicated in both sociability and mood (Bao et al., 2005); similarly areas such as the cornu ammonis subfield 2 of the hippocampus have also been implicated (Alexander et al., 2016, Cao et al., 2017). These similarities are seen throughout many areas of the brain implicated in both sociability and mood.

Many studies have shown both social function and mood can influence and alter appetite (Higgs, 2015, Simmons et al., 2016). Such alterations in appetite can lead to food related disorders such as obesity, which are as a result of increased food intake and reduced energy expenditure (Barry et al., 2009). Obesity has been associated with significant increases in depression, bipolar disorder, anxiety disorders and altered social behaviour (Simon et al., 2006, Hryhorczuk et al., 2013, Al-Agha et al., 2016). However, anxiety and mood disorders do not always result from overeating and obesity; it is more common that obesity presents as a result of mood disorder, anxiety or social setting (Godart et al., 2000, Singh, 2014). Such that adolescents with

major depression are predisposed to a higher risk of obesity in adulthood (Richardson et al., 2003).

Eating disorders such as anorexia nervosa (AN) and bulimia nervosa (BN) are also severe psychiatric disorders associated with a marked alteration in food intake (Morris and Twaddle, 2007, Russell, 1979). Studies have shown that patients with AN and BN have a significantly higher chance of suffering from mood disorders than that of healthy individuals (Godart et al., 2015). It was also found that mood and anxiety disorders expressing co-morbidly can result in an increase in clinical severity of AN and in addition, psychological symptoms (Brand-Gothelf et al., 2014).

Disorders such as social anxiety and phobias have a high comorbidity with eating disorders such as AN and BN (Halmi, 1991); sociability tends to be reduced in clinical groups of AN and BN (Hinrichsen et al., 2003), while increased social standard (how an individual perceives there social surroundings) has also been implicated as a common feature of eating disorders (Gunnard et al., 2012). When examining the circuitry of appetite regulation, a crossover between appetite, social function and mood can be observed. Subregions of the hypothalamus, amygdala, cortex and hippocampus appear to interlink between these three behaviours (Holsen et al., 2012). Moreover, pathways such as the dopaminergic, serotonergic, oxytocinergic and ghrelinergic system are known to play key parts in regulating social function, appetite and mood (Skuse and Gallagher, 2009, Lesch, 2007, Volkow et al., 2011). Many of the neuropeptides within these pathways as described in table 1.5 are known to form an intricate relationship, regulating secretion and receptor expression (de la Mora et al., 2016).

Another area which links these signalling peptides and their subsequent effect on behaviour is the popular area of study into the brain-gut axis. It is well documented that ghrelin (Cabral et al., 2017) and glucagon-like peptide 1 (Holst, 2007) are synthesised within the gut, while tryptophan the serotonin precursor is metabolised within the gut (Agus et al., 2018). These peptides and their associated receptors are known to influence gut function such as motility, inflammation and permeability (Welch et al., 2014, Sakata and Sakai, 2010, Yang et al., 2017, Kelly et al., 2015). The brain-gut axis focuses on the cross-talk between the brain and the gut and how this

can influence centrally-regulated functions and vice versa (Cussotto et al., 2018). Disorders such as autism spectrum disorder (ASD) which are identified through multiple symptoms, some of these being alterations to an individual's social functioning, mood and appetite are now being investigated for dysfunction within the brain-gut axis (van De Sande et al., 2014, Fowlie et al., 2018, Golubeva et al., 2017). ASD presents with many associated gut issues, such as an increase in gut permeability, which is theorised to lead to disturbances in neurodevelopment and cognition (Fowlie et al., 2018). The change in gut function has been linked to differences in gut microbiome function and increased bacterial metabolite movement from the gut. These metabolites are known to interact with GPCRs (Cohen et al., 2017, Husted et al., 2017), therefore it could be hypothesised that these metabolites may influence GPCR expression in a ligand mediated manor and subsequently alter GPCR dimerization both centrally and peripherally, subsequently leading to the observed alterations in social behaviour, appetite and mood.

Therefore, the simultaneous alterations to social function, mood and appetite that appears to exist in patients presenting with disorders in these areas, may indicate a central mechanism which underlies the pathologies associated with such disorders as obesity, AN, bipolar, anxiety and social dysfunction.

Table 1.5: Neuromodulators of sociability, mood and food intake

Oxytocin	Social Behaviour	- Social buffering of stress is mediated by endogenous oxytocin - Intranasal oxytocin improves social functioning in psychiatric disorder - Oxytocin enhances social preference and reduced social avoidance - Oxytocin improves social cognition in patients with schizophrenia - Reduced circulating oxytocin levels are associated with increased social avoidance	(Smith and Wang, 2014) (Gibson et al., 2014) (Lukas et al., 2011) (Woolley et al., 2014) (Brown et al., 2014)
	Appetite Regulation	- Increased oxytocin levels reduce food intake, irrespective of leptin resistance - Administration alters food preference, reducing salt intake - Hedonic food intake is regulated by oxytocin treatment - Oxytocin reduced food intake through vagal afferents - Reduction in weight induced by oxytocin is associated with increased lipid utilisation and enhanced satiation signalling - Oxytocin is involved in both homeostatic and hedonic feeding; the elicitation of either response being controlled by the discrete localization of the receptor within the amygdala	(Morton et al., 2012a) (Stricker and Verbalis, 2004) (Ott et al., 2013a) (Iwasaki et al., 2015) (Blevins et al., 2016) (Klockars et al., 2018) (Lawson et al., 2015) (Kim et al., 2015)

		- Oxytocin administration reduces overall caloric intake in men at 366kcal/ day which may indicate a possible role of oxytocin in obesity treatments - In patients with bulimia nervosa, administration of intranasal oxytocin was shown to further reduce food intake over a 24-hour period	
	Mood	- Dysregulation of peripheral oxytocin synthesis is associated with depression onset in woman - Bi-polar disorder is associated with altered oxytocin levels which were associated with manic episodes - Post-partum depression is associated with reduced oxytocin levels during the third trimester and during breast feeding - Treatment for manic-depressive state was associated with decreases in oxytocin levels associated with reductions in symptoms	(Cyranowski et al., 2008) (Lien et al., 2017) (Stuebe et al., 2013) (Turan et al., 2013)
Ghrelin	Social Behaviour	- Hippocampal ghrelin rat knockout attenuates the social transmission of food intake - Social isolation results in reduced circulating ghrelin levels - Increased ghrelin was correlated with reduced social withdrawal in Prader-Willi syndrome	(Hsu et al., 2018) (Nonogaki et al., 2007) (Tauber et al., 2017)

	Appetite Regulation	- Intravenous ghrelin is shown to induce food intake in men - Weight loss following a gastrectomy is associated with long term reduced ghrelin levels - Ghrelin is a viable treatment for cachexia associated with cancer. - Ghrelin administration and increases in endogenous ghrelin increase hedonic feeding	(Wren et al., 2001) (Koizumi et al., 2014) (Ali et al., 2013) (Goldstone et al., 2014)
	Mood	- Increased ghrelin is associated with onset of depression in woman - Ghrelin levels are correlated to neuronal changes in the reward pathway in depressed patients - Obesity and subsequent depression is associated with circulating ghrelin levels - Increased weight gain associated with olanzapine treatment is connected to increases in ghrelin receptor - In GHSR knockout mice, a reduction in both social interaction and food intake was observed - ghrelin was found to have an anxiolytic effect, when either the GHSR was overexpressed within the amygdala or ghrelin was administered	(Akter et al., 2014) (Matsuo et al., 2012) (Tuncel et al., 2016) (Zhang et al., 2014) (Lutter et al., 2008) (Jensen et al., 2016)

5-HT	Social Behaviour	- Increased 5-HT neurotransmission reduced social interaction - Social isolation alters the effect of 5-HT reuptake inhibitors - Tryptophan knockout mice reveal an autistic like social phenotype - 5-HT_{2C} receptor antagonism restores the social deficits associated with Pten gene deletion - Decreased tryptophan is known to enhance aggressive behaviour while decreasing social comparison and enhancing antisocial behaviour - Dietary depletion of tryptophan results in reduced 5-HT turnover; subsequently, this results in decreased preference for social interaction, which is reversed with tryptophan supplementation - Targeting the 5-HT_{1A} receptor specifically showed marked increases in social behaviour associated with alterations to the lateral amygdala	(Homberg et al., 2007) (Dankoski et al., 2014) (Kane et al., 2012) (Sejourne et al., 2015) (Bilderbeck et al., 2014) (Beis et al., 2015) (Zhang et al., 2015b) (Wang et al., 2013)
	Appetite regulation	- Activation of 5-HT neurons in the hypothalamus result in reduced food intake - Agonists of the 5-HT_{2C} receptor decrease appetite and could be useful as an anti-obesity treatment - 5-HT transporter are involved in the symptoms and recovery of bulimia nervosa	(Boisvert et al., 2011) (Halford and Harrold, 2012) (Pichika et al., 2012)

	Mood	- Gut-brain axis controls mood through synthesis of tryptophan - Reduced expression of 5-HT_{1A} receptor in the mesiotemporal cortex was associated with depression - Tryptophan rich diets can reduce the onset and symptoms of depression - 5-HT reuptake inhibitors are a common treatment for major depressive disorder - The 5-HT_{2A} receptor has reduced binding capacity for 5-HT in patients with depression - In patients with bulimia nervosa, a positive correlation was seen between increased 5-HT levels and decreases in anxiety and depression upon feeding - Reduction of the gut-derived 5-HT precursor tryptophan resulted in significant onset of depressive-like symptoms in women who had recovered from major depressive disorder	(Jenkins et al., 2016) (Wang et al., 2016) (Shabbir et al., 2013) (Clevenger et al., 2018) (Wang et al., 2016) (Gauthier et al., 2014) (Smith et al., 1997)
Neuropeptide Y	Social Behaviour	- Social competence in schizophrenic patients was associated with NPY levels in the cerebral spinal fluid - Age dependent caregiving and maltreatment was associated with altered NPY levels in adolescent mice	(Stalberg et al., 2014) (Hill et al., 2014) (Hostetler et al., 2013)

		- Central expression of NPY and its effects on social behaviour is species specific	
	Appetite Regulation	- GLP-1R mediates its anorexigenic effect by decreasing NPY expression - NPY downregulated the anorexigenic α-melanocyte-stimulating hormone, to elicit its orexigenic effect - Intracerebroventricular injection of NPY increases food intake in mice	(Yang et al., 2014) (Cyr et al., 2013) (Geerling et al., 2013)
	Mood	- The antidepressant effect associated with exercise is mediated by the expression of NPY and the NPY Y1 receptor within the hippocampus - Increased cortisol and decreased NPY was associated with suicide attempt in depressed patients - Intracerebroventricular injection of NPY results in attenuation of the depressive like effects induced by the amyloid-β peptides - NPY has been shown to reduce depressive-like states in animal models - NPY in the frontal cortex has is associated with mood disorders such as bipolar and schizophrenia, where a reduction in NPY mRNA can be observed in both conditions	(Bjornebekk et al., 2010) (Westrin et al., 1999) (dos Santos et al., 2013) (Stogner and Holmes, 2000) (Kuromitsu et al., 2001)

GLP-1	Appetite Regulation	- GLP-1 is reduces both food intake and subsequently body weight through peripheral administration, where c-fos immunohistochemistry shows this action to be mediated through areas such as the amygdala and brainstem - Administration of Exendin-4, an agonist of the GLP-1R, decreases circulating ghrelin levels and decreases food intake in fasted rats, showing the potent effect of the Exendin-4 agonist in the GLP-1 pathways - Co-administration of GLP-1 and PYY can both reduce circulating ghrelin levels but also result in a further decrease in food intake when compared to administration alone	(Parker et al., 2013) (Perez-Tilve et al., 2007b) (Schmidt et al., 2014)
	Mood	- The length of GLP-1 treatment has been associated with different effects on mood, such that acute GLP-1 induces anxiety-like behaviours, while chronic treatment resulted in an antidepressant-like effect. Interestingly this difference was associated with increased 5-HT turnover and 5-HT receptor synthesis - GLP-1 has been shown to influence the impaired cognitive function associated with mood disorders - Liraglutide a GLP-1R agonist significantly improves the cognitive function in bi-polar and depressed patients	(Anderberg et al., 2016) (McIntyre et al., 2013) (Mansur et al., 2018) (Mansur et al., 2017)

1.10 Objectives of the thesis

It is clear the oxytocinergic system plays critical roles in many behaviours and that these behaviours are further modulated through the central crosstalk of many hormonal pathways such as ghrelin, 5-HT and GLP-1. Furthermore, centrally-regulated behaviours such as appetite, mood and social behaviour involve intricate crosstalk between these hormonal pathways. Current knowledge of GPCR crosstalk and heterodimerisation is associated with novel alterations in GPCR signalling, many dimerization studies showing alterations to the signalling of OTR, GHSR, GLP-1r, 5-HT_{2A} and 5-HT_{2C}. Subsequently, this signalling modulation can lead to changes in behaviour, therefore, GPCR crosstalk and dimerization may conceivably lead to changes in appetite, mood and social behaviour.

Due to the vast role of the oxytocinergic system across many behaviours and disorders, and the current knowledge on the role of the oxytocin receptor crosstalk and heteromerization, it can be hypothesised that the oxytocin receptor is likely to form multiple heterodimers with receptors such as GHSR, 5-HT2A, 5-HT2C and GLP-1R. The identification of novel OTR-GPCR interactions may elicit novel signalling pathways which could be implicated in the normal or abnormal functioning of appetite, mood and social behaviour. Thus, the overall goal of this thesis is to investigate novel OTR heterocomplexes and the influence these interactions may have on the downstream signalling of the OTR. Such findings are poised to contribute to the discovery of novel pharmacotherapies targeting behaviours associated with the oxytocinergic system.

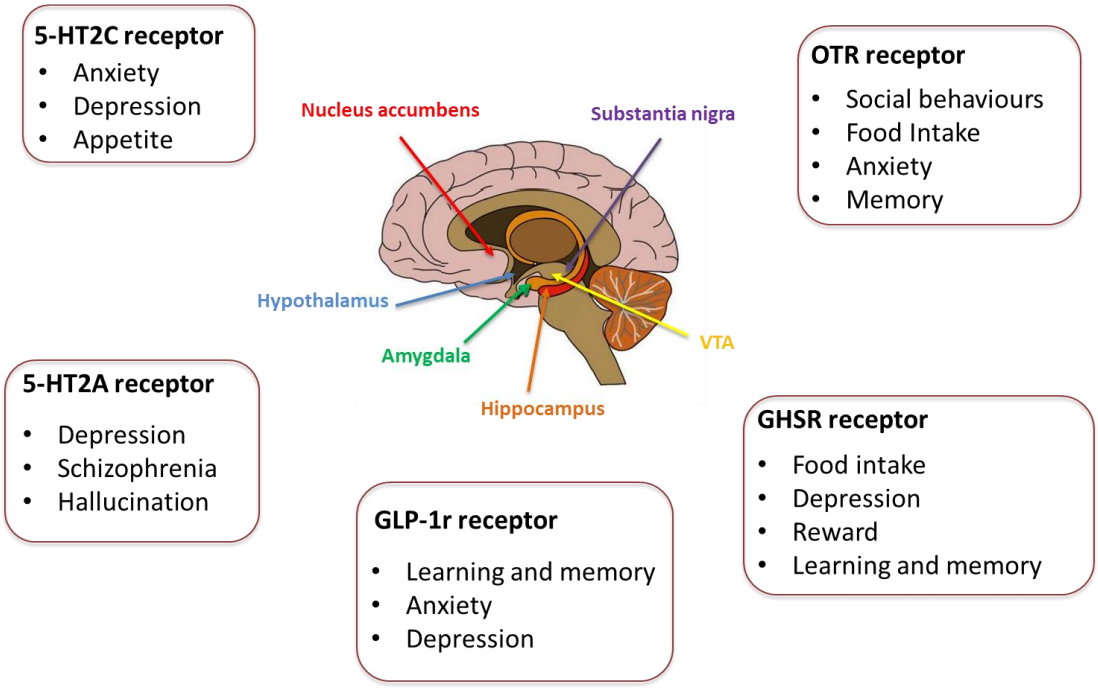

Figure 1.8: *Schematic demonstration demonstrating both the expressional and functional overlap between GHSR, 5-HT2A, 5-HT2C, GLP-1R and OTR.*

Aim 1: Investigate the crosstalk and possible heterodimerization of the oxytocin receptor and ghrelin 1a receptor. (Chapter 2)

The OTR and GHSR are known to play similar and opposing roles in many centrally-regulated behaviours, such as mood, sociability and appetite regulation (Morton et al., 2012b, Myers et al., 2014, Sala et al., 2013, Lukas et al., 2011, Slattery and Neumann, 2010), Although there is some functional evidence of crosstalk between these systems, where increases in oxytocin levels have been shown to reduce circulating ghrelin levels in humans (Vila et al., 2009), and conversely oxytocin administration is known to enhance the secretion of ghrelin in vitro in a ghrelin expressing cell line (Iwakura et al., 2011), while ghrelin addition results in increased secretion of oxytocin *in-vitro* (Gálfi et al., 2016).

Whether crosstalk or heterodimerization between the OTR and GHSR occurs is unclear. Thus, using a multitude of *in-vitro* methods such as co-localization, flowcytometry based FRET, trafficking and calcium mobilization assays, *ex-vivo* primarily neuronal cultures the possible interaction of these receptors in the brain will be directly investigated.

Aim 2: Identify possible crosstalk between the oxytocin receptor and glucagon like peptide 1 receptor (Chapter 3)

Central GLP-1R signalling has been shown to crosstalk with neuronal oxytocin signalling, where pre-treatment with a GLP-1R antagonist prior to an anorexigenic dose of oxytocin resulted in attenuation of the oxytocin-mediated anorexigenic response compared to no GLP-1R pre-treatment (Rinaman and Rothe, 2002). Furthermore, administration of GLP-1R ligands to the intra-paraventricular nucleus (PVN) was shown to increase calcium influx in oxytocin neurons, again highlighting the ability of GLP-1R to crosstalk with oxytocin-mediated signalling pathways (Katsurada et al., 2014).

To explore the mechanism behind such interactions, the heterodimerization and crosstalk between the OTR and GLP-1R was investigated (Chapter 3). *In-vitro* methods were used to analyse this such as co-localization assays, flow cytometry-based FRET, Gαq signalling, Gαs signalling and co-trafficking assays.

Aim 3: Investigate the relationship between the oxytocinergic and serotonergic pathway in terms of the oxytocin receptor, 5-HT 2a receptor and 5-HT 2c receptor (Chapter 4&5).

Crosstalk between the serotonergic and oxytocinergic pathways has been previously shown across multiple studies (Emiliano et al., 2006, Eaton et al., 2012). Interestingly this OT-5-HT crosstalk has been further linked to interactions with the 5-HT 2a receptor (5-HT2A), where activation of the 5-HT2A with agonist DOI directly injected into the paraventricular nucleus of the hypothalamus results in increased levels of circulating oxytocin (Zhang et al., 2002, Van de Kar et al., 2001). Moreover, oxytocin is known to diminish stress via its anxiolytic effect (Peters et al., 2014), where interaction of the serotonergic pathway through antagonism of 5-HT2C blocked the increase in oxytocin secretion associated with stress (Jorgensen et al., 2002). Contrastingly, agonism of the 5-HT2C receptor resulted in an increase in circulating oxytocin levels (Jorgensen et al., 2003b, Jorgensen et al., 2003a).

As shown the oxytocinergic pathway is shown to have an intricate relationship with 5-HT especially via the 5-HT2A and 5-HT2C, therefore the possible interaction

between the OTR with these two 5-HT receptors was investigated. Through a number of techniques (co-localization and FRET, PLA) and functional assays (calcium mobilization, inositol monophosphate and ligand mediated trafficking) a novel functional heterocomplex is investigated between OTR-5-HT2A (Chapter 4) and OTR-5-HT2C (Chapter 5).

Chapter 2

A ghrelin receptor and oxytocin receptor heterocomplex impairs oxytocin mediated signalling

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2.1 Abstract

Oxytocin mediates its behavioural effects via the centrally expressed oxytocin receptor (OTR). Oxytocin signalling has been implicated in multiple disorders involving centrally-regulated pathways, including obesity, ASD, schizophrenia and depression. The OTR has been described to have a complex downstream signalling pathway and an increased understanding of oxytocinergic signalling is needed for the development of novel and better treatments for centrally-regulated disorders. The ghrelin receptor (GHSR), known primarily for its role in centrally-regulated energy balance and food intake, has in more recent years also been shown to play a role in mood disorders, including anxiety and depression. Although there have been suggestions of crosstalk between both signalling systems, these have largely been unexplored to date. Here we show, to our knowledge for the first-time, compelling evidence for the formation of an OTR and GHSR heterocomplex, resulting in significant modulation of OTR downstream signalling. Co-localized expression of the OTR and GHSR is shown in a heterologous cellular expression system and in primary cultures from the hypothalamus and hippocampus of Sprague-Dawley pups. A physical interaction between the OTR and GHSR is confirmed using flow-cytometry based fluorescence resonance energy transfer (fcFRET). Co-expression of the GHSR results in a significant attenuation of OTR-mediated Gαq signalling and changes in receptor trafficking within the cell. Together, these data demonstrate a potential functional relevance of an OTR/GHSR heterocomplex and its ability to alter OTR signalling, which is poised to have important implications for future therapeutic strategies, involving oxytocinergic signalling.

2.2 Introduction:

G-protein coupled receptors (GPCRs) represent one of the largest family of receptors involved in cell membrane signal transduction and have become one of the leading therapeutic targets worldwide (Chakraborty and Chattopadhyay, 2015, Bouvier, 2001, Sriram and Insel, 2018). GPCR signalling produces a diverse array of functions including neurotransmission, cellular metabolism, cell differentiation and also inflammatory and immune responses (Betke et al., 2012, Steury et al., 2017, Weiss et al., 2017, Sebastiao and Ribeiro, 2009).

Classically, GPCRs were assumed to function as monomeric structures. However, the concept of homodimerization, heterodimerization and even higher-order oligomerization of GPCRs has emerged over the past three decades (Kern et al., 2015, Chen et al., 2015, Schellekens et al., 2013f, Wellman and Abizaid, 2015, Agnati et al., 1980, Borroto-Escuela et al., 2017a). Accumulating studies show that the GPCR heteromerization significantly impact downstream signalling pathways, for example, via preferentially regulated G protein coupling, ligand-mediated allostery or agonist-promoted GPCR endocytosis and co-internalization, all with marked consequences for biological functionality (Cudmore et al., 2012, Rocheville, 2000, Borroto-Escuela et al., 2013b, Siddiquee et al., 2013b, Schellekens et al., 2013c).

The oxytocin receptor (OTR), a class A GPCR, is a 7-transmembrane receptor expressed widely throughout the periphery and central nervous system in areas such as the reproductive system (Kimura et al., 2013, Thackare et al., 2006), and centrally in areas of the hippocampus, hypothalamus and amygdala (Raam et al., 2017b, Calcagnoli et al., 2014, Gimpl and Fahrenholz, 2001b) respectively. The OTR is known for its diverse function upon activation with its endogenous ligand oxytocin. Upon binding of oxytocin, OTR-mediated signalling occurs primarily through the Gαq alpha subunit (Gαq), but is also known to signal through the Gαi alpha subunit (Busnelli and Chini, 2018). Activation of Gαq subunit results in a cascade of events leading to inositol trisphosphate (IP3) and diacylglycerol (DAG) synthesis (Tuteja, 2009b). Activated IP3 subsequently interacts with its receptor and combined activation of the ryanodine receptor results in an influx of calcium (Ca^{2+}) mobilization from intracellular stores within the endoplasmic reticulum (Murtazina et al., 2011). This is

followed by allosteric activation of protein kinase C (PKC) by DAG (Huang, 1989). Ca^{2+} release is also known to play an important role in the activation of specific isoforms of PKC (Luo and Weinstein, 1993). The release of Ca^{2+} from intracellular stores has a wide array of functions (Berridge, 2002), including regulation of protein synthesis (Viero et al., 2010, Wong et al., 1993), cell proliferation and cell death (Capiod, 2011, Pinton et al., 2008), memory and learning (Nakamura et al., 2017) and membrane trafficking (Li et al., 2013).

The endogenous OTR ligand, oxytocin, is also known to regulate and interact with other central hormones (Crowley et al., 1991, Jorgensen et al., 2003b), including the gut-derived hormone, ghrelin, which is critically involved in appetite regulation and feeding (Howick et al., 2017, Schellekens et al., 2010, Pradhan et al., 2013). Increases in oxytocin levels have been shown to reduce circulating ghrelin levels in humans (Vila et al., 2009), while contrastingly oxytocin administration is known to enhance the secretion of ghrelin *in-vitro* in a ghrelin expressing cell line (Iwakura et al., 2011). Furthermore, in a neurohypophyseal diabetes cell line, addition of ghrelin resulted in increased secretion of oxytocin (Gálfi et al., 2016). Such crosstalk between oxytocinergic and ghrelinergic systems may indicate a possible interaction between the OTR and the ghrelin receptor type 1a (GHSR), due to OTR influence on oxytocin secretion (Viero et al., 2010).

The ghrelinergic system consists of the neuroendocrine peptide, ghrelin, which signals via the ghrelin receptor type 1a, referred throughout this manuscript as GHSR, and a truncated ghrelin type 1b receptor (Albarran-Zeckler and Smith, 2013). The GHSR, similarly to the OTR, also belongs to the class A GPCRs and is known to primarily signal through the Gαq protein, which results in the release of Ca^{2+} from intracellular stores (Schellekens et al., 2013f). The GHSR is also known to activate other signalling pathways, including the Gαi-dependent signalling pathway, which inhibits cyclic adenosine monophosphate (cAMP), a regulatory subunit of protein kinase A (PKA) (M'Kadmi et al., 2015). It is also known to activate the Gα12/13 subunit, upon activation it regulates the serum response element associated with transcriptional activity via hydrolysis of guanine triphosphates (Suzuki et al., 2009). Different GHSR ligands have also been shown to have functional selectivity and

biased signalling, which may result in more selective functional outputs when targeting the GHSR (Holst et al., 2004, Sivertsen et al., 2011, M'Kadmi et al., 2015, Ramirez et al., 2018).

The OTR and GHSR are known to play similar roles in centrally-regulated behaviours, where the OTR is implicated in mood, sociability and appetite regulation (Morton et al., 2012b, Myers et al., 2014, Sala et al., 2013, Lukas et al., 2011, Slattery and Neumann, 2010), with the GHSR primarily involved in appetite and metabolism (Howick et al., 2017, Schellekens et al., 2010, De Vriese and Delporte, 2007) and more recently also in anxiety and mood (Chuang and Zigman, 2010, Schellekens et al., 2012). Both the OTR and GHSR have the ability to form heterodimers with other GPCRs as seen in table 1.1 and 1.2 which results in alterations in receptor signalling (Schellekens et al., 2015b, Wrzal et al., 2012, Terrillon et al., 2003, Romero-Fernandez et al., 2013, Kern et al., 2012, Rediger et al., 2011). An overlap in receptor expression can be observed across multiple brain regions including the hippocampus (Zigman et al., 2006b, Lin et al., 2017a, Lin et al., 2018), the paraventricular and ventromedial sub regions of the hypothalamus (Yoshimura et al., 1993, Zigman et al., 2006b), the basolateral amygdala and the piriform cortex (Mani et al., 2014, Marusak et al., 2015).

Considering the similarities in central OTR and GHSR expression patterns, the behavioural overlap and shared involvement in similar disorders, we hypothesize that the previously documented crosstalk between the ghrelinergic and oxytocinergic systems may involve a direct interaction between the OTR and GHSR receptors and novel heteromer formation. Therefore, this study will investigate if a direct physical interaction exists between the OTR and GHSR and the functional consequences on downstream signalling associated with the formation of an OTR/GHSR heterocomplex.

2.3 Materials and methods

2.3.1 Receptor ligands

Endogenous agonists ghrelin (SP-GHRL-1, Innovagen), oxytocin (1910/1, Tocris), synthetic agonist carbetocin (SML0748, Sigma) and antagonist JMV 2959 (345888, Merck) were used to examine OTR and GHSR signalling. All compounds were prepared in assay buffer (1x Hanks Balanced Salt Solution supplemented with 20mM HEPES).

2.3.2 Transfection and cell culture

Human embryonic kidney cells (HEK293A) (Invitrogen, R70507) were stably transfected with plasmid containing the human growth hormone secretagogue 1a receptor sequence (GHSR) (Genecopeia, X0963; Accession code, U60179.1), were C-terminally tagged with an Enhanced Green Fluorescent Protein (HEK293A-GHSR-EGFP) and a neomycin resistance gene. Cell lines with the stable expression of the GHSR-EGFP were generated and previously described by our group (Ramirez et al., 2018, Schellekens et al., 2015b, Schellekens et al., 2013d, Schellekens et al., 2013f). Similarly, a plasmid construct containing the human oxytocin receptor (OTR) fused with a turbo green fluorescent protein (tGFP) (RG211797, Origene) and a neomycin resistance marker was stably transfected into HEK293A cells using Lipofectamine LTX reagent (15338500, Thermofisher) in accordance with the manufacturers specifications. Cells were antibiotic selected using G418 (Sigma, G418-RO) at a concentration of 500ng/ml. Further selection was performed with the use of flow assisted cell sorting (FACS) and monoclonal selection in 96 well plates, after which cells with the highest expression of the OTR-tGFP were chosen.

Through the course of the study cell lines were sustained in maintenance media using high glucose Dulbecco modified Eagle Media (DMEM) (Sigma, D5796) with the addition of 10% fetal bovine serum (FBS) (Sigma, F7524) and 1% Non-essential amino acids (NEAA) (Sigma, M7145). Medium was supplemented with G418 (Sigma, G418-RO), at the concentration 300 ng/ml and 500ng/ml for HEK-GHSR-EGFP and HEK-OTR-tGFP cell lines, respectively. Cells were regularly checked using flow cytometry to ensure maintained expression of the GFP tags. Cells were kept in an environment of 5% CO_2 at 37^{o}C. At a confluence of 80-90% cells were passaged to a lower density.

Both the OTR and GHSR were stably expressed in HEK293A cells to yield HEK293A-GHSR-EGFP (as previously described (Schellekens et al., 2013f))and HEK293A-OTR-tGFP lines (as described below). Co-expression of the opposing receptor was transiently incorporated into these cell lines using lentiviral vectors, as described below.

2.3.3 Lentiviral production and lentiviral transduction

A 2nd generation lentiviral packaging system (Follenzi and Naldini, 2002, Vigna and Naldini, 2000, Schellekens et al., 2013f) was used to transiently co-express either the GHSR or the OTR tagged with a C-terminal red fluorescent protein (tRFP) in cells stably expressing the OTR -tGFP or GHSR-EGFP. Briefly, both genes of interest were cloned into a pHR-SIN-BX-tRFP plasmid, a replication deficient HIV expression vector which as previously described in our lab (Schellekens et al., 2013f). Using HEK293T-17 cells, the cloned lentiviral plasmid was packaged alongside a pMD.D-VSV.G envelope construct and a 3rd generation lentiviral packaging construct pCMV ΔR8.91, to generate viral expression vector. Stable HEK293A-GPCR-GFP cell lines were transduced with these lentiviral vectors, Lv-GPCR-tRFP. To do so packaged viral vectors were diluted in transduction media (high glucose DMEM supplemented with 2% FBS, 1% NEAA and 8ug/ml of Polybrene (Sigma, H9268)) and incubated with cells. Following 24-hours' incubation the virus was diluted ½ with the addition of transduction media. After a further 48-hours incubation media was replaced with maintenance media for another 24 hours. Fluorescence was measured by flow cytometry to confirm successful transduction and GPCR expression.

2.3.4 Co-localization and trafficking of the receptors

HEK293A cells stably expressing GHSR-EGFP transduced with lentiviral plasmid Lv-OTR-tRFP were used to analyse co-localization and ligand-mediated co-internalization of receptors, as previously shown (Schellekens et al., 2015b). Cells were seeded at a concentration of 7*10^4 cells/well on 24 well plates (83.3922.005, Sarstedt) containing Poly-L-Lysine treated borosilicate discs and incubated for 24-30 hours at 5% CO_2 at 37^{o}C. Cells were then fixed to borosilicate discs by incubating cells for 30 minutes with 4% paraformaldehyde. The borosilicate discs containing cells were then mounted onto microscope slides and receptor co-localization was

visualised using a 60x objective on a laser scanning confocal fluorescent microscope (FV 1000 Confocal System, Olympus). Cells were further analysed using the Olympus fluoview FV3000 software. Images from 4 independent experiments, with 2-6 images of individual cells taken per experiment, were subsequently merged to observe possible overlay of receptors using the Image J software, merge coloured channels option.

Ligand-mediated trafficking was used to determine any alterations to receptor trafficking and internalization. Cells were seeded 5*10^4 cells/well in a 24 well plate (83.3922.005, Sarstedt) 48 hours prior to assay. Media was changed to serum free maintenance media 24 hours prior to assay. Trafficking of both receptors was assessed following ligand pre-treatment for 60 minutes. Cells were then fixed using paraformaldehyde and subsequently imaged using fluorescent microscopy (IX71, Olympus). Image J software was used to separately trace the subcellular region and membrane region of the cells. The subcellular region was measured as the fluorescence within the cytosol and perinuclear space, while the membrane region was measured as the outer area lining the cell. The fluorescent intensity values for GFP and for RFP in each area were obtained and used to calculate the ratio of perinuclear/membrane fluorescent intensity. Once the ratios were attained GraphPad (Prism 5.0; GraphPad Software Inc.) was used to graph results. For each treatment group, 4 independent experiments were performed with treatments in triplicate per experiment. Within each well, three images were captured using fluorescent microscopy, where 3-4 cells per image were quantitatively analysed.

2.3.5 Intracellular calcium mobilization assays

Intracellular Ca^{2+} mobilization was monitored with the use of the FLIPR Tetra® High-Throughput Cellular Screening System (Molecular Devices), as previously described in our lab (Ramirez et al., 2018). All cell lines were seeded at 3.5x105 cells/ml in black clear bottom 96 well microtiter plates (3904, Corning) and incubated overnight at standard culture conditions. Maintenance media was changed to serum free media 24 hours prior to assay. On the day of the assay, ligand-mediated intracellular Ca^{2+} influx was measured according to the manufacturer's protocol (R8186, Molecular Devices). Briefly, media was removed from wells and replaced with 80µl of Ca^{2+} 5 dye

(R8186, Molecular Devices) diluted in assay buffer. Cells were incubated for 1.5 hours in a humidified environment at 37°C with 5% CO_2. Next, 40 µL of 3x concentrated compounds were prepared in assay buffer and added to the cell plate using the liquid handling mode of the FLIPR Tetra® High-Throughput Cellular Screening System (Molecular Devices). Fluorescent readings were taken at an excitation of 485nm and emission of 525nm. The percentage of relative fluorescent units (RFU), calculated as the difference between the maximum (Vmax) and baseline (Vmin) fluorescence (Vmax-Vmin) and corrected for background fluorescence in the cell, was normalized to the maximum response (100% signal) obtained with 3.3% fetal bovine serum (FBS). All data was analysed using GraphPad Prism software (PRISM 5.0; GraphPad Software Inc.) using nonlinear regression analysis and variable slope.

2.3.6 Inositol monophosphate one accumulation assay

Receptor mediated changes in inositol monophosphate accumulation (IP-ONE) were measured using Cisbio HTRF technology (62IPAPEB, Cisbio) and analysed using the Flex-station II (Molecular Devices). Compounds were prepared at a 2x concentration in assay buffer containing 50 mM lithium chloride (LiCl). A total of 35 µl was then added to the corresponding wells of a white, flat bottom, 96 well plate (675074, Greiner Bio-One). Cells were prepared by removing media and washing with phosphate buffered saline (PBS), after which the cells were mechanically detached using PBS, spun down at 800 rpm and re-suspended in assay buffer. Cell were added at a concentration of $3.0x10^4$ cells/well (35 µl) and incubated for 1hr at 37°C. Next, 75 µl of IP1-d2 acceptor and 75 µl of anti- cryptate conjugate donor (62IPAPEB, Cisbio) were added to each well and then incubated for 1 hour at room temperature in the dark. Here IP1-d2 competes with native IP-ONE for binding with the anti-cryptate conjugate. Using the Flex-station II (Molecular Devices) integration settings were adjusted to 400 µs and an integration delay at 50 µs. Excitation is set at 314 nm and emission at 620 nm and 665 nM. The accumulation of IP-ONE was calculated as the HTRF ratio of $665/620*10^4$ and expressed as a percentage of the control (untreated cells). Results were calculated using GraphPad Prism software (PRISM 5.0; GraphPad Software Inc).

2.3.7 Flow cytometry based fluorescence resonance energy transfer

To assess physical interaction between receptors under investigation, all cell lines were washed and mechanically detached from the wells using PBS and centrifuged for 3 minutes at 800 rpm. The cell pellet was then re-suspended in 400 µl of suspension buffer (2nM Ethylenediaminetetraacetic acid (EDTA) in PBS). The cells were passed through a 100 µm nylon mesh cell strainer and collected in a 5ml round bottom polystyrene tube. The BD FACSAria™ Fusion (BD Biosciences) was used to analyse fluorescence energy resonance transfer (FRET). Non-transfected and non-transduced HEK293A cells were used to define the instrument settings, adjusting cell gating based on their size and granularity (forward scatter, side scatter and photomultiplier tube voltage). Cells expressing either OTR-tGFP alone or GHSR-tRFP alone were used to further set the photomultiplier tube voltage and compensate for spectral bleed through. A total of $1.0x10^4$ cells of interest were then analysed for FRET signal and recorded. The tGFP, used as the donor was excited at 488 nm and detected at 525/550 nm, while tRFP, the acceptor was excited at 560 nm and detected using 610/620 nm. The FRET signal between the tGFP/tRFP pair was analysed using an excitation of 488 nm and detected at 610/620 nm. A two-dimensional dot plot of tGFP versus tRFP was used to gate cells co-expressing both receptors, which was subsequently used to construct the final dot plot of tGFP versus the fcFRET signal and histograms of number of cells versus fluorescent intensity of fcFRET.

2.3.8 Primary neuronal cultures

The hypothalamus and hippocampus of postnatal day 1 (P1) Sprague Dawley rats were dissected following rapid decapitation. The dissection of the hypothalamus and hippocampus was performed as previously described, modified for P1 pups (Staal et al., 2007). The brain was removed and placed on an ice-cold petri-dish. The ventral side was faced up; the meninges were then removed from around the hypothalamus. Using a C5 forceps the frontal lobe was pushed down to allow the hypothalamus to be pinched out using a C7 forceps. A scalpel was then used to remove the frontal lobe and to separate the right hemisphere from the left. With the medial side facing up the C7 forceps was placed under the corpus callosum and the thalamus and striatum gently removed revealing the hippocampus, the C7 forceps was then placed under

the ventral hippocampus and the hippocampus was gently rolled out until removed. Following dissection, brain regions were stored in harvest media (50 ml hibernate A (A12475-01, Invitrogen), 125 µl 100x glutamax (35050038, Invitrogen)). Following this the tissue was dissociated with the use of papain media (10 mg papain (P4762, Sigma), 5 ml Hibernate A (-CaCl2) (HA, Brainbits), 12.5 µl 100x glutamax (35050038, Invitrogen), 50 µl 100 mM L-Cysteine (C7352, Sigma), 500 µl 5mM EDTA/PBS) incubated with tissue for 30 minutes with gentle shaking at 37^{o}C. To ensure a single cell suspension was obtained, tissue was triturated with a 18G needle in triturate media (46 ml Neurobasal media (21103, Invitrogen), 2.5 ml Heat inactivated FBS, 125 µl 100x glutamax, 500 µl 100x penicillin/streptomycin and 500 µl 100x NEAA) and centrifuged at 1100 rpm, this step was repeated until a single cell suspension was attained. Subsequently cells were plated on 24 well plates (83.3922.005, Sarstedt) pre-treated with Poly-L-Lysine. Cells were kept in neurobasal maintenance media (47.5 ml neurobasal media, 125 µl 100x glutamax, 1 ml B27 (17504-044, Invitrogen), 500 µl 100x penicillin/streptomycin, 50 µl Mito/serum extender (355006, Biosciences) and 500 µl of 100x NEAA) in a humidified environment at 37^{o}C with 5% CO_2. Media was changed every 4 days until cells were at the required density. Cells were fixed using 4% paraformaldehyde for 30 minutes at room temperature.

2.3.9 Immunocytochemistry of neuronal cells

The fixed neuronal cultures were incubated for 2 hours at room temperature with blocking solution, containing 5% bovine serum albumin (BSA) (A2153, Sigma) dissolved in PBS and 0.1% Triton. Next, primary antibodies were diluted in PBS supplemented with 1% BSA and 0.1% Triton. Monoclonal goat anti-OTR (ab87312, Abcam, 1:200) and monoclonal rabbit anti-GHSR (ab95250, Abcam, 1:300) were added to corresponding well, and incubated overnight at 4^{o}C. The specificity of these antibodies for their corresponding receptors have been previously confirmed (Gonzalez-Iglesias et al., 2015, Yoshimoto et al., 2017). The following day cells were washed 4 times with PBS and 0.1% Triton. Subsequently the donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11055, Thermoscientific, 1:500) and Chicken anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (A-21442, Thermoscientific, 1:500) were diluted

in PBS with 1% BSA and 0.1% Triton. The secondary antibodies were then added to each well and incubated for 2 hours at room temperature in a dark environment. Cells were then washed 3 times in PBS with added 0.1% Triton. Next cells were counterstained with bisbenzimide (1:2000) for 4 minutes and washed 2 times with PBS and 0.1% Triton. Finally, the borosilicate discs containing stained cells were fixed onto microscope slides. Co-localization was then analysed using laser scanning confocal fluorescent microscopy (FV 1000 Confocal System, Olympus).

2.3.10 Statistical analysis

Statistical analysis of Ca^{2+} mobilization, IP-ONE accumulation and ligand-mediated internalization assays was performed using Prism Software (GraphPad Prism 5.0). All data is represented as the mean ± SEM. The number of independent experiments is described in each graph legend. D'Agostino & Pearson was used to test for normality. Data, which followed Gaussian distribution, was analysed using Two-way ANOVA with a Bonferroni post-hoc test. A non-parametric Mann Whitney was used for data which did not follow Gaussian distribution. Significance was defined as follows; * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.

2.4 Results

2.4.1 Co-localization of the oxytocin and ghrelin receptors

The first step in identifying the formation of a heterocomplex between the OTR and GHSR was to check if these receptors co-localize within the cell. Confocal microscopy revealed receptor co-localization following transient transfection of HEK-OTR-tGFP with Lv-GHSR-tRFP within the same confocal plane, indicating a close proximity of receptor expression within the same cells, which would make a physical interaction between OTR and GHSR a possibility. When images of cells expressing HEK-OTR-tGFP (Figure 2.1a) and Lv-GHSR-tRFP (Figure 2.1B) are merged a yellow overlay can be seen indicating co-localization of the receptors, blue DAPI (4',6-diamidino-2-phenylindole) staining (yellow overlay only) was used to highlight the nucleus of the cell (Figure 2.1C). Furthermore, a ubiquitous expression of each individual receptor can be seen throughout the cell cytoplasm; a pattern previously seen for these receptors (Di Benedetto et al., 2014, Mear et al., 2013).

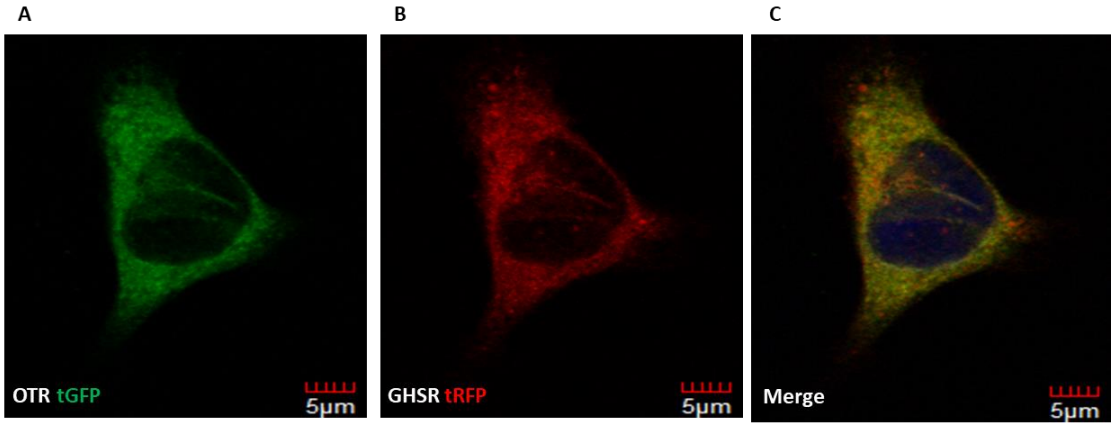

Figure 2.1: Co-localization of the OTR and GHSR co-expressed in HEK293A. (A) *Expression of HEK-OTR tGFP in green co-localizes with* ***(B)*** *expression of Lv-GHSR tRFP in red as indicated by* ***(C)*** *merged images showing overlay (yellow) of the HEK-OTR-tGFP and Lv-GHSR-tRFP. This image represents the co-localization seen across three independent lentiviral transductions.*

2.4.2 Flow cytometry based fluorescence resonance energy transfer shows an interaction between the oxytocin and ghrelin receptors

To study the possible physical interaction between the OTR and GHSR, flow-cytometry based fluorescence resonance energy transfer (fcFRET) was used. The analysis of fcFRET signal was performed according to the protocols previously described by our and other groups (Schellekens et al., 2015b, Chruścicka et al., 2018, Banning et al., 2010, Wang et al., 2006). The fluorescent tags of HEK-OTR-tGFP and Lv-GHSR-tRFP were utilised for fcFRET analysis. The tGFP (donor) and tRFP (acceptor) are known to significantly overlap in donor emission and the acceptor absorption spectrum. Therefore, they are widely accepted for the use in FRET experiments including flow cytometry-based FRET analysis. As seen in Figure 2.2A an average positive fcFRET signal of 29.5% is observed across the cell population co-expressing both the HEK-OTR-tGFP and Lv-GHSR-tRFP. Due to FRET occurring between donor and acceptor molecules located within 100 Å, this positive fcFRET signal indicates that the physical interaction required to form an OTR and GHSR heterocomplex is present. Transduction of HEK-OTR-tGFP with a control Lv-tRFP plasmid showed a negligible average fcFRET signal of 0.3%, indicating no false FRET signal is produced due to random collision from over-expression of these two fluorescent proteins. The HEK-OTR-tGFP and Lv-GHSR-tRFP expressed alone did not show a fcFRET signal, indicating no artificial signal was produced from tGFP at an emission reading of 610/620 nm, and similarly no adsorption of tRFP at an excitation of 488 nm. Figure 2.2B further shows alteration to the median fluorescent intensity of the fcFRET signal. When HEK-OTR-tGFP and Lv-GHSR-tRFP are co-expressed (HEK-OTR-tGFP-GHSR-tRFP) a 5-fold change in fluorescent intensity is seen compared to HEK-OTR-tGFP and Lv-GHSR-tRFP expressed alone. Changes in median fluorescent intensity due to the overexpression of receptors was controlled for by using HEK-OTR-tGFP-Lv-tRFP control cells. The median fluorescent intensity of HEK-OTR-tGFP-Lv-tRFP was similar to the median fluorescent intensity of HEK-OTR-tGFP and Lv-GHSR-tRFP expressed alone, indicating that observed changes in median fluorescent intensity are a result of receptor co-expression and not overexpression of receptors. This result adds evidence for the formation of an OTR and GHSR heterocomplex.

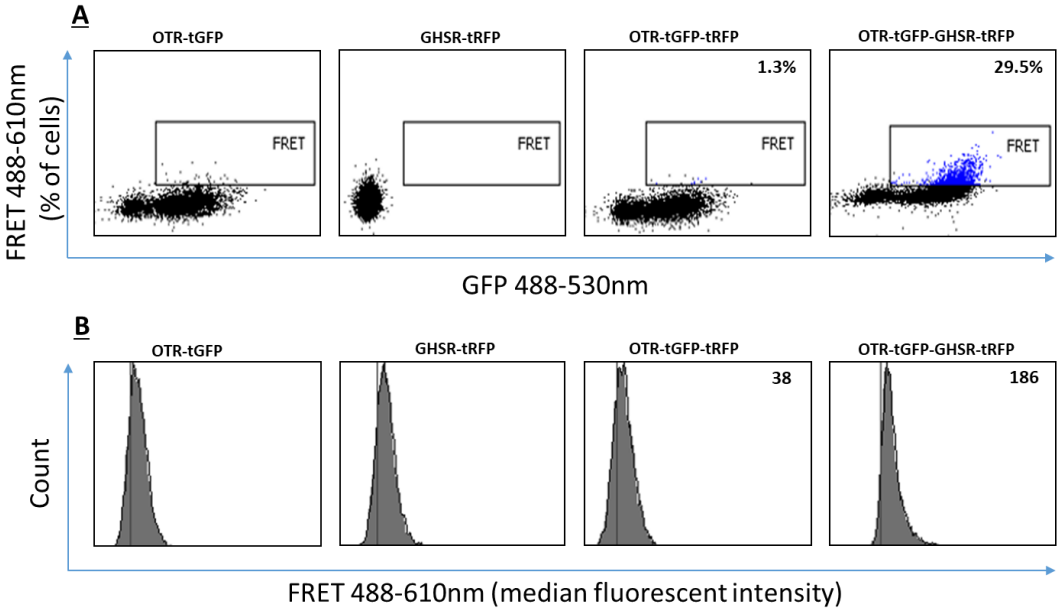

Figure 2.2: Flow Cytometry based FRET between OTR and GHSR. (A) *A FRET signal was seen across 29.5% of total population of HEK cells co-expressing OTR-tGFP and GHSR-tRFP, following subtraction of 1.3% the FRET signal seen in negative control OTR-tGFP-tRFP.* ***(B)*** *A shift in fluorescent intensity of FRET signal can be seen when both OTR and GHSR are expressed in the one cell, this is compared to both receptors expressed alone and control cells OTR-tGFP-tRFP. Each graph is representative of 3 independent experiments with values being an average of the 3 independent experiments.*

2.4.3 Receptor trafficking analysis reinforces an interaction between the oxytocin and ghrelin receptors

Ligand-mediated internalization was used to analyse the influence of receptor co-expression on the trafficking of the OTR and GHSR. Receptor agonists, ghrelin (Figure 2.3B), oxytocin (Figure 2.3C) and carbetocin (Figure 2.3D) were used to analyse this effect. Interestingly Figure 2.3A reveals high subcellular localization, possibly due to a high basal activity of these receptors, when co-expressed under control conditions, in concurrence with Figure 2.1. When treated with the OTR and GHSR agonists (Figure 2.3B, C) respective receptor expression becomes increasingly granular as indicated by the arrows in Figure 2.3 B, C & D, which is indicative for altered receptor trafficking following ligand treatment and receptor internalization into intracellular vesicles (Oakley et al., 2001). Changes to ligand-induced receptor trafficking can be

observed when both receptors are co-expressed in cells. As expected, cells treated with oxytocin or the synthetic OTR agonist carbetocin, demonstrate increased OTR trafficking and internalization in cells expressing the HEK-OTR-tGFP alone or co-expressing Lv-GHSR-tRFP (Figure 2.3F). Interestingly, Lv-GHSR-tRFP upon co-expression with HEK-OTR-tGFP also demonstrates a significant change in receptor expression following oxytocin and carbetocin treatment (Figure 2.3E, $P < 0.0001$), while these ligand-mediated changes in GHSR trafficking are not seen when Lv-GHSR-tRFP is expressed alone (Figure 2.3E). This suggests coordinated movement of the OTR/GHSR pair when co-expressed and reinforces a direct physical interaction between the OTR and GHSR. In addition, ghrelin induces internalization of the GHSR, when expressed alone or co-expressed in HEK-OTR-tGFP (Figure 2.3E). Noteworthy, significant changes are also observed to the trafficking of the OTR following ghrelin treatment, but only when HEK-OTR-tGFP and Lv-GHSR-tRFP are co-expressed (Figure 2.3F, $P<0.0001$), while this is not observed when HEK-OTR-tGFP is expressed alone (Figure 2.3F), again highlighting co-ordinated movement of these receptors following co-expression. These results confirm ligand-mediated coordinated trafficking of both receptors within the cell, which reinforces the possible formation an OTR/GHSR heterocomplex.

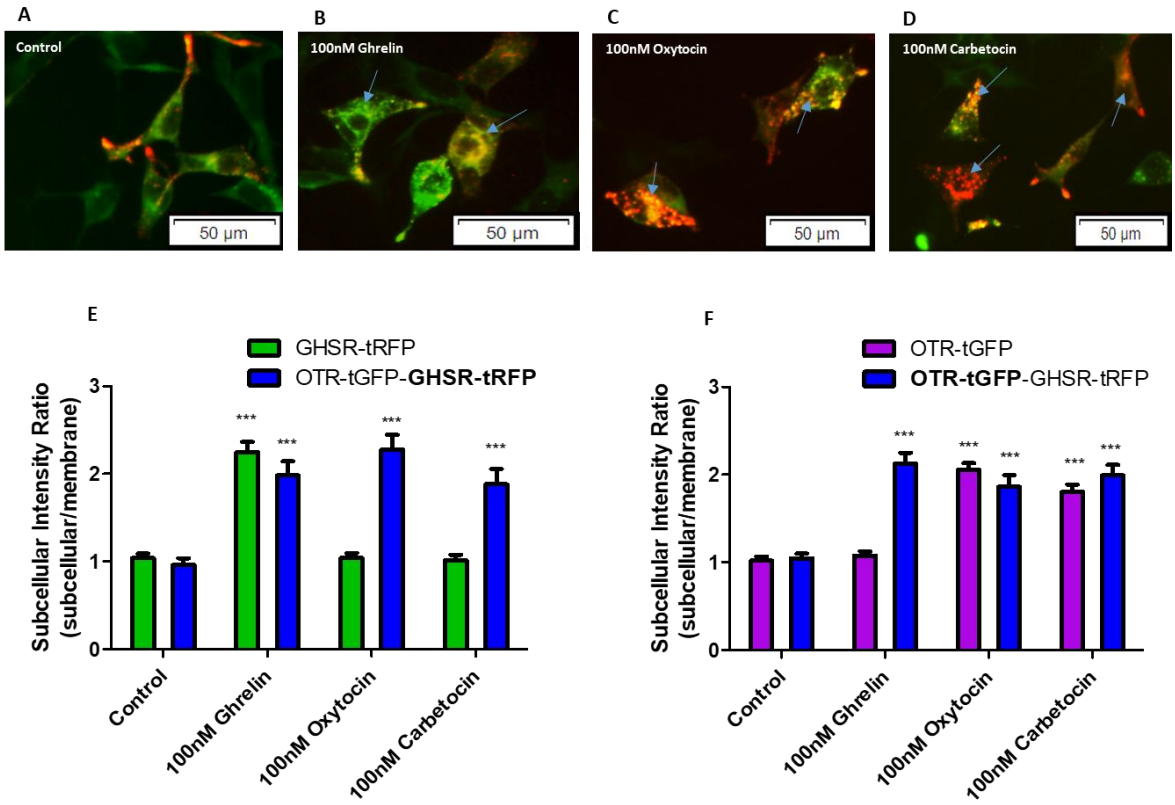

Figure 2.3: Dual ligand-mediated receptor trafficking following co-expression of OTR and GHSR. (A) *Untreated HEK-OTR-tGFP co-expressing Lv-GHSR-tRFP (**B-D**) Addition of ghrelin, oxytocin and carbetocin results in increased trafficking of HEK-OTR-tGFP-Lv-GHSR-tRFP as indicated by arrows.* ***(E)*** *Quantitative analysis shows co-trafficking of Lv-GHSR-tRFP when co-expressed with HEK-OTR-tGFP.* ***(F)*** *Quantitative analysis shows co- trafficking of HEK-OTR-tGFP when co-expressed with Lv-GHSR-tRFP. Data is representative of 3 independent experiments with each treatment in triplicate for each experiment. Results are represented as ±SEM, statistical significance was analysed using a Two-way ANOVA followed by Bonferoni post-hoc test comparing between treatment groups and there relevent control, * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.*

Together, the dual ligand-mediated receptor trafficking, positive fcFRET signal, and co-internalization of the OTR and GHSR pair indicate the possible organisation of these receptors into a heterocomplex (Figure 2.1, 2.2 &2.3). Next, we investigated the functional consequences of such interaction on the downstream signalling pathways of these receptors.

2.4.4 Attenuation of the oxytocin receptor mediated calcium signalling via inositol monophosphate accumulation

Ligand-mediated Ca^{2+} mobilization was analysed using the FLIPR Tetra high throughput screening platform. In cells co-expressing HEK-OTR-tGFP and Lv-GHSR-tRFP, significant attenuation of OTR-mediated signalling was observed compared to cells expressing the HEK-OTR-tGFP alone (Figure 2.4A, $F(10,224) = 14.01$, $P<0.0001$). A significant 19-fold shift in oxytocin EC_{50} concentration was seen when HEK-OTR-tGFP and Lv-GHSR-tRFP are co-expressed (Figure 2.4B, $P= 0.0317$), indicating changes to the potency of oxytocin and efficacy of the OTR upon co-expression. Comparing Lv-GHSR-tRFP co-expressed with HEK-OTR-tGFP to Lv-GHSR-tRFP expressed alone, no alteration in GHSR-mediated Ca^{2+} signalling was observed when treated with GHSR endogenous ligand ghrelin (Figure 2.4D, $P>0.1000$). Furthermore, no significant change to ghrelin EC_{50} was seen (Figure 2.4E, $P= 0.1508$). These results highlight a significant attenuation effect of GHSR expression on the Gαq-dependent signalling of the OTR.

To ensure the attenuation observed in OTR mediated Gαq signalling was not due to the system used to express the receptor or due to the expression of specific fluorescent proteins, the OTR and GHSR were cloned into differing expression vectors containing different fluorescent tags, as described in the methods section. This resulted in the expression of HEK-GHSR-EGFP and Lv-OTR-tRFP compared to HEK-OTR-tGFP and Lv-GHSR-tRFP (Figure 2.4A, B, D&E). Ca^{2+} mediated signalling was again analysed using the FLIPR Tetra high throughput screening platform. Similar to our previous result, Lv-OTR-tRFP signalling was significantly attenuated when co-expressed with HEK-GHSR-EGFP compared to Lv-OTR-tRFP alone (Figure 2.4C, $F(1,32) = 3.394$, $P=0.0747$). Moreover, HEK-GHSR-EGFP signalling remained unchanged when co-expressed with Lv-OTR-tRFP compared to expression of HEK-GHSR-EGFP alone (Figure 2.4F, $F(1,32) = 0.9500$, $P=0.3370$). These findings highlight the robust attenuation associated with OTR-mediated Gαq signalling upon co-expression with the GHSR.

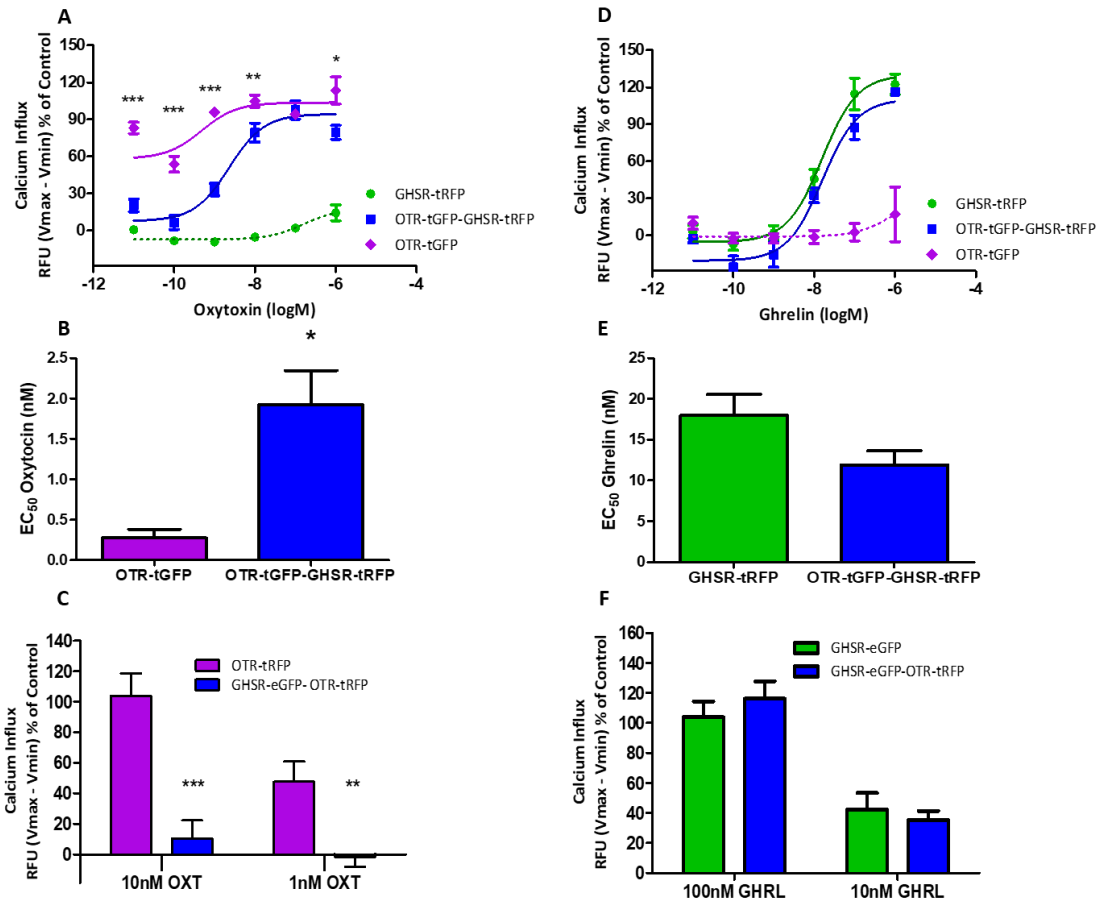

Figure 2.4: Co-expression attenuates OTR mediated calcium signalling. (A) *HEK-OTR-tGFP induced* Ca^{2+} *signalling is downregulated when co-expressed with Lv-GHSR-tRFP in the presence of endogenous agonist oxytocin.* ***(B)*** *Potency, as indicated by* $EC_{50,}$ *is decreased from 0.1nM in OTR to 1.9nM when HEK-OTR-tGFP and Lv-GHSR-tRFP are co-expressed. Similar attenuation of OTR mediated signalling is observed* ***(C)*** *when fluorescent tags are reversed, expressing HEK-GHSR-EGFP and Lv-OTR-tRFP.* ***(D)*** *There is no change in GHSR induced* Ca^{2+} *signalling Lv-GHSR-tRFP is co-expressed with HEK-OTR-tGFP* ***(E)*** *the* EC_{50} *remains unchanged at 12.3nM for Lv-GHSR-tGFP compared to 14.3nM for Lv-GHSR-tRFP co-expressed with HEK-OTR-tGFP.* ***(F)*** *Reversal of fluorescent tag to express HEK-GHSR-EGFP and Lv-OTR-tRFP resulted in no changes to GHSR signalling when HEK-GHSR-EGFP is expressed alone or co-expressed with Lv-OTR-tRFP. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed (B, E) at each concentration (A, C, D, F), * indicating* $p < 0.05$*; ** indicating* $p < 0.01$*; or *** indicating* $p < 0.001$*. Graphs are representative data of 3 independent experiments with each treatment in triplicate for each experiment.*

To ensure that alterations to Ca^{2+} mobilization were specific for the Gαq signalling pathway, accumulation of inositol monophosphate, another downstream signalling molecule produced from Gαq activation, was also analysed. Cells expressing the HEK-OTR-tGFP alone or co-expressing both the HEK-OTR-tGFP and Lv-GHSR-tRFP were treated with OTR endogenous agonist, oxytocin. A significant attenuation in OTR-mediated IP-ONE accumulation can be observed upon co-expression with the GHSR (Figure 2.5A, $F(2, 68) = 11.66$, $P<0.0001$), which is in line with the attenuated Ca^{2+} influx (Figure 2.4A). Expression of the Lv-GHSR-tRFP alone or co-expressed with the HEK-OTR-tGFP showed no effect on GHSR mediated IP-ONE accumulation when treated with endogenous agonist, ghrelin (Figure 2.5C, $F(2,69) = 2.764$, $P=0.0701$).

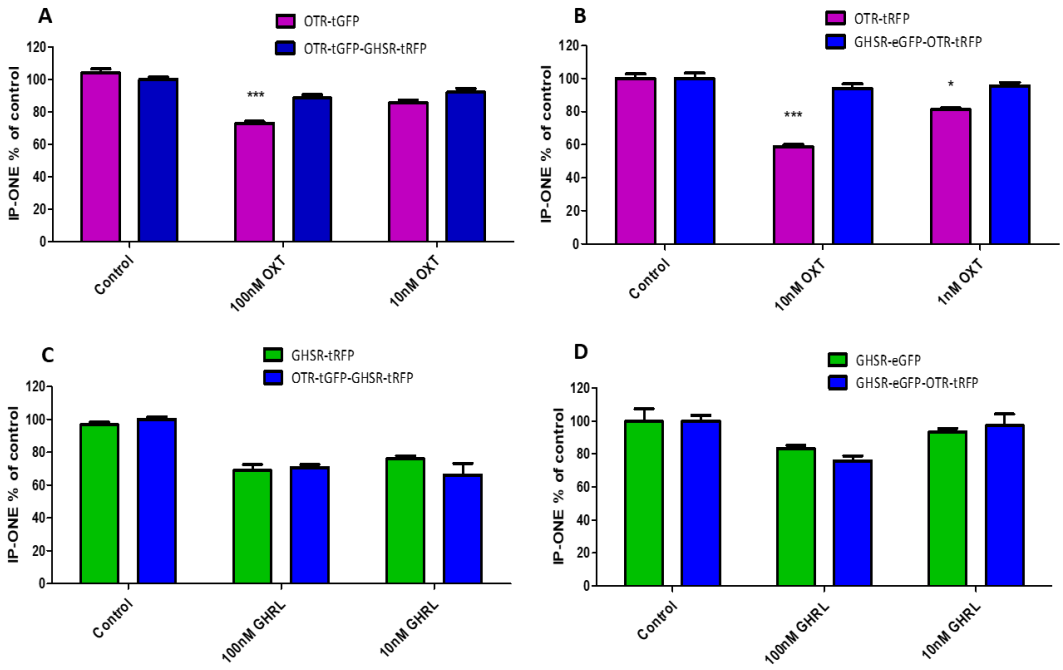

Figure 5: OTR-mediated inositol monophosphate accumulation is attenuated in the presence of the GHSR (A) *Reduced IP-ONE accumulation can be seen when HEK-OTR-tGFP was co-expressed with Lv-GHSR-tRFP.* ***(B)*** *Attenuated OTR signalling is still observed when receptors tags are changed so as to express HEK-GHSR-EGFP and Lv-OTR-tRFP.* ***(C)*** *No significant alteration is seen to Lv-GHSR-tRFP IP-ONE accumulation when co-expressed with HEK-OTR-tGFP.* ***(D)*** *Similarly, no change to GHSR IP-ONE accumulation is seen when receptors tags are changed to express HEK-GHSR-EGFP and Lv-OTR-tRFP. Results are represented as ±SEM, statistical significance between groups comparing receptor alone versus co-expressed, at each concentration, * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$. Data is representative of 2-3 independent experiment with treatments in triplicate per experiment.*

Next, IP-ONE accumulation was performed in stable HEK-GHSR-EGFP expressing cells transiently transduced with the Lv-OTR-tRFP. Here, the oxytocin-mediated IP-ONE accumulation in HEK cells transduced with Lv-OTR-tRFP was only attenuated when Lv-OTR-tRFP was co-expressed in HEK-GHSR-EGFP cells (Figure 2.5B, $F(2,32) = 24.55$, $P<0.0001$). Moreover, ghrelin-induced IP-ONE accumulation was unchanged in HEK-GHSR-EGFP co-expressing Lv-OTR-tRFP, which is in line with the OTR specific attenuation upon GHSR co-expression (Figure 2.5D, $F(2,32)=0.8066$, $P=0.4552$). These data also show that the decrease in OTR-mediated Gαq signalling following GHSR co-expression, is not due to the expression system used (i.e stable versus

transient, transfection versus transduction) or the fluorescent tags used (i.e GFP versus RFP). These data further highlight the ability of GHSR co-expression to mediate the OTR-mediated Gαq signalling.

2.4.5 Ghrelin receptor antagonist pre-treatment inhibits oxytocin receptor calcium signalling

Next, cells co-expressing the HEK-OTR-tGFP and Lv-GHSR-tRFP were pre-treated with a specific GHSR antagonist, JMV 2959, prior to Ca^{2+} mobilization analysis. Here, OTR signalling was shown to be further attenuated in the presence of GHSR antagonist JMV 2959 compared to oxytocin treatment alone, but only upon co-expression with the GHSR (Figure 2.6A, $F(3, 73) = 1.355$, $P=0.2634$). However, no significant alteration to the EC_{50} of oxytocin was observed following pre-treatment with JMV 2959 (Figure 2.6B), indicating the ability of JMV 2959 to alter the efficacy of oxytocin following OTR and GHSR co-expression. In addition, JMV 2959 had no effect on HEK-OTR-tGFP signalling when OTR is expressed alone (Figure S2.1a), but JMV 2959 was able to inhibit Lv-GHSR-tRFP signalling when expressed with HEK-OTR-tGFP (Figure 2.6C, $P=0.0076$, $P=0.0048$) with significant alteration to ghrelin EC_{50}, as expected (Figure 2.6D, $P= 0.0500$). A similar effect of attenuated GHSR Ca^{2+} mobilization was observed on Lv-GHSR-tRFP cells exposed to JMV 2959 (Figure S2.1b).

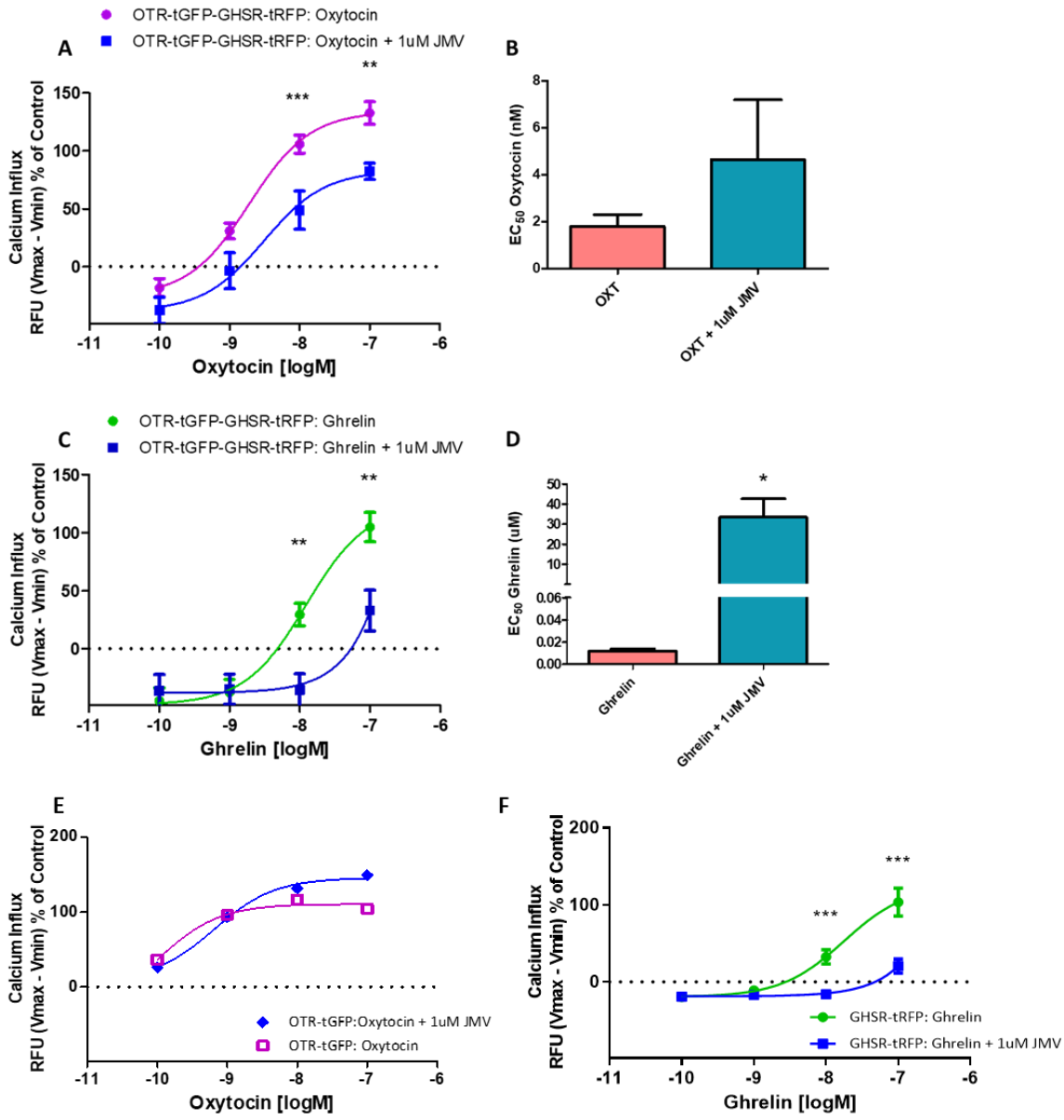

Figure 2.6: GHSR antagonist pre-treatment reduces efficacy of the OTR mediated calcium signalling. ***(A)*** *Pre-treatment with GHSR antagonist JMV 2959 results in a further attenuation of OTR mediated Ca^{2+} signalling when HEK-OTR-TGFP and Lv-GHSR-tRFP are co-expressed.* ***(B)*** *EC_{50} remains similar at 1.9nM for oxytocin and 3.0nM for oxytocin + 1uM JMV 2959* ***(C)*** *GHSR antagonist JMV 2959 blocks GHSR mediated Ca^{2+} signalling when Lv-GHSR-tRFP and HEK-OTR-tGFP are co-expressed.* ***(D)*** *EC_{50} is altered from 0.01uM for ghrelin and 20uM for ghrelin + 1uM JMV 2959.* ***(E)*** *JMV 2959 has no effect of OTR mediated Ca^{2+} signalling when OTR is expressed alone.* ***(F)*** *As expected JMV2959 attenuates the effect of GHSR-tRFP when expressed alone. Results are representative of three independent experiments with each treatment in triplicate per experiment*

*Results are depicted as ±SEM. Statistical significance is compared between groups at all concentration (A, C) and between groups (B, D), * indicating p < 0.05; ** indicating p < 0.01; or *** indicating p < 0.001. Graphs are a representative of 4 independent experiments with each treatment being in triplicate per experiment.*

2.3.6 Co-localization of the oxytocin receptor and ghrelin receptor in hypothalamic and hippocampal primary cultures

Next, we investigated co-localized expression of the OTR and GHSR under endogenous expression levels in in hypothalamic and hippocampal postnatal day 1 rat neuronal cultures. Confocal microscopy images show that GHSR (shown in red) (Figure 2.7B) and OTR (shown in green) (Figure 2.7C) co-localize, as indicated by the by the yellow overlay observed in the merged image, which indicated that these receptors co-localize on the same confocal plane (Figure 2.7D). Furthermore, a ubiquitous co-localized expression of both receptors throughout the cell body and dendrites of the hippocampal neurons can be seen. The ubiquitous sub-cellular expression of both receptors is in line with that observed in the heterologous expression system in Figure 1.

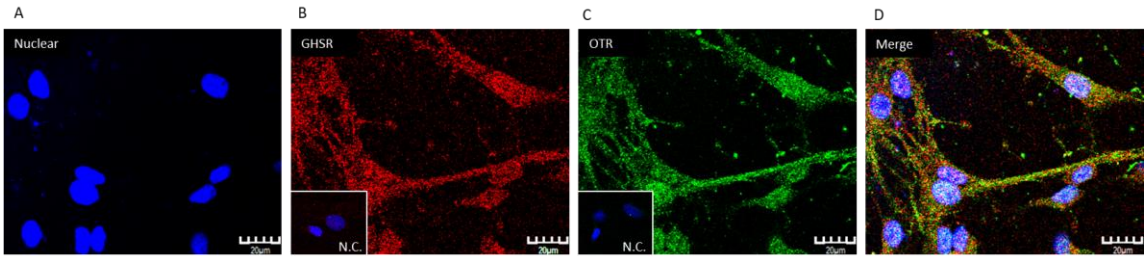

Figure 2.7: Co-localization of OTR and GHSR in rat hippocampal neurons. *Hippocampal neurons with a* ***(A)*** *nuclear stain were shown to express both* ***(B)*** *GHSR (red) and* ***(C)*** *OTR (green).* ***(D)*** *Co-localization is shown in the merged image, indicated by yellow. Data is representative of 3 independent staining experiments where hippocampal neurons from postnatal day 1 rats were pooled (N-4) from 2 separate litter groups.*

Next, OTR and GHSR expression was investigated in the hypothalamus. The hypothalamus plays a prominent role in integrating signals from central and peripheral pathways and is involved in behaviours such as appetite, mood and sociability (Sohn, 2015, Drevets et al., 2008). As can be seen in Figure 2.8, co-localization on a single confocal plane is shown between the OTR (green) (Figure

2.8C) and the GHSR (red) (Figure 2.8B) as observed by the yellow overlay of the merged image (Figure 2.8D). Expression of both OTR and GHSR is also clearly localized on the cell membrane. Interestingly, this expression pattern differs slightly from that seen in the hippocampus where expression is seen mainly subcellular, throughout the cell body and neuronal dendrites.

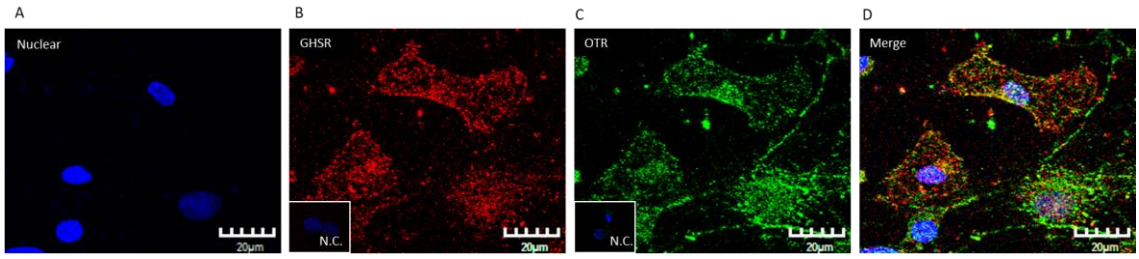

Figure 2.8: Co-localization of OTR and GHSR in rat hypothalamic neurons. *Primary hypothalamic neurons with* ***(A)*** *nuclear stain from postnatal day 1 rat pups were shown to express both* ***(B)*** *the GHSR (red) and* ***(C)*** *the OTR (green).* ***(D)*** *Co-localization is indicated by the yellow overlay seen in the merged image. Data is representative of 3 independent staining experiments where hippocampal neurons from postnatal day 1 rats were pooled (N-4) from 2 separate litter groups.*

2.5 Discussion

The concept of heterodimerization adds a novel dimension to GPCR pharmacology and paves the way forward for new GPCR-targeting therapeutics (Hübner et al., 2016, Qian et al., 2018). The influence of heterocomplex formation on downstream GPCR signalling and functionality is vast but always directly dependent on the receptors that are present within the complexes (Terrillon and Bouvier, 2004b, Romero-Fernandez et al., 2013, Scarlett et al., 2018, Schellekens et al., 2013b). Ligands specific to one GPCR present within the complex, will be able to modulate downstream signalling of the other GPCRs within the heterocomplex, to which they have no innate affinity (Ward et al., 2011b, Ellis et al., 2006, Borroto-Escuela et al., 2014b, Wrzal et al., 2012, Schellekens et al., 2015b). Therefore, the discovery of novel GPCR-GPCR interactions is poised to lead to a better understanding of the pathophysiology of disorders associated with alterations in GPCR signalling and function (Borroto-Escuela et al., 2017a). Here we show, to our knowledge for the first time, compelling evidence for an interaction between the OTR and GHSR, which is likely to occur via the formation of a heterocomplex and leads to a significant attenuation of OTR-mediated Gαq signalling. This represents an exciting novel finding, which contributes to the advancement of the GPCR dimerization field overall, but in particular contributes to the intricate signalling crosstalk between oxytocinergic and ghrelinergic systems. Aberrant ghrelinergic signalling via the GHSR is associated with a multitude of disorders, spanning from obesity to depression (Howick et al., 2017, Tuncel et al., 2016, Huang et al., 2017). Likewise, oxytocinergic signalling via the OTR, plays a pivotal role in social behaviour and is involved in mood disorders, including anxiety, depression, but also addiction and obesity (Blevins et al., 2015, Ott et al., 2013b, Thienel et al., 2016, Zhou et al., 2014, Brown et al., 2014, Jobst et al., 2014, McQuaid et al., 2014, Jobst et al., 2015). Thus, the ghrelinergic and oxytocinergic system share some interesting overlapping functionalities and play key roles in the pathophysiology of certain brain disorders, which may be explained by the formation of an OTR/GHSR heteromer.

Here, we demonstrate co-localized expression of the OTR and GHSR, a positive fcFRET and dual receptor trafficking, indicative of the direct physical interaction of

the OTR/GHSR pair. We observe a ubiquitous subcellular co-localization of GHSR and OTR under basal conditions, which is in contrast with the usually reported localization of GPCRs on the cell membrane (McNeely et al., 2012, Nevins and Marchese, 2018) However, high ligand-independent constitutive activity under basal conditions has previously been reported to occur for the OTR and the GHSR also, which can explain a mainly cytosolic GPCR expression pattern (Di Benedetto et al., 2014, Mear et al., 2013). High constitutive expression and agonist-dependent endocytosis, associated with a ubiquitous subcellular expression, have also been shown with the β2 adrenergic and M3 muscarinic receptors (Scarselli and Donaldson, 2009). In addition, altered basal ligand-independent and agonist-mediated changes in receptor trafficking have also reported upon heteromerization of the GHSR and 5-HT2c receptor (Schellekens et al., 2013f). Here, we observe no changes in basal OTR trafficking following GHSR co-expression, but we demonstrate unique ligand-mediated trafficking of the GHSR/OTR pair upon co-expression, indicating receptor co-trafficking, which is absent under control conditions.

Moreover, the ubiquitous expression of the OTR and GHSR was also observed in hippocampal and hypothalamic primary culture, indicating that this expression pattern is likely due to basal activity of these receptors. GPCR trafficking under control conditions has been linked to biased signalling associated with GPCRs and is known to prolong GPCR signalling (Pavlos and Friedman, 2017). Therefore, the ubiquitous expression of OTR and GHSR may indicate a potential differential or biased signalling at the OTR-GHSR heterocomplex and may have similar consequences for the duration of OTR/GHSR heterocomplex signalling. The ligand-mediated co-trafficking observed between the OTR and GHSR upon co-expression, may also indicate biased signalling of these receptors, due to the significant increased movement of the GHSR into the subcellular region following co-expression and vice versa, and it would be interesting to investigate this further. Indeed, ligand-mediated biased signalling of the GHSR has recently been documented (Ramirez et al., 2018)

Potentially the most interesting finding is the attenuation of OTR Gαq mediated signalling following OTR/GHSR co-expression and potential heterocomplex formation. The alteration in OTR Gαq signalling may also be influenced by the

increased receptor trafficking observed following ligand treatment when the GHSR and OTR are co-expressed in cells. Indeed, the reduction in OTR Gαq signalling may be due to slower recycling of the receptors back to the membrane following formation of the GPCR complex. However, while increased trafficking can be seen with both the OTR and GHSR, no alteration is seen to the GHSR Gαq signalling. Another potential mechanism behind OTR attenuation may include OTR's ability to activate the Gαi signalling pathway (Busnelli and Chini, 2018). The Gαi signalling pathway is known to inhibit production of cAMP and hence activation of PKA (Birnbaumer, 2007), which are both indirect regulators of calcium influx from intracellular stores through co-ordinated crosstalk between the Gαq and Gαi proteins (Borodinsky and Spitzer, 2006, Howe, 2011, Reiken et al., 2003). Therefore, an inhibition of OTR-mediated Gαi activity, may inhibit cAMP and PKA activity, subsequently reducing OTR-mediated calcium influx through the ryanodine receptors, which is the major calcium release channel on the sarcoplasmic reticulum (Reiken et al., 2003). This would further back up the hypothesis that increased receptor trafficking under OTR and GHSR co-expression may induce biased signalling of the OTR and switching to a Gαi mediated activation, and further investigations are warranted. Moreover, we also observed an additional attenuation of OTR-mediated calcium signalling following pre-incubation with GHSR antagonist. A negative binding cooperativity has been shown for heterodimer pairs (Vischer et al., 2011), and thus, it is tempting to speculate that the GHSR has the ability to alter the ligand binding site of the OTR resulting in a reduced affinity of oxytocin for the OTR (El-Asmar et al., 2005). However, further experiments such as radiolabelled binding assays would need to be performed to confirm this hypothesis.

Co-expression within the same confocal plane of the OTR/GHSR was also observed in postnatal day 1 rat neuronal cultures of the hippocampus and hypothalamus, highlighting the possibility of the formation of an OTR/GHSR heterocomplex, which is poised to play an important physiological role. The co-localization of OTR and GHSR in the hippocampus and hypothalamus is of particular interest, with reduced OTR signalling within the hippocampus being linked to reduced adult hippocampal neurogenesis (Lin et al., 2017a). Neurogenesis is a key factor in memory formation

(Deng et al., 2010) and OTR signalling is known to induce long-term spatial memory in the hippocampus (Tomizawa et al., 2003) and plays a role in social discrimination (Raam et al., 2017b). Within the hypothalamus, the OTR is known to play a role in food intake, where increased OTR signalling is linked to nutrient excess, notably reduced OTR signalling being linked to the onset of obesity and reduced activity (Blevins and Ho, 2013). Future studies are needed to corroborate central OTR/GHSR heterocomplex formation, such as proximity ligation assays, to determine if the functional consequence of heterocomplex formation lead to changes in hippocampal or hypothalamic function.

In conclusion, we show, to our knowledge for the first-time, compelling evidence for functional OTR and GHSR crosstalk, which is likely via the formation of a novel OTR/GHSR heterocomplex, with important downstream signalling consequences. This OTR/GHSR interaction is poised to play an important physiological role and it will be interesting to investigate this interaction further to confirm whether the observed crosstalk is indeed through the formation of a heterocomplex or through a more short-term dynamic interaction. In addition, investigations into the functionality of this interaction in future behavioural studies, are now warranted, as these will lead to better understanding of disorders associated with downregulated OTR signalling. The findings from this study represent an intriguing potential for the development of novel therapeutics targeting central disorders involving oxytocinergic signalling, including appetite regulation, depression, schizophrenia and autism, and will subsequently lead to a better treatment efficacy compared to single GPCR targeted drugs with less associated side effects (Uhrig et al., 2016, Blevins et al., 2015, McQuaid et al., 2014, Penagarikano et al., 2015).

2.6 Supplementary Figures

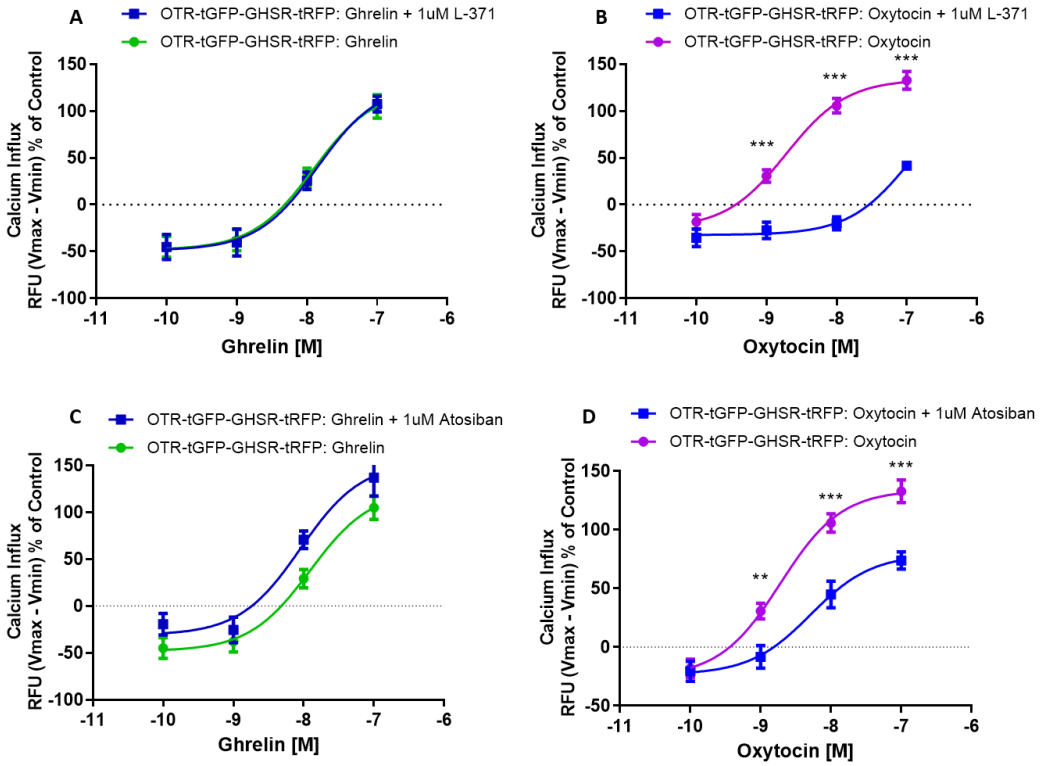

Figure S2.1 OTR antagonists Atosiban and L-371-257 have no effect on GHSR-mediated signalling when co-expressed with the OTR. (A,C) *GHSR pre-treated with L-371 and Atosiban has no significant effect on GHSR mediated signalling when co-expressed with the OTR.* ***(B, D)*** *As anticipated OTR antagonist L-371 and Atosiban significantly reduce OTR mediated signalling. Results are depicted as ±SEM. Statistical significance is compared between groups at all concentration (A, C) and between groups (B, D), * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$. Graphs are a representative of 4 independent experiments with each treatment being in triplicate per experiment.*

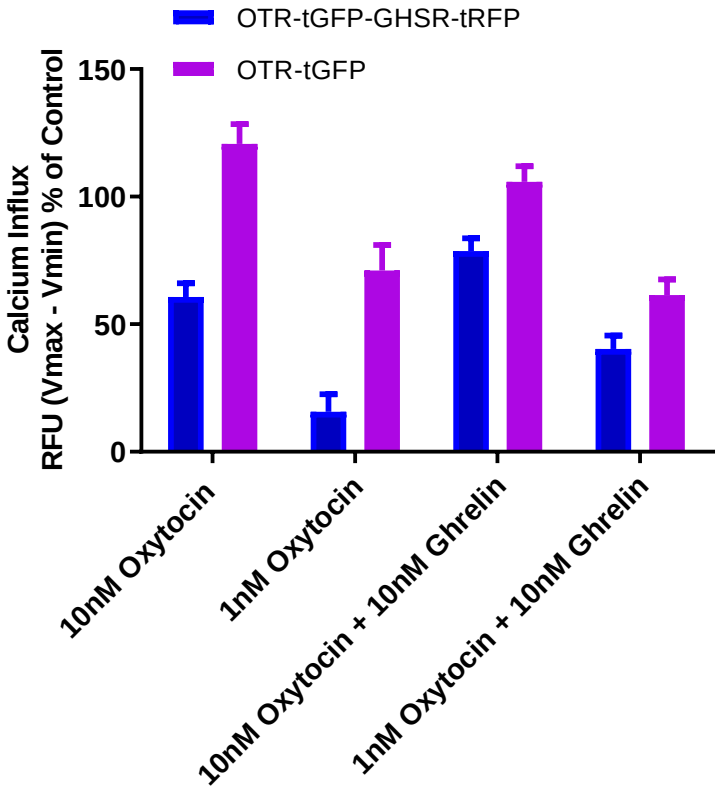

Figure S2.2 Co-administration of oxytocin and ghrelin has no synergistic effect on OTR-mediated signalling.

Chapter 3

A novel oxytocin receptor heterocomplex mediates glucagon like peptide 1 receptor Gαq dependent signalling

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3.1 Abstract

The oxytocin receptor (OTR) and glucagon like peptide 1 (GLP-1R) receptor have been implicated in many of the same disorders such as diabetes, obesity, anxiety and depression. This overlapping functionality may be partly due to their convergence in central expression. Indeed, both receptors are expressed in many areas of the brain, including the hippocampus, hypothalamus and amygdala. Moreover, functional crosstalk between oxytocin and GLP-1-mediated pathways has been previously reported, highlighting a potential interaction between these two neuropeptides signalling pathways and their receptors.

Here we investigate if functional crosstalk through dimerization occurs between the OTR and the GLP-1R, using co-localization assays, flow cytometry-based fluorescence resonance energy transfer, Gαq signalling, Gαs signalling and co-internalisation assays. We show data demonstrating a potential physical interaction between the OTR/GLP-1R pair, with significant changes to downstream GLP-1R-mediated Gαq signalling and a reduction in OTR and GLP-1R trafficking, which combined reinforce the existence of a functional OTR/GLP-1R heterocomplex.

In addition, strong co-localization of the receptor pair in primary neuronal cultures points to a potential physiological relevance of such an OTR/GLP-1R interaction. In conclusion, we demonstrate compelling evidence for an interaction and crosstalk between the OTR and GLP-1R, which is likely to have significant consequences and pharmacotherapeutic relevance for the future development of treatments for metabolic disorders such as diabetes, obesity and mood disorders, including anxiety and depression.

3.2 Introduction

G-protein coupled receptors (GPCRs) facilitate many, if not most physiological responses upon activation by their corresponding endogenous ligands, including circulating hormones and neurotransmitters (Leung and Wong, 2017, Alavi et al., 2018, Steury et al., 2017). Indeed, downstream signalling following GPCR modulation, controls a wide variety of cellular activities including, gene expression, hormonal synthesis, cell metabolism, cell proliferation and regulation of trophic factors (Sebastiao and Ribeiro, 2009, Weiss et al., 2017, Ho et al., 2009, Bouvier, 2001). Interestingly, while GPCRs can function as monomers, it is becoming clear they also function as homodimers, heterodimers and even higher order GPCR oligomers (Iglesias et al., 2017, Terrillon and Bouvier, 2004a, Armando et al., 2014, Schellekens et al., 2013e). These interactions have significant impacts on the subsequent cellular signalling pathways and may even result in novel pharmacological signalling (Xue et al., 2018, Szlachta et al., 2018, Scarlett et al., 2018, Marquez-Gomez et al., 2018). The identification of novel dynamic GPCR interactions and crosstalk, with or without the formation of dimers, will contribute to a better understanding of GPCRs function and the development of more accurate and specified treatments targeting such receptor oligomeric complexes.

Both the oxytocin receptor (OTR) and glucagon like peptide 1 (GLP-1R) receptors have been implicated in disorders such as obesity (Isaacs et al., 2016, Takayanagi et al., 2008), diabetes (Samson and Garber, 2013, Zhang et al., 2013), anxiety (Gottschalk and Domschke, 2018, Ghosal et al., 2013) and depression (Slattery and Neumann, 2010, Anderberg et al., 2016). Interestingly, central GLP-1R signalling has been shown to crosstalk with neuronal oxytocin signalling, where pre-treatment with a GLP-1R antagonist prior to an anorexigenic dose of oxytocin resulted in attenuation of the oxytocin-mediated anorexigenic response (Rinaman and Rothe, 2002). Furthermore, administration of GLP-1R ligands to the intra-paraventricular nucleus (PVN) was shown to increase calcium influx in oxytocin neurons, again highlighting the ability of GLP-1R to crosstalk with oxytocin-mediated signalling pathways (Katsurada et al., 2014). Furthermore, co-localization of GLP-1 and the oxytocin

peptide has been demonstrated within the paraventricular and supraoptic nucleus (Zueco et al., 1999).

The OTR is a class A GPCR, known to primarily signal through the Gαq alpha subunit resulting in the accumulation of inositol triphosphate and subsequent calcium influx from intracellular stores into the cytoplasm (Busnelli and Chini, 2018). The OTR can also bind to the Gαi alpha subunit, which inhibits the production of cyclic adenosine monophosphate (Strakova and Soloff, 1997). Moreover, the OTR is known to form constitutive functional homo and heterodimers (Terrillon et al., 2003, de la Mora et al., 2016, Romero-Fernandez et al., 2013). Formation of such complexes results in the alteration to the downstream signalling of the OTR and the ability to regulate other GPCR functions (Wrzal et al., 2012, Terrillon et al., 2003, Devost and Zingg, 2004a). OTR when co-expressed with the β2-adrenergic receptor, results in an attenuation of the OTR-mediated ERK1/2 signalling, but only upon pre-treatment with β2-adrenergic receptor ligands. Interestingly, pre-treatment with the OTR antagonist resulted in alterations to β2-adrenergic receptor-mediated ERK1/2 signalling (Wrzal et al., 2012). Furthermore, heterodimerization of the OTR and the dopamine 2 receptor (DRD2) enhances the DRD2 ability to attenuate PKA synthesis and increases MAPK signalling, as well as an increase in the OTR-mediated Gαq pathway. Moreover, this OTR-DRD2 dimer formation results in functional alterations OTR-mediated anxiety like behaviour, revealing the biological relevance of such heteroreceptor complexes (de la Mora et al., 2016).

In contrast, the GLP-1R is a class B GPCR and known to interact primarily with the Gαs alpha subunit, increasing levels of intracellular cAMP (Harikumar et al., 2012, Wang et al., 2001). However, the GLP-1R is also known to bind to the Gαq alpha subunit, with subsequent elevations in intracellular calcium levels (Zhang et al., 2015a, Coopman et al., 2010). The GLP-1R couples to the β-arrestin protein, which controls receptor desensitization and internalisation, regulating GLP-1R expression levels on the cell membrane (Hager et al., 2016). Similar, to the OTR, the GLP-1R is known to interact and heterodimerize with two other receptors, including the gastric inhibitory peptide receptor and the glucose-dependent insulinotropic polypeptide receptor,

which results in alteration to the GLP-1R Gαq signalling and binding of β-arrestin, enhancing cell surface expression (Schelshorn et al., 2012, Whitaker et al., 2012).

Considering, the vast and overlapping central expression of the OTR and GLP-1R, and their converging functionalities as described above, we hypothesized that the OTR and GLP-1R are likely to have a functional physical interaction or crosstalk. As both receptors are already known to form dimers with other GPCRs as seen in table 1.1 and 1.4. Here we identify a physical interaction between the OTR and GLP-1R which results in alterations to the GLP-1R mediated signalling in a G-protein specific manner, moreover, changing the OTR and GLP-1R trafficking.

The identification of this novel OTR and GLP-1R heterocomplex is poised to have significant consequences on the pathophysiology of metabolic and central disorders associated with these receptors, as down regulation of GLP-1R can have effects in disorders such as diabetes and obesity. Moreover, allow for better understanding of the OTR and GLP-1R signalling pathways, which may lead to improved pharmacotherapeutic, increasing the specificity and potency of such therapeutics.

3.3 Materials and methods

3.3.1 Receptor ligands

To investigate the interaction between the OTR and GLP-1R, OTR endogenous agonist oxytocin (1910/1, Tocris), synthetic agonist carbetocin (SML0748, Sigma), and OTR antagonists; L-371-257 (2410, Tocris) and atosiban (A3480, Sigma) were used. All compounds were prepared in assay buffer (1x Hanks balanced salt solution (HBSS) supplemented with 20mM HEPES). Similarly, GLP-1R endogenous ligand glucagon-like peptide 1 (7-37) (Tocris, 5374) and synthetic agonist Exendin-4 (Tocris, 1933) were prepared in assay buffer.

3.3.2 Transfection and cell culture

The expression vector containing the OTR sequence with a C-terminally tagged turbo green fluorescent protein (tGFP) and neomycin resistance gene was purchased from Origene (RG211797). The plasmid was used to stably express OTR-tGFP in Human Embryonic Kidney Cells (HEK293A) (Invitrogen, R70507). Transfection of cells was performed with the use of Lipofectamine LTX reagents as specified by the manufacturer (15338500, Thermofisher). Cells expressing the receptor were selected using the geneticin at a concentration of 500ng/ml (Sigma, G418-RO). Cells stably expressing the OTR-tGFP were further selected using flow assisted cell sorting (FACS) and monoclonal selection.

Cells were maintained in Dulbecco's modified eagle media (DMEM) (Sigma, D5796) supplemented with 10% fetal bovine serum (FBS) (Sigma, F7524), 1% Non-essential amino acids (NEAA) (Sigma, M7145), and 500ng/ml of G418. Cells were sustained in an environment of 5% CO_2 at 37^{o}C, where at 80-90% confluency were routinely passaged.

3.3.3 Cloning and lentiviral transduction

The GLP-1R sequence was amplified from its plasmid (R&D systems, RDC0294) using AmpliTag Gold 360 Master Mix (4398876 Applied Biosystem) and designed primers with EcoRV and XhoI restriction sites incorporated; GLPR1_Forward 5'-AG CTT GGA TCC GATA TC GCTA GC-3' with EcoRV; GLPR1_Reverse 5'-TGC TTA CTC GAG GCT GCA GGA GGC CTG GC-3' with XhoI. After amplification the GLP-1R sequence was cut with

these restriction enzymes. A pHR-SIN-BX-tagRFP target vector previously used in our lab (Schellekens et al., 2013e) was cut between the SFFV promoter and tagRFP sequence using BamHI enzyme. Following a blunting reaction (Anza DNA Blunt End Kit, IVGN2404, Invitrogen), the pHR-SIN-GLP-1R-tagRFP vector was subsequently cut with XhoI. In the next step, the amplified GLP-1R sequence was ligated into the pHR-SIN-BX-tagRFP vector with a molar ratio of insert: vector (5:1) (Invitrogen 15224-017). The pHR-SIN-GLP-1R-tagRFP vector construct was subsequently amplified and confirmed by restriction analysis and sequencing (Eurofins).

The pHR-GLP-1R-tagRFP viral plasmid was packaged using a 2nd generation lentiviral systems as previously described (Follenzi and Naldini, 2002, Vigna and Naldini, 2000, Schellekens et al., 2015a). In brief, a 3rd generation packaging construct, pCMV ΔR8.91; the envelope construct, pMD.G-VSV-G and pHR-GLP-1R-tagRFP were used to transfect HEK293AT-17 cells as previously described in our lab (Schellekens et al., 2015a, Schellekens et al., 2013e).

Across 5 days the HEK-OTR-tGFP stable cell line was transduced using the packaged pHR-GLP-1R-tRFP vector to obtain cells with the co-expression of a stable OTR and transient GLP-1R expression (HEK-OTR-tGFP-Lv-GLP-1R-tRFP). The packaged pHR-SIN-GLP-1R-tRFP was diluted in transduction media (DMEM with the addition of 2% FBS, 1% NEAA and 8ug/ml of Polybrene (Sigma, H9268)) and incubated with HEK-OTR-tGFP cells for 24hrs. Following this, the virus was diluted ½ using transduction media and cells were incubated for another 24hrs. Transduction efficiency was analysed using FACS Calibur (BD Biosciences).

3.3.4 Co-localization

Cells co-expressing OTR-tGFP and GLP-1R-tRFP, (HEK-OTR-tGFP-Lv-GLP-1R-tRFP) were used to analyse co-localization of receptors. A 24-well cell culture plates (83.3922.005, Sarstedt) containing borosilicate disks were treated with poly-l-lysine to ensure maximum attachment of cells. The HEK-OTR-GFP-Lv-GLP-1R-tRFP cells were then seeded and incubated for 24-30 hrs at 37^{o}C in a 5% CO_2 environment. Cells were then fixed using 4% paraformaldehyde (PFA) for 20mins. The cells were then visualised across 3 independent experiments with a 60x magnification on laser

scanning confocal fluorescent microscopy (FV 1000 Confocal System, Olympus) and subsequently analysed using Olympus fluoview FV3000 software.

3.3.5 Ligand mediated receptor trafficking

Cells of interest were seeded onto 96 well clear plates (83.3924.005, Sarstedt). Media was changed to serum free 24 hours prior to assay. Cells were then treated with receptor ligands for 30 mins, washed using phosphate buffered saline (PBS) and fixed using 4% PFA for 20mins. Using three independent experiments with each treatment group in triplicate, ligand mediated receptor trafficking was analysed using the IN-Cell Analyser 1000 (Agilent Technologies). Using 5 images from each treatment group the IN-cell software (Agilent Technologies) calculated the internalisation ratio; which was subsequently used to calculate the perinuclear intensity as percentage of the control (Assay buffer). Results were interpreted using GraphPad (Prism 5.0; GraphPad Software Inc.).

3.3.6 Intracellular Calcium Mobilization assays

The cells were seeded on a black clear bottom 96 well plates (3904, Corning) and grown for 24 hours in maintenance media. Media was changed to serum free media 24 hours prior to the assay being performed. Media was then replaced with 80ul of calcium 5 dye according to manufacturer instruction (R8186, Molecular Devices). Cells were then incubated for 1.5 hours at 37^{o}C in an environment of 5% $CO_{2.}$ Using the liquid handling mode of the FLIPR Tetra® III, High-Throughput Screening System (Molecular Devices), 40ul of the 3x compounds was added to the 80ul of cell/calcium 5. Fluorescence was measured in real time for 120 seconds at excitation of 484nm and an emission of 525nm. The percentage of relative fluorescence units (RFU) was calculated using the maximum value (Vmax) and subtracting the baseline (Vmin), expressed as a % of the control (3.3 % FBS). All graphs were prepared in GraphPad (Prism 5.0; GraphPad Software Inc.) using nonlinear regression analysis with variable slope.

3.3.7 Cyclic adenosine monophosphate assay

Cyclic Adenosine Monophosphate (cAMP) accumulation was analysed using Cisbio homogeneous time-resolved fluorescence (HTRF) technology (62AM4PEB, Cisbio). Cells 24hrs prior to the assay were deprived of FBS. Directly before the assay cells

were mechanically detached from wells using PBS, centrifuged at room temperature for 4mins at 800 x g, resuspended in Assay buffer and counted. Following this $4.0x10^3$ of cells were added to the wells of a 96 well, white, flat bottom plate (675074, Greiner Bio-One) containing 5 µl of compounds diluted in assay buffer with 0.5 µM Phosphodiesterase inhibitor (IBMX) (62AMXADC, Cisbio). Cells were incubated with compounds at 37^oC for 30 mins. Finally, 5 µL of cAMP-d2 conjugate (donor) and 5 µL of anti-cAMP cryptate conjugate (acceptor) diluted in lysis buffer were added to each well. Here, cAMP-d2 conjugate competes with accumulating endogenous cAMP from the cells. This was then further incubated for 1 hour at room temperature. Fluorescence at 620nm and 665nm was recorded using the Flexstation II (Molecular Devices). The percentage of relative fluorescent units (RFU) was calculated as the ratio of 665nm/620nm and multiplied by 10^4 as per manufacturers instruction. The fluorescent signal is inversely proportional to percentage of endogenous cAMP detected in the sample.

3.3.8 Flow cytometry based fluorescence resonance energy transfer

The fluorescence resonance energy transfer (FRET) signal between the HEK-OTR-tGFP and Lv-GLP-1R-tRFP was analysed according the protocol developed in our lab (Chruścicka et al., 2018) using the BD FACSAria™ Fusion (BD Biosciences). Before analysis cells were spun down at 800 rpm for 3 minutes and the pellet re-suspended in 400 µl of suspension buffer (2nM Ethylenediaminetetraacetic acid (EDTA) in PBS). The instrument settings were defined using non-transfected HEK293A cells, to set up forward scatter, side scatter and photomuliplier tube voltage, based on their size and granularity. HEK-OTR-tGFP and Lv-GLP-1R-tRFP were used to further adjust the voltage of the photomultiplier tube and to compensate for any spectral bleed through. HEK-OTR-tGFP-Lv-GLP-1R-tRFP was then analysed for FRET. Excitation at 488nm was applied with detections at 610-620nm, exciting the tGFP (donor) while reading emission at the tRFP (acceptor) spectrum. To control for artificial FRET signal HEK-OTR-tGFP was transduced with an empty tRFP vector. Two-dimensional dot plot was utilised gating cells expressing both tGFP and tRFP. To ensure cell only expressing both tags were analysed. A tGFP versus FRET signal dot plot was then used to determine the FRET percentage.

3.3.9 Calcium imaging

Calcium imaging which was previously described in our lab (Pastor-Cavada et al., 2016, Torres-Fuentes et al., 2018) has been now modified to allow for a higher throughput. $2.0x10^5$ cells/well were plated on a 12 well clear plate. Media was changed to serum free 1 day prior to the assay. On the day of the assay media was removed, cells were washed with PBS and incubated for 1 hour at 37^oC with 7uM Fura 2-AM (F1221, Biosciences) in Assay buffer. Within the cell, intracellular esterase's cleave the acetoyxmethyl segment (AM) of Fura 2-AM resulting in its active form. Upon calcium release from intracellular stores to the cytoplasm, calcium binds to FURA dye resulting in a shift in maximum excitation wavelength from 368nm to 335nm with emission remaining unchanged (~510nm). Therefore, excitation is read at 368nm and 335nm, while emission is read at 510nm. The ratio of both excitation/emission positively correlates to calcium released from intracellular stores. Following incubation, Fura 2-AM was replaced with Assay buffer. Cells were analysed using an Olympus BX50W1 set up with a Mercury arc burner (MT20 illumination system, Cell R, Olympus). Cells were viewed and regions of interest (single cells) were set using the digital epiflourescent system and its corresponding computer software (Cell R, Olympus). Compounds were manually pipetted into each well of the 12 well plate and an excitation spectrum of 380nm and 340nm were recorded with a fixed emission of 510nm in a real time.

3.3.10 Primary neuronal cultures

Postnatal day 1 (P1) Sprague Dawley rats were sacrificed using rapid decapitation. The hypothalamus and hippocampus was dissected using a method previously described but modified for P1 (Staal et al., 2007). The brain was removed from the skull and placed on an ice-cold petri dish. The meninges from around the hypothalamus were removed, using a C5 forceps before the hypothalamus was pinched out. The frontal lobe was removed using a scalpel and then the left and right hemisphere were separated. Each hemisphere was then set with the medial side facing up and again using a C7 forceps the corpus callosum, thalamus and striatum were removed. The C7 forceps was then placed under the ventral hippocampus and rolled out. This was then repeated on the other hemisphere. Once dissected the

hippocampus and hypothalamus were placed in separate 50ml falcon tubes containing harvest media (50ml hibernate A (A12475-01, Invitrogen), 125µl 100x glutamax (35050038, Invitrogen)). The tissue was then dissociated using a papain media to aid in the separation of cells (10mg papain (P4762, Sigma), 5ml Hibernate A (-CaCl2) (HA, Brainbits), 12.5µl 100x glutamax (35050038, Invitrogen), 50µl 100mM L-Cysteine (C7352, Sigma), 500µl 5mM EDTA/PBS). Cells were incubated with shaking for 30mins with papain containing media. A single cell suspension was obtained through cell trituration where papain media was removed, and triturate media was added (46ml Neurobasal media (21103, Invitrogen), 2.5ml Heat inactivated FBS, 125µl 100x glutamax, 500µl 100x penicillin/streptomycin and 500µl 100x NEAA). Cells were triturated using an 18G needle and centrifuged at 1100 rpm. this step was repeated until a single cell suspension was attained. Cells were then plated on 24 well plates containing poly-l-lysine treated borosilicate discs. The cells were maintained in neurobasal maintenance media (47.5ml neurobasal media, 125µl 100x glutamax, 1ml B27 (17504-044, Invitrogen), 500µl 100x penicillin/streptomycin, 50µl Mito/serum extender (355006, Biosciences) and 500µl of 100x NEAA) in a humidified environment at 37^{o}C with 5 % CO_2. Media was replaced every 4 days until cells were at the desired confluency. Cells were then fixed to discs using 4% paraformaldehyde for 30mins at room temperature.

3.3.11 Immunocytochemistry of neuronal cells

Following fixation, neuronal cultures were stored in PBS at 4^{o}C until staining was performed. On the day of staining wells were blocked for 2 hours at room temperature with 5% bovine serum albumin (BSA) (A2153, Sigma) dissolved in PBS and 0.1% Triton. Following this, the blocking agent was removed from the wells (minus control wells) and replaced with the primary antibody. To detect the OTR goat monoclonal anti-OTR (ab87312, Abcam, 1:200) was used and for the GLP-1R, rabbit polyclonal anti-GLP-1R (NBP1-97308, Novusbio, 1:50) was added to its corresponding well. Cells were then incubated overnight at 4^{o}C. The following day both primary antibodies and blocking solution (control wells) were removed from all wells and secondary antibody was added to the corresponding wells. Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A-11055,

Thermoscientific, 1:500) was used to detect OTR and Chicken anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (A-21442, Thermoscientific, 1:500) was used to detect GLP-1R. All antibodies were diluted in PBS with 1% BSA and 0.1% Triton. Secondary antibodies were incubated with cells for 2 hours at room temperature in the dark. Cells were then washed 3 times using PBS with added 0.1%. Bisbenzimide (1:2000) was then added to each well for 4 minutes and subsequently washed 2 times with PBS and 0.1% Triton. Following this, cells were fixed onto microscope slides and subsequently visualised using laser scanning confocal fluorescent microscopy (FV 1000 Confocal System, Olympus).

3.3.12 Statistical analysis

Statistical analysis of calcium mobilization, cAMP assays and ligand-mediated internalisation assays were performed using Prism Software (GraphPad Prism 5.0). All data is represented as the mean ± SEM, the number of independent experiments is described in each graph legend. The number of independent experiments is described in each graph legend. D'Agostino & Pearson was used to test for normality. Data, which followed Gaussian distribution, was analysed using Two-way ANOVA with a Bonferroni post-hoc test. A non-parametric Mann Whitney was used for data which did not follow Gaussian distribution. Significance was defined as follows; * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.

3.4 Results

3.4.1 Co-localization and positive FRET signal between OTR-tGFP and GLP-1R-tRFP

The HEK-OTR-tGFP cell line transiently co-expressing Lv-GLP-1R-tRFP was analysed, using confocal microscopy, to investigate potential co-localized expression of the OTR and GLP-1R across the same confocal plane. Both the OTR, shown in green (Figure 3.1a), and the GLP-1R, shown in red (Figure 3.1B), demonstrated co-localized expression on the membrane and within the cytoplasm, as indicated by the yellow/orange overlay of receptors seen in the merged image (Figure 3.1C).

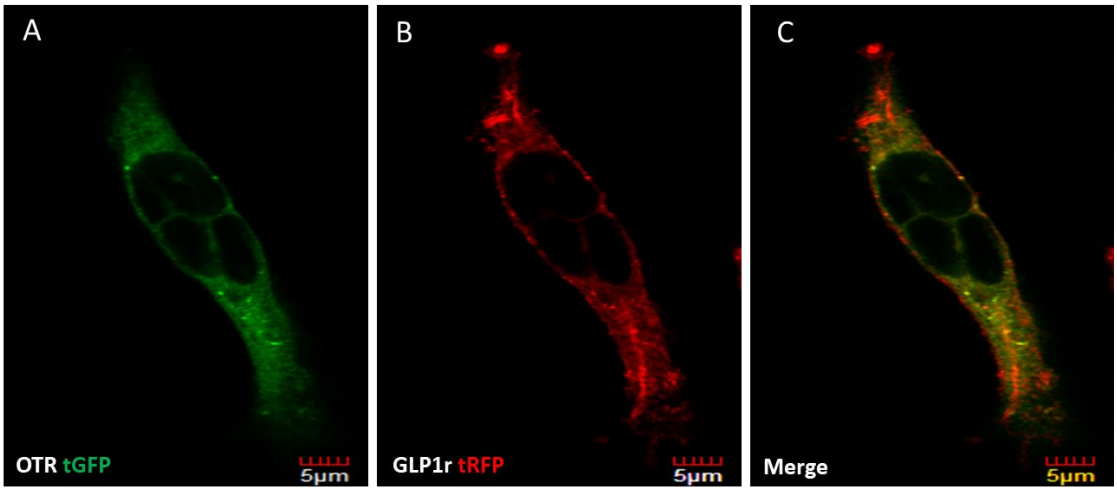

***Figure 3.1**: **Co-localization of HEK-OTR-tGFP co-expressing Lv-GLP-1R-tRFP**. **(A)** The OTR receptor expression is shown in green **(B)** with the GLP-1R been shown in red. **(C)** Overlapping expression of receptors is depicted by the yellow overlay shown in merged image. Images are representative of 3 separate experiments.*

Next, flow cytometry-based fluorescence resonance energy transfer (fcFRET) revealed the receptors to be in a close proximity as shown by a positive FRET signal (Figure 3.2). Control HEK cells stably expressing either HEK-OTR-tGFP or Lv-GLP-1R-tRFP alone failed to give a FRET signal (Figure 3.2A), highlighting that no artificial FRET signal is produced from any single fluorescent protein (Figure 3.2A). Similarly, cells expressing HEK-OTR-tGFP transduced with a control Lv-tRFP vector, resulted in a minimal 1.5% FRET signal (Figure 3.2A), demonstrating the specificity of the result obtained, ensuring that tGFP and tRFP overexpression gives no artificial FRET signal.

In contrast, when HEK-OTR-tGFP was co-expressed with Lv-GLP-1R-tRFP a positive FRET signal was shown for 28.8% of the selected cell population (Figure 3.2A), indicating the close proximity and substantial overlay between the tGFP and tRFP absorbance spectrum. Figure 3.2B further showed the median fluorescent intensity of the FRET signal. The HEK-OTR-tGFP or Lv-GLP-1R-tRFP expressed alone and HEK-OTR-tGFP co-expressed with control Lv-tRFP resulted in a similar low median fluorescent intensity count (Figure 3.2B), which again shows there is no artificial effect from the overexpression of tGFP and tRFP. But interestingly, a shift in median fluorescent intensity was seen when the OTR and GLP-1R were co-expressed, again showing an interaction between the OTR and GLP-1R (Figure 3.2B). Together these findings demonstrate a potential protein-protein interaction between OTR and GLP-1R which highly indicative for a potential heterocomplex formation.

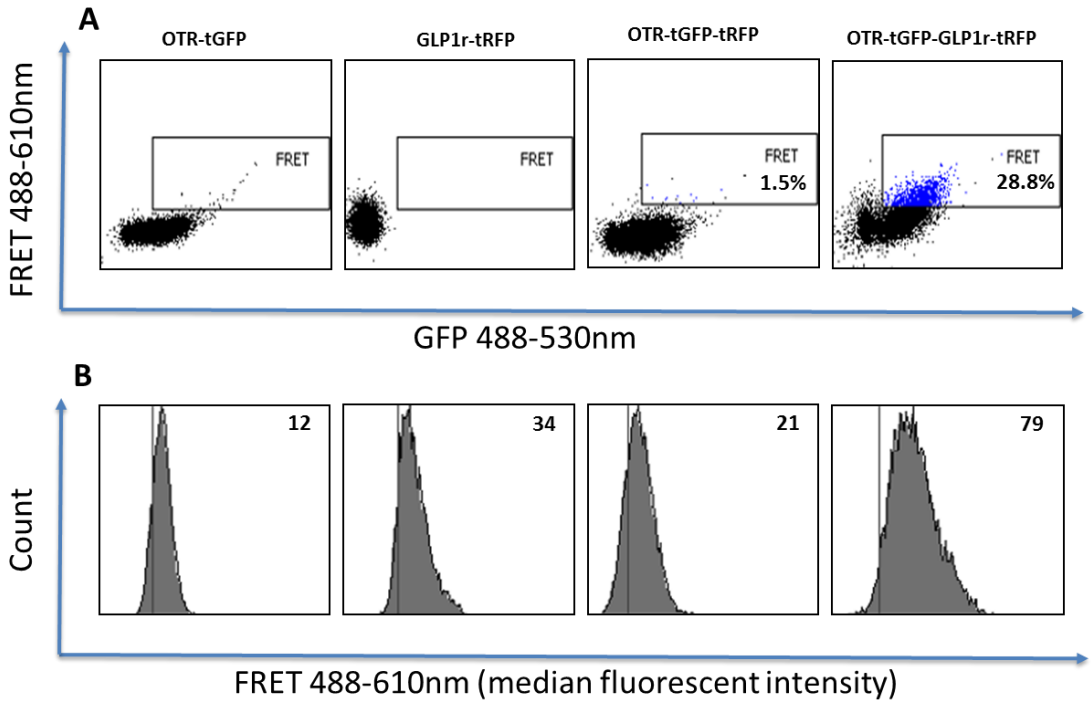

Figure 3.2: Flow cytometry-based fluorescence resonance energy transfer between OTR and GLP-1R. (A) *HEK-OTR-tGFP and Lv-GLP-1R-tRFP expressed alone results in no FRET signal, HEK-OTR-tGFP co-expressed with an empty Lv-tRFP vector results in minimal 1.5% FRET signal, HEK-OTR-tGFP co-expressed with Lv-GLP-1R-tRFP results in a positive FRET signal of 28.8%.* ***(B)*** *HEK-OTR-tGFP and Lv-GLP-1R-tRFP expressed alone and HEK-OTR-tGFP co-expressed with an empty Lv-tRFP vector results in similar median fluorescent intensity, HEK-OTR-tGFP co-expressed with Lv-GLP-1R-tRFPin comparison results in a ~2.5-fold increase in median fluorescent intensity. The graph is representative of 3 independent experiments, with the values being the average across all three experiments.*

3.4.2 Co-expression of OTR and GLP-1R attenuates GLP-1R-mediated signalling in calcium mobilization assays

Heterodimerization of GPCRs is known to influence the downstream signalling pathways of the GPCRs within the dimer complex (Schellekens et al., 2013e, Harikumar et al., 2012). The primary signalling pathway of the OTR is through the Gαq alpha subunit, resulting in an intracellular calcium influx following receptor activation (Billups et al., 2006). In contrast, GLP-1R downstream signalling is mainly shown through the Gαs subunit, which leads to increased intracellular levels of

cAMP, and to a lesser extent via Gαq -mediated signalling (Thompson and Kanamarlapudi, 2015). Interestingly, co-expression of HEK-OTR-tGFP and Lv-GLP-1R-tRFP resulted in a significant reduction in GLP 7-37-mediated signalling, a potent GLP-1R agonist, compared to Lv-GLP-1R-tRFP alone (Figure 3.3A, $F (5, 96) = 7.272$, $P < 0.0001$)). A significant ~6-fold increase in the EC_{50} of GLP 7-37, as well as a decrease in efficacy, was seen when Lv-GLP-1R-tRFP was expressed alone versus co-expressed with HEK-OTR-tGFP (Figure 3.3A and B, $F = 124.9$, $P =0.0480$). As expected, there was no effect of GLP 7-37 on the HEK-OTR-tGFP expressed alone, demonstrating specificity of GLP 7-37 for the GLP-1R and no cross-reactivity in the assays performed (Figure 3.3A, $F (5, 96) = 7.272$, $P < 0.0001$)). Furthermore, when HEK-OTR-tGFP and Lv-GLP-1R-tRFP were co-expressed, no alteration was seen to OTR-mediated calcium signalling when induced by the OTR endogenous agonist oxytocin, indicating that the effect of co-expression is on GLP-1R-mediated Gαq signalling only (Figure 3.3C $F (5, 165) = 0.3703$, $P= 0.8684$). The Lv-GLP-1R-tRFP expressed alone showed no effect of oxytocin on GLP-1R-mediated calcium signalling, showing specificity of oxytocin for the OTR (Figure 3.3C $F (5, 165) = 0.3703$, $P= 0.8684$). Finally, no change to the EC_{50} of oxytocin was observed when HEK-OTR-tGFP was expressed alone versus co-expressed with Lv-GLP-1R-tRFP (Figure 3.3D, $F = 7.527$, $P =0.1932$). This data indicates the functional relevance of the OTR/GLP-1R heterocomplex and the ability of the OTR to mediate downstream GLP-1R-mediated Gαq signalling.

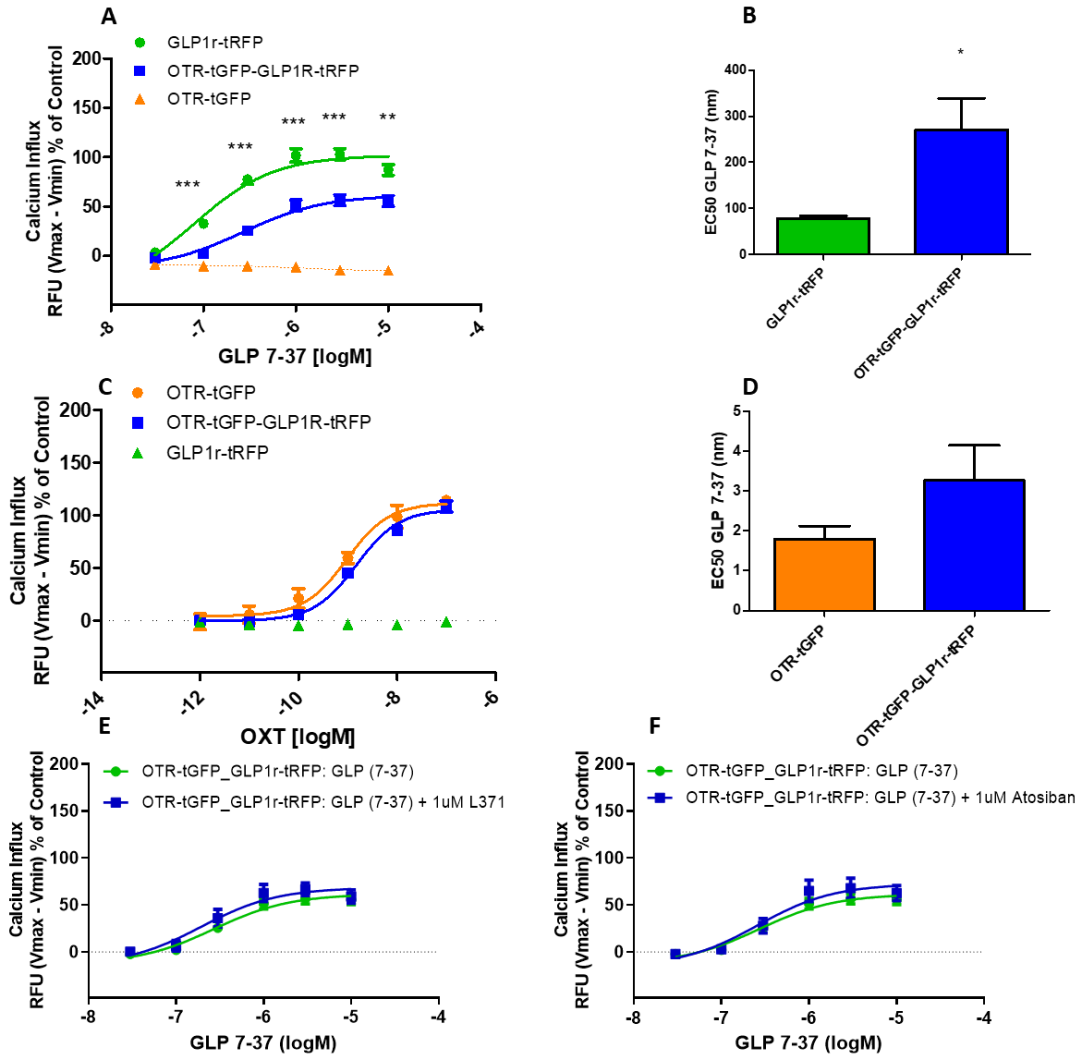

Figure 3.3: Attenuated GLP-1R-mediated Gαq signaling, as seen by intracellular calcium influx, upon co-expression of the OTR and GLP-1R. (A) *The Lv-GLP-1R-tRFP co-expressed with HEK-OTR-tGFP results in a significant attenuation of GLP-1R-mediated calcium signaling* ***B)*** *A significant increase in the EC_{50} of GLP 7-37 was observed when HEK-OTR-tGFP and Lv-GLP-1R-tRFP are co-expressed.* ***(C)*** *The HEK-OTR-tGFP-mediated calcium influx remained unchanged regardless of being expressed alone or co-expressed with Lv-GLP-1R-tRFP.* ***(D)*** *No change was seen to the EC_{50} of oxytocin when HEK-OTR-tGFP and Lv-GLP-1R-tRFP co-expressed.* ***(E)*** *The OTR antagonist L-371-257 had no effect on GLP-1R-mediated calcium signaling.* ***(F)*** *The OTR antagonist Atosiban had no effect on GLP-1R-mediated calcium signaling. The data is representative of 3 independent experiments with each treatment in triplicate for each experiment. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed (B, D) at each concentration (A, C), * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.*

Next, it was investigated if the attenuation of GLP-1R-mediated calcium influx following co-expression with the OTR could be restored following inhibition of OTR specific signalling. Restoration of calcium signalling upon antagonist pre-treatment has been previously seen in heterodimerization studies (Schellekens et al., 2013e). Therefore, the specific OTR antagonists, L-371-257 and atosiban, were utilised in cells co-expressing HEK-OTR-tGFP and Lv-GLP-1R-tRFP. However, no restoration to the GLP-1R-mediated Gαq signalling was observed when comparing GLP 7-37 treatment alone versus GLP 7-37 addition following preincubation with the OTR antagonist L-371-257 (Figure 3.3E, $F= (5, 65) = 0.7337$, $P =0.6008$)). Similarly, GLP 7-37 treatment alone versus pre-treatment with the OTR antagonist atosiban failed to restore the attenuated GLP-1R-mediated calcium signalling when co-expressed with HEK-OTR-tGFP (Figure 3.3F, $F= (5, 65) = 1.031$, $P =0.4069$)). Suggesting that the attenuation of GLP-1R-mediated signalling is not via ligand induced heterocomplex formation.

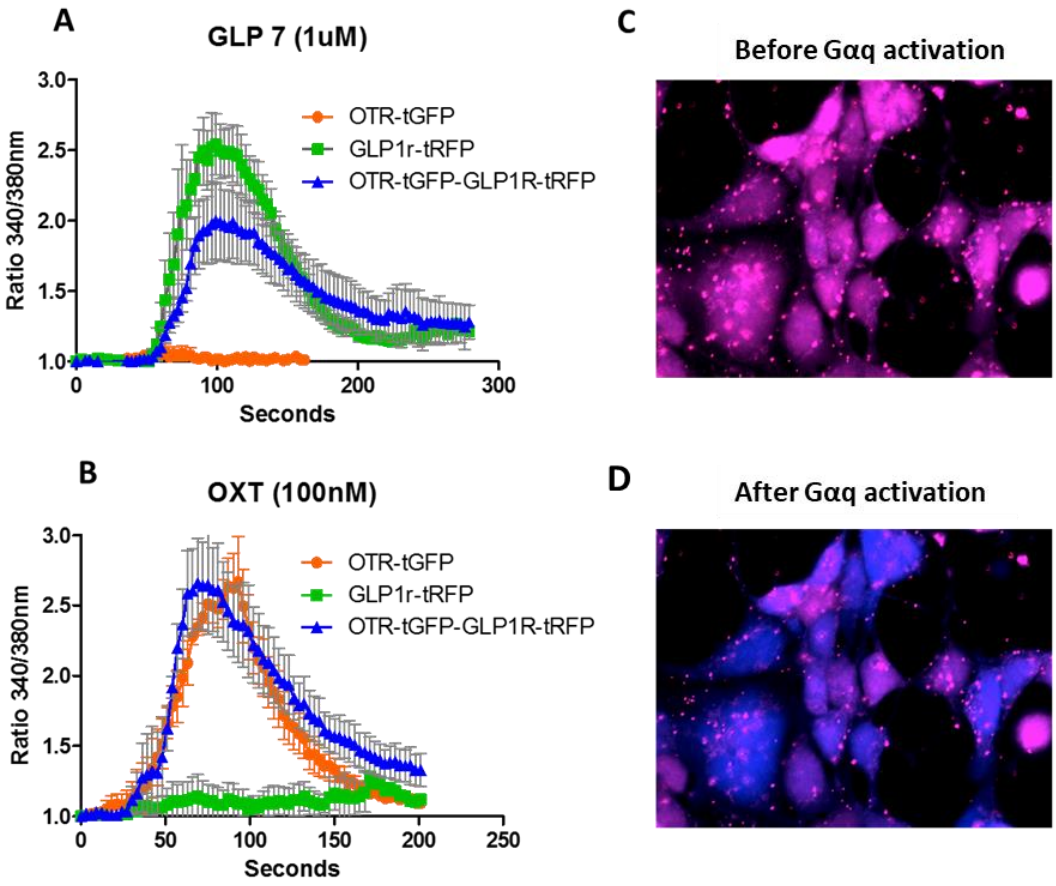

Figure 3.4: Attenuated GLP-1R-mediated Gαq signaling, in calcium imaging, upon co-expression of the OTR and GLP-1R. (A) *A reduction in GLP-1R-mediated calcium signalling can be seen when Lv-GLP-tRFP is co-expressed with HEK-OTR-tGFP.* ***(B)*** *No effect is seen to the OTR-mediated calcium signalling when HEK-OTR-tGFP is co-expressed with Lv-GLP-1R-tRFP.* ***(C)*** *A shift in fluorescence can be seen following addition of receptor agonist from pink (no calcium release) to blue (calcium release). Graphs are representative of 3 independent experiments with each treatment group performed in triplicate across each experiment.*

Following this, calcium release from intracellular stores upon Gαq activation using calcium imaging was investigated to ensure consistency of the Gαq results obtained (Figure 3.4). Co-expression of HEK-OTR-tGFP and Lv-GLP-1R-tRFP when treated with GLP 7-37 revealed attenuation of GLP-1R-mediated calcium signalling compared to Lv-GLP-tRFP expressed alone, reinforcing results obtained in the calcium mobilization assay (Figure 3.4A). As expected, GLP 7-37 had no effect on HEK-OTR-tGFP calcium influx (Figure 3.4A). Upon treatment with oxytocin, no alteration to HEK-OTR-tGFP calcium signalling was seen when compared to co-expression with Lv-GLP-1R-tRFP,

indicating the heterocomplex between OTR and GLP-1R had no effect on the OTR-mediated Gαq signalling. Moreover, as expected, no effect of oxytocin was seen in cells solely expressing Lv-GLP-1R-tRFP (Figure 3.4B). Upon activation of the Gαq alpha subunit, the calcium released binds to the active state of FURA dye resulting in a shift in maximum excitation from 368nm (unbound calcium) to 335nm (bound calcium), this shift can be seen in Figure 3.4C.

3.4.3 Co-expression of OTR and GLP-1R results in decreased receptor trafficking

Activation of the Gαq signalling pathway can be affected by the amount of receptor expression on the cell membrane, which is dependent on trafficking of the receptor from the membrane to the cytoplasm and *vice versa* (Smith and Rajagopal, 2016). Therefore, due to the observed attenuation of GLP-1R-mediated calcium signalling (Figure 3.3, Figure 3.4), OTR and GLP-1R trafficking was investigated. Quantitative analysis of the data yields a modest oxytocin-mediated internalisation of the HEK-OTR-tGFP at ~20%, which is reduced upon co-expression of the Lv-GLP-1R-tRFP (Figure 3.5A, F= (2, 75) = 4.344, P=0.0164)). Moreover, no co-internalisation is seen between HEK-OTR-tGFP an Lv-GLP-1R-tRFP following GLP 7-37 treatment (Figure 3.5A, F= (2, 75) = 4.344, P=0.0164)).

Furthermore, when comparing Lv-GLP-1R-tRFP alone versus co-expressed with OTR, a significant reduction in Lv-GLP-1R-tRFP trafficking is seen when co-expressed in HEK cells stably expressing OTR-tGFP (Figure 3.5B, F= (2, 54) = 2.175, P= 0.1235)). Furthermore, no co-internalisation of HEK-OTR-tGFP with Lv-GLP-1R-tRFP is seen upon treatment with oxytocin (Figure 3.5B, F= (2, 54) = 2.175, P= 0.1235)).

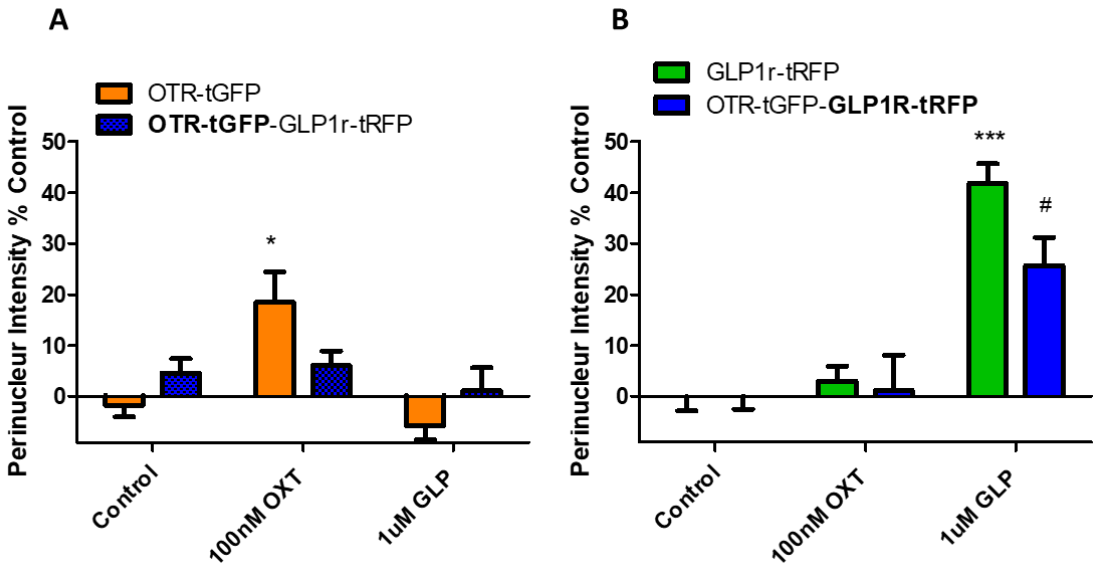

Figure 3.5: Co-expression of OTR and GLP-1R results in decreased receptors trafficking. (A) *Quantitative analysis reveals significant OTR receptor trafficking in HEK-OTR-tGFP cells when expressed alone, which is reduced upon co-expression of Lv-GLP-1R-tRFP.* ***(B)*** *A significant reduction in GLP-1R-mediated internalisation was seen upon treatment with GLP when Lv-GLP-1R-tRFP was co-expressed with HEK-OTR-tGFP. All graphs are representative of 3 independent experiments with each treatment group in at least triplicate for each experiment. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed (B, D) at each concentration (A, C), * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.*

3.4.4 Cyclic adenosine monophosphate accumulation is unaltered following GLP-1R/OTR heterocomplex.

The GLP-1R is known to primarily signal through the Gαs alpha subunit, resulting in accumulation of cAMP (Donnelly, 2012). Heterodimerization of GLP-1R has been previously shown to attenuate the ability of the GLP-1R to activate the Gαs-mediated pathway (Harikumar et al., 2012). Moreover, OTR is known to block cAMP accumulation through activation of the Gαi alpha subunit (Zhou et al., 2007b). Therefore, it was hypothesized that the heterocomplex formation between OTR and GLP-1R could result in changes to cAMP accumulation. However, no changes in cAMP accumulation were seen when both receptors were co-expressed (Figure 3.6). The HEK-OTR-tGFP alone and co-expressed with Lv-GLP-1R-tRFP were treated with oxytocin (Figure 3.6B) and carbetocin (Figure 3.6A), two OTR specific agonists. As

expected, neither treatment with oxytocin (Figure 3.6B, F= (3, 52) = 0.2781, P= 0.8409)) nor carbetocin (Figure 3.6A, F= (3, 52) = 1.535, P= 0.2165)) altered cAMP synthesis. Next, GLP-1R-mediated cAMP accumulation was analysed, using three GLP-1R specific agonists, GLP 7-37 (Figure 3.6C, F= (4, 31) = 0.8189, P= 0.5230)), GLP 3 (Figure 3.6D, F= (4, 31) = 0.7036, P= 0.5955)) and Exendin 3 (Figure 3.6E, F= (4, 31) = 0.1695, P= 0.9523)), which were all equally able to increase cAMP accumulation, without altering signalling between cells expressing Lv-GLP-1R-tRFP alone or co-expressed with HEK-OTR-tGFP. These findings highlight that the OTR/GLP-1R heterocomplex does not alter Gαs-dependent signalling.

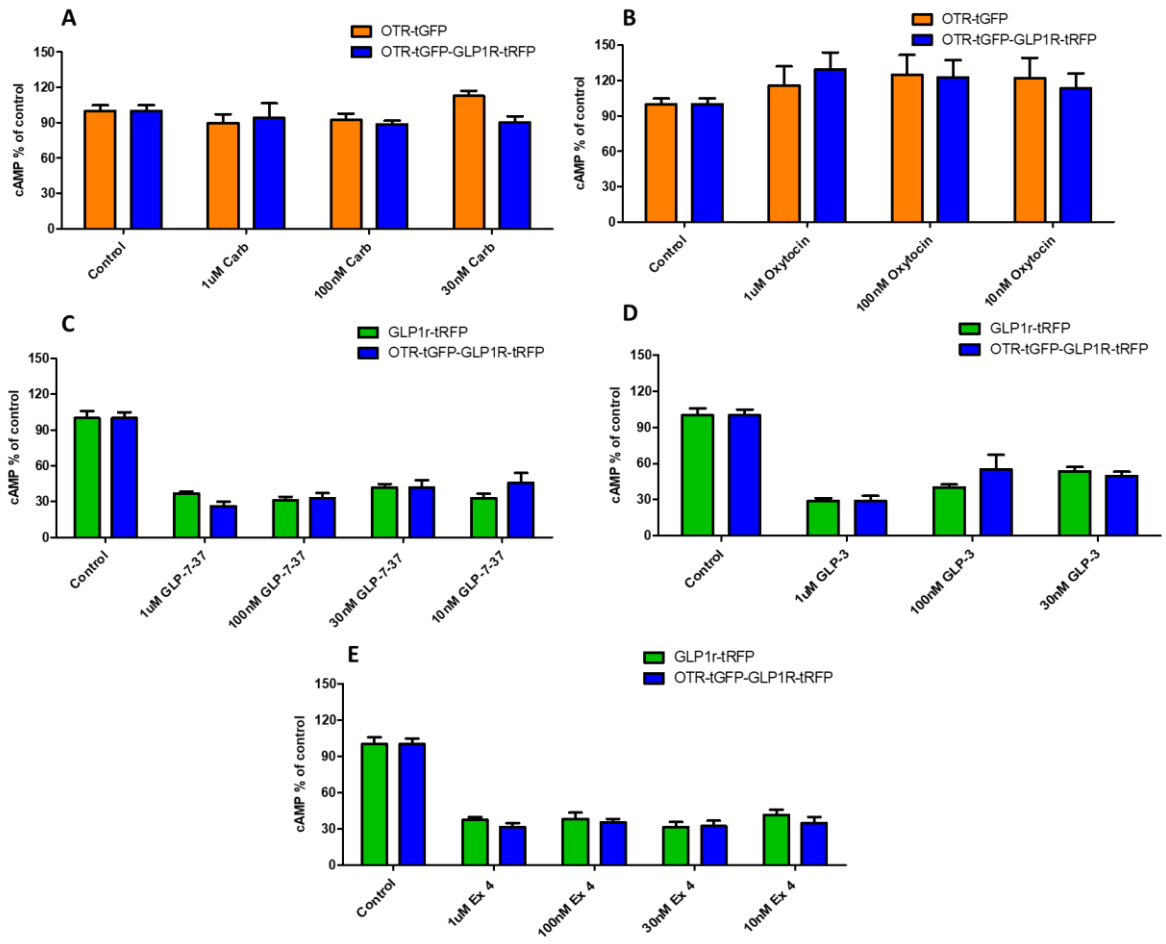

Figure 3.6: Co-expression of the OTR and GLP-1R has no effect on intracellular accumulation of cAMP. (A) *Treatment with OTR synthetic agonist carbetocin and endogenous OTR agonist,* ***(B)*** *oxytocin on HEK-OTR-tGFP alone and co-expressed with Lv-GLP-1R-tRFP resulted in no alteration to OTR-mediated cAMP attenuation. HEK-OTR-tGFP co-expressing Lv-GLP-1R, when treated with GLP-1R agonist,* ***(C)*** *GLP 7-37,* ***(D)*** *GLP 3 and* ***(E)*** *exendin 4 had no effect on the ability of GLP-1R to promote cAMP accumulation. Both graphs are representative of 3 independent experiments with each treatment group being in triplicate for each experiment.*

3.4.5 Co-localization of OTR and GLP-1R in ex-vivo neuronal cultures

The OTR and the GLP-1R have many known centrally-regulated physiological functions (Dumais and Veenema, 2016, Baggio and Drucker, 2014). Moreover, co-expression of the OTR and GLP-1R has been demonstrated within the hippocampus and hypothalamus (Heppner et al., 2015, Cork et al., 2015, Freeman et al., 2014). The GLP-1R within the hippocampus and hypothalamus is known to impact such functions as hippocampal memory, learning and appetite regulation (Hsu et al., 2015, During et al., 2003), moreover, hypothalamic energy homeostasis (Lopez-Ferreras et al., 2018, Ten Kulve et al., 2016). Within the hippocampus the OTR promotes neurogenesis and impacts social memory (Raam et al., 2018, Lin et al., 2017b), while hypothalamic OTR is known to alter food intake (Noble et al., 2014). These similar receptor expression profiles and overlapping functionalities across similar brain regions, has prompted the idea of interaction and crosstalk between the OTR and GLP-1R, for which has been demonstrated in numerous in vitro assays, described above. Therefore, it was next investigated if both receptors are co-expressed in primary neurons, as an initial indication of a potential functional relevance of a novel OTR/GLP-1R heterocomplex.

High expression of both the GLP-1R (Figure 3.7B) and OTR (Figure 3.7C) was observed in primary hippocampal neuronal cultures. Overlay of the images from the conjugated GLP-1R and OTR reveal co-localization of the receptor within single confocal planes (Figure 3.7D). Most of co-localization was observed on the membrane of the cell body. Interestingly, primary neuronal cultures from the hypothalamus showed a different expression pattern throughout the neuron compared to the hippocampus (Figure 3.7E-H). Co-localization of the GLP-1R and OTR within the hypothalamus, revealed co-expression present across the cell body and on the neuronal axons (Figure 3.7H). Such co-localization in primary neuronal cultures may indicate a physiological consequence of centrally expressed OTR and GLP-1R.

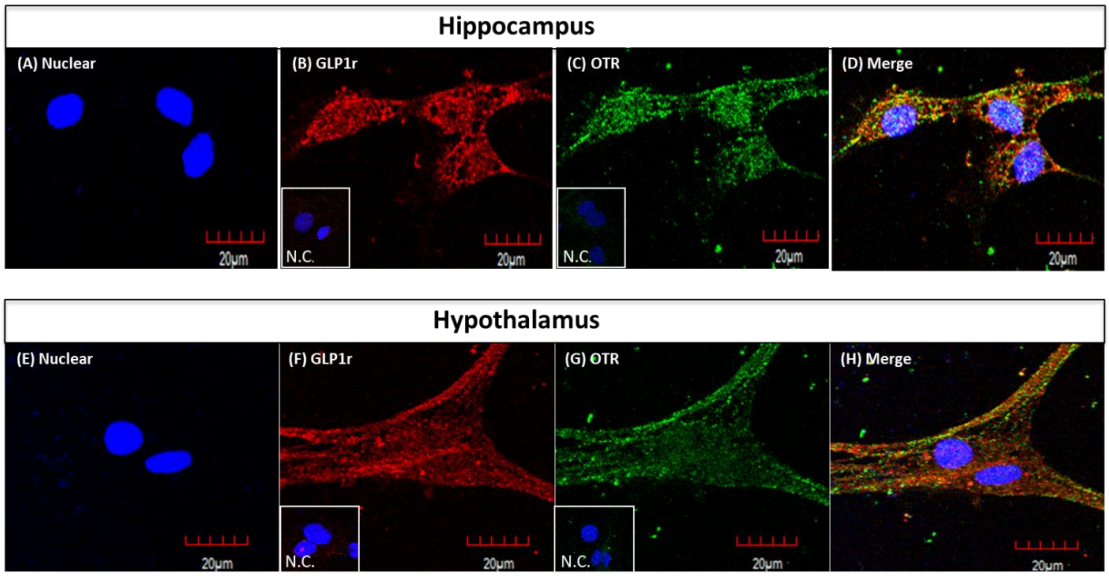

Figure 3.7: Co-localization of OTR and GLP-1R in hippocampal and hypothalamic neuronal cultures. (A) *Hippocampal primary cultures were nuclear stained (blue)* ***(B)*** *GLP-1R was bound with a secondary antibody conjugated to an Alexa Fluor 594 (red) and* ***(C)*** *OTR was bound with a secondary antibody conjugated to an Alexa Fluor 488 (green).* ***(D)*** *Overlay of the images reveals co-localization of OTR and GLP-1R (yellow).* ***(E)*** *Hypothalamic primary cultures were nuclear stained (blue),* ***(F)*** *GLP-1R can be seen in red due to its attached secondary antibody conjugated to an Alexa Fluor 594,* ***(G)*** *OTR was bound with a secondary antibody conjugated to an Alexa Fluor 488 (green).* ***(H)*** *Merged images show co-localization of OTR and GLP-1R in the hypothalamic neurons (yellow).*

3.5 Discussion

GPCRs are targeted by over a third of pharmaceuticals currently on the market and novel GPCR drugs continue to be identified (Hauser et al., 2018, Bologna et al., 2017). However, GPCRs have until recently only been targeted as monomeric structures, where side effects are often caused by off-target effects and the high doses needed for a physiological effect (Sriram and Insel, 2018). It can be theorised that, due to the lack of knowledge regarding GPCR heterocomplex signalling, targeting of GPCRs as monomeric structures can result in these off-target effects. For example, the increased weight gain associated with antipsychotics such as olanzapine (Lord et al., 2017) can be tentatively-linked to the heterodimerization of serotonin 2c receptor and the ghrelin receptor resulting in increased ghrelin signalling and, subsequently, appetite (Schellekens et al., 2015a, Huang et al., 2018). Here emerges the concept of GPCR heterodimerization, where dimer formation results in novel pathways of GPCR activation not seen in their monomeric state (Goupil et al., 2013, Terrillon and Bouvier, 2004a). Therefore, investigating the effect of GPCR heterocomplex formation on the receptor's signalling pathways may be beneficial to improving treatment efficacy and reducing the likelihood of GPCR targeted side effects.

Here, we identify, to our knowledge for the first time, a significant effect of an OTR and GLP-1R interaction via formation of a heterocomplex on the downstream signalling of GLP-1R. The use of *in-vitro* overexpression systems, as done here via the expression of OTR-tGFP and GLP-1R-tRFP in HEK293A cells, has now become a standard method for heterocomplex identification (Schellekens et al., 2015a, Schellekens et al., 2013e, Moutkine et al., 2017, Lukasiewicz et al., 2011, Rediger et al., 2011). Confocal microscopy revealed co-localization within the same confocal plane of OTR-tGFP and GLP-1R-tRFP in the HEK293A cells. which indicated a potential physical interaction of OTR and GLP-1R. Furthermore, the observed co-localization presented on the cell membrane reinforces the existence of a functional OTR/GLP-1R heterocomplex (Jean-Alphonse and Hanyaloglu, 2011). Other common methods used to identify a physical interaction between GPCRs are FRET, bioluminescence resonance energy transfer and co-immunoprecipitation (Boyer and Slesinger, 2010, Cottet et al., 2012, Achour et al., 2011, Li et al., 2012). Here, we use a flow-cytometry

based FRET methodology, developed in our lab (Chruścicka et al., 2018), which confirmed the physical interaction needed for formation of a heterocomplex between the OTR and GLP-1R.

As previously mentioned heterocomplex formation can affect the functionality of GPCRs (Wrzal et al., 2012, Schelshorn et al., 2012). Therefore, the downstream signalling effect of the OTR and GLP-1R heterodimerization was assessed. Calcium mobilization assays and calcium imaging were used to analyse the Gαq signalling pathway as both the OTR and GLP-1R are known to signal via this alpha subunit (Busnelli and Chini, 2018, Coopman et al., 2010). Alteration to GLP-1R-mediated calcium signalling was observed, with significant attenuation in GLP 7-37 efficacy and EC_{50}. Interestingly, antagonism of the OTR had no effect on restoring or further decreasing GLP-1R-mediated calcium signalling, indicating no effect of OTR antagonist binding on the GLP-1R and OTR heterocomplex formation, where such effects have been previously seen in GPCR dimerization (Schlessinger, 2002). The OTR-mediated calcium signalling was not affected, indicating a modulatory effect of OTR on the GLP-1R. This data matches alterations seen upon co-expression of gastric inhibitory peptide receptor and GLP-1R leading to alterations to the GLP-1R-mediated Gαq signalling pathway (Schelshorn et al., 2012). The GLP-1R and OTR are also known to signal through the Gαs and Gαi alpha subunit, effecting the accumulation of cAMP (Wang et al., 2001, Strakova and Soloff, 1997). cAMP assays showed no changes to cAMP accumulation in either the OTR or GLP-1R-mediated signalling. Due to the alterations in Gαq specific GLP-1R signalling, it could be hypothesized that GPCR scavenging from the OTR is occurring - a phenomenon whereby a GPCR acquires more G proteins than another from a shared pool of available G-proteins (Nijmeijer et al., 2010). As the Gαq pathway is the primary signalling pathway of the OTR, it is possible the OTR is scavenging shared pools of Gαq proteins to ensure no changes occur in OTR-mediated Gαq signalling (Vischer et al., 2011).

Furthermore, the coupling of the G-protein subunits to the corresponding GPCR can often be affected by β-arrestin coupling and subsequent internalisation of the receptor from the membrane into the cytoplasm (Takenouchi et al., 2018, Smith and

Rajagopal, 2016). In this study, a decrease in both the OTR and GLP-1R trafficking was observed which has been previously shown to indicate the reduced coupling of β-arrestin to OTR and GLP-1R (Takenouchi et al., 2018). The enhanced expression of GLP-1R on the cell membrane has been previously reported when GLP-1R is co-expressed with the glucose-dependent insulinotropic polypeptide receptor which resulted in altered trafficking of the GLP-1R , where the GLP-1R was shown to remain longer on the cell membrane (Whitaker et al., 2012), indicating the ability of GLP-1R to modulate the internalisation of other GPCRs. The increased expression of GPCRs on the cell membrane can often result in receptor desensitization, with GPCR heterodimerization been shown to impact this desensitization (Pfeiffer et al., 2001). GPCR desensitization is caused by overstimulation of GPCRs leading to uncoupling of this receptor from the respective G-protein causing a decrease in receptor signalling (Bologna et al., 2017). Such desensitization may indicate why a decrease in the GLP-1R Gαq-mediated signalling is observed, although further investigation of other downstream signalling would further back up this hypothesis.

Finally, co-expression of the OTR and GLP-1R was observed in primary neuronal cultures, within the hippocampus and the hypothalamus. Both the OTR and the GLP-1R have shown to play major roles in physiological functions associated with the hypothalamus and hippocampus (Hsu et al., 2015, Lin et al., 2017b, Kasahara et al., 2013, Burmeister et al., 2017). The GLP-1R in these areas is known to play a critical role in memory and learning (During et al., 2003, Hansen et al., 2015), diabetes (Hansen et al., 2009, Samson and Garber, 2013) and energy homeostasis (Hayes et al., 2010, Adams et al., 2018). Therefore, any alteration to the expression or downstream signalling pathways of GLP-1R in these areas can have major implications (Ten Kulve et al., 2016). This result highlights the possible physiological relevance of an OTR-GLP-1R heterocomplex, although future *ex-vivo* and *in-vivo* studies are required to fully elucidate this hypothesis.

3.6 Conclusion

Collectively, these findings yield compelling evidence for a novel functional heterocomplex between the OTR and the GLP-1R, with significant downstream signalling consequences. This heterocomplex formation results in a Gαq-specific

alteration to the GLP-1R signalling pathway, with prolonged cell membrane expression of both the OTR and GLP-1R. Such a formation may have important physiological implications in GLP-1R and OTR-mediated effects in areas such as diabetes, obesity, anxiety and depression. Overall, we highlight another novel signalling pathway induced by GPCR heterocomplex formations, further emphasising the importance of GPCR heterocomplex research in fully understanding the complex network of GPCR signalling.

3.7 Supplementary Figures

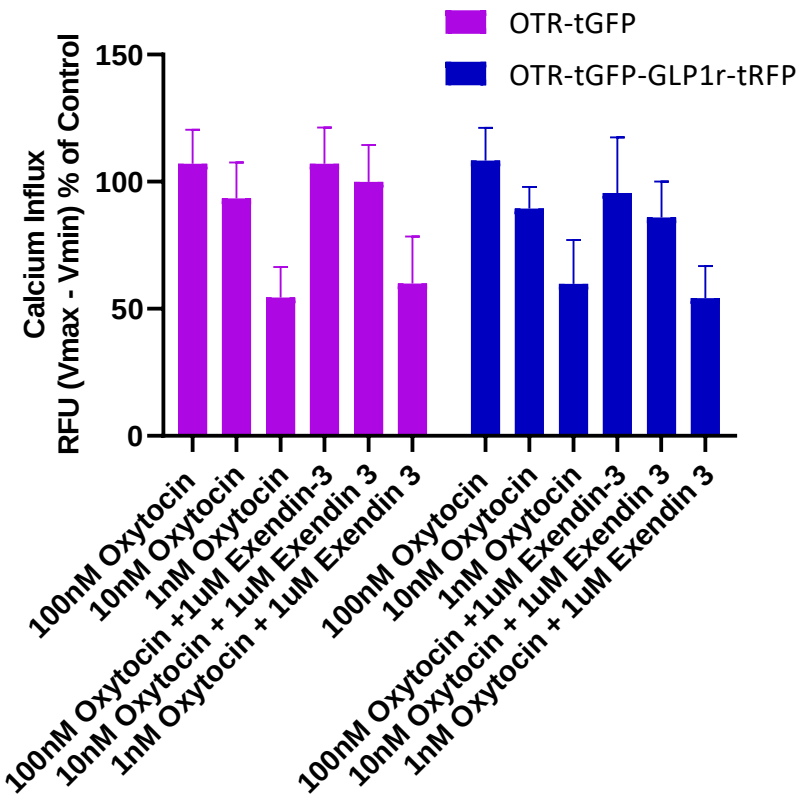

Figure S3.1: Pre-treatment with GLP-1R antagonist Exendin 3 has no effect on OTR-mediated calcium signalling. Acute addition of three concentrations of oxytocin alone or following pre-treatment with GLP-1R antagonist Exendin 3, we compared and revealed no significant effect of GLP-1R antagonist treatment on OTR-mediated signalling. Data is representative of 4 independent experiments with 3 replicates in each.

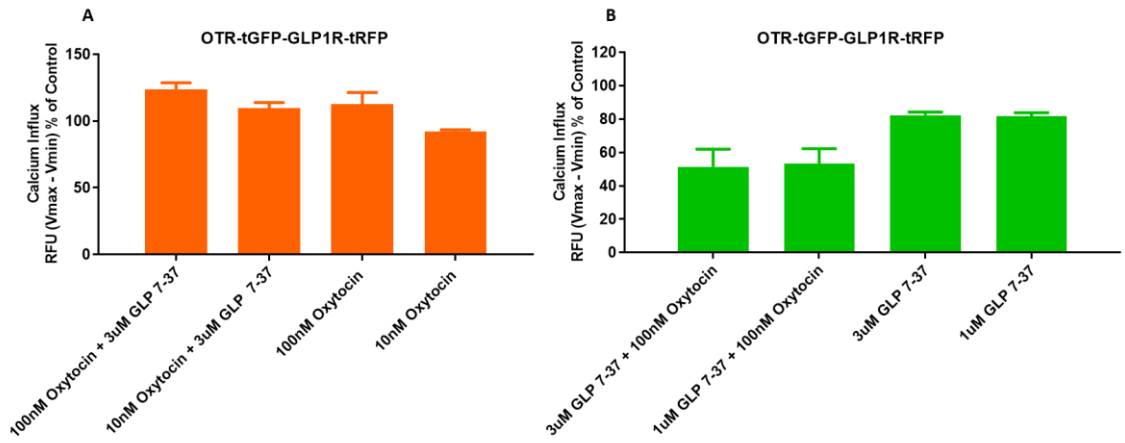

Figure S3.2: Co-addition of oxytocin and GLP 7-37 have no synergistic effect on GLP-1R or OTR-mediated calcium signalling. Data is representative of 1 experiment with 3 replicates for each condition.

Chapter 4

Attenuation of oxytocin and serotonin 2A receptor signalling through novel heteroreceptor formation.

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4.1 Abstract

The oxytocin receptor (OTR) and the 5-hydroxytryptamine 2A receptor (5-HT_{2A}) are expressed in similar brain regions modulating central pathways critical for social and cognition-related behaviours. Signalling crosstalk between their endogenous ligands, oxytocin 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-5-HT_{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-HT_{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-5-HT_{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-5-HT_{2A} downstream signalling crosstalk showed a reduction in potency and efficacy of oxytocin and OTR synthetic agonists, carbetocin and WAY267464 on OTR-mediated Gαq signalling. Similarly, the activation of 5-HT_{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-trafficking of the OTR/5-HT_{2A} pair and their potential heteromerization. Overall, these results constitute a novel mechanism of specific interaction between the oxytocin and 5-HT neurotransmitters via OTR-5-HT_{2A} heteroreceptor formation and provide potential new therapeutic strategies in the treatment of social and cognition-related diseases.

4.2 Introduction

Oxytocinergic signalling represents one of the major neuroendocrine systems in mammals (Du Vigneaud et al., 1953). Oxytocin, the nine-amino acid peptide hormone is produced in paraventricular and supraoptic nuclei of the hypothalamus. Peripherally-secreted, oxytocin is mainly known to stimulate uterine smooth muscle contractions associated with parturition and milk ejection during lactation (Graf, 1969, Gimpl and Fahrenholz, 2001a, Young et al., 1996, Arrowsmith and Wray, 2014). The known actions of oxytocin are mediated by the one type of oxytocin receptor (OTR), which belongs to the largest subclass of the rhodopsin-β adrenergic receptor family (class A) of G-protein coupled receptors (GPCRs) (Zingg and Laporte, 2003, Kimura et al., 1992a). Detection of the OTR in kidney, ovary, testis, thymus, bone cells, osteoblasts, myoblasts, adipocytes, cardiomyocytes, vascular endothelial cells and different types of cancer cells has expanded the possible spectrum of oxytocin activities from male and female fertility regulation to regulating of the immune system, control of the cardiovascular system, formation of bones and muscles, and control of growth of certain cancer cells (Welch et al., 2014, Li et al., 2016, Imanieh et al., 2014).

Oxytocin is released in a number of brain regions where this peptide functions as a neurotransmitter (Lee et al., 2009, Mustoe et al., 2018). Central oxytocin 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 (De Dreu, 2012, Meyer-Lindenberg et al., 2011, Borland et al., 2018, Neumann and Slattery, 2016). Furthermore, dysfunction in the oxytocin system is associated with several mental disorders characterized by social impairments in particular; autism spectrum disorders (ASD), social anxiety disorder, borderline personality disorder and recently also schizophrenia (Woolley et al., 2014, Davis et al., 2013, Husarova et al., 2016). In spite of the fact that pre-clinical studies have shown beneficial effects of oxytocin administration on social cognition and prosocial behaviour, pointing towards its promising therapeutic potential, clinical evidence for its efficacy has not yet been established (Bartz et al., 2011, Gauthier et al., 2016). The effects of oxytocin administration to healthy subjects but also to

patients with schizophrenia and ASD demonstrated a great diversity in response and inconsistency throughout several different studies, which may be indicative of changing heterocomplex levels between different disorders (Leppanen et al., 2017, Keech et al., 2018). This highlights the complex nature of the oxytocin signalling system that may result from the oxytocin system's ability to interact and modulate multiple neurotransmitter signalling systems in the brain.

In several rodent studies, the ability of oxytocin to modulate serotonergic (5-HT) signalling has been demonstrated (Dölen et al., 2013, Yoshida et al., 2009, Eaton et al., 2012, Lefevre et al., 2017). The 5-HT signalling represents one of the main neurotransmission systems in the brain, involved in mood, stress, and social behaviours (Kheirouri et al., 2016, Lesch, 2007). Interestingly, oxytocin administration has been shown to significantly increase 5-HT axon length and density in the amygdala and hypothalamus of prairie vole males, showing the effect of oxytocin signalling on 5-HT innervation during development (Eaton et al., 2012). The oxytocin neuropeptide is also known to regulate the 5-HT synthesis and release from serotonergic neurons with an origin in the midbrain raphe nuclei, which was subsequently shown to reduce anxiety-like behaviour in rodents (Yoshida et al., 2009). The modulation of 5-HT release by the oxytocin peptide is mainly driven through OTR, expressed on the serotonergic neurons. In addition, regulation of 5-HT release also involves signalling of the serotoninergic receptors in particular, the serotonin 2A/2C receptors ($5\text{-HT}_{2A/2C}$). Further studies have yielded very exciting results, which showed that the coordinated activity of the oxytocin and 5-HT neurotransmitters in the nucleus accumbens of mice is crucial for rewarding properties of social interactions (Dölen et al., 2013). This specific interaction between oxytocin and 5-HT systems was then validated in nonhuman primates and in humans, where oxytocin administration influenced 5-HT signalling in the amygdala, insula, hippocampus, dorsal raphe nucleus, and orbitofrontal cortex, key limbic regions implicated in the control of stress, mood, and social behaviours (Lefevre et al., 2017, Kheirouri et al., 2016, Lesch, 2007, Mottolese et al., 2014). The OTR and $5\text{-HT}_{1A/1B}$ (serotonin 1A/1B receptors) have been suggested to be primarily involved in these processes (Lefevre et al., 2017, Kheirouri et al., 2016, Lesch, 2007, Mottolese et al.,

2014). Interestingly, 5-HT_{2A} activation in the hypothalamus has been shown to affect oxytocin neurons resulting in an increased level of circulating oxytocin (Zhang et al., 2002, Van de Kar et al., 2001). These studies clearly demonstrate bi-directional relations between the oxytocin and 5-HT neurotransmitters and reiterated the involvement of OTR and 5-HT_{2A} crosstalk in this interaction.

Similarly to the OTR, the 5-HT_{2A} also belongs to class A GPCRs, being primarily coupled to Gαq proteins following its activation (Raote et al., 2007, Raote et al., 2013). Both GPCRs are critical signal transducers in the brain and have received much attention as promising therapeutic targets for social and cognition related disorders (Muguruza et al., 2013b, Rasmussen et al., 2016, Uhrig et al., 2016). In addition, both OTR and 5-HT_{2A} are well known to function as constitutive homodimers, but also heterodimers with other GPCRs as seen in table 1.1 and 1.3 (Borroto-Escuela et al., 2010a, Romero-Fernandez et al., 2013, Terrillon et al., 2003, Felsing et al., 2018). Oligomerization of GPCRs is known to modulate their downstream signalling and exert a significant impact on receptor physiology and function (Borroto-Escuela et al., 2010a, Ramirez et al., 2018, Łukasiewicz et al., 2016). Interestingly, the changes in the formation and function of GPCRs heterodimers are associated with many neuropsychiatric disorders (Fuxe et al., 2014a, Fuxe and Borroto-Escuela, 2016, Gonzalez-Maeso et al., 2008, Kolasa et al., 2018). The OTR has been recently shown to form specific heteroreceptor complex with dopamine 2 receptor (DRD2) resulting in changes in downstream signalling of both receptors (Romero-Fernandez et al., 2012). Moreover, this specific receptor-receptor interaction is involved in the OT-mediated anxiolytic effect (de la Mora et al., 2016, Ramirez et al., 2018, Fuxe et al., 2014a, Fuxe and Borroto-Escuela, 2016, Gonzalez-Maeso et al., 2008, De la Mora et al., 2012). 5-HT_{2A} also forms heterodimers with the DRD2. This heterodimerization has been shown to increase 5-HT_{2A} mediated Gαq signalling and reduce DRD2 mediated Gαi signalling after stimulation of both receptors in cells (Borroto-Escuela et al., 2010a). Furthermore, administration of the 5-HT_{2A} hallucinogenic agonists 2,5-Dimethoxy-4-iodoamphetamine (DOI) and lysergic acid diethylamide (LSD) increased the affinity of DRD2 in the ventral and dorsal striatum, highlighting the role of 5-HT_{2A}-DRD2 heterodimers in the mechanism of action of these hallucinogenic drugs (Fuxe et al.,

2014b, Borroto-Escuela et al., 2014b). The mechanism of atypical antipsychotics is also a good example that compounds interacting simultaneously with multiple GPCRs are clinically more effective, compared to drugs specific for one receptor. Thus, physical interaction between different GPCRs and subsequent changes in their downstream signalling is gaining attraction to become an accepted approach for the development of new therapies for many central nervous system (CNS) disorders, especially for these with multifactorial and polygenic aetiology.

Since the OTR and 5-HT_{2A} are both involved in social and cognition related behaviours and their location overlaps within brain regions associated with these processes, we hypothesize that the OTR and 5-HT_{2A} may form heteroreceptor complexes with subsequent downstream signalling changes. In this study, we first evaluate the possible formation of OTR-5-HT_{2A} heteroreceptor complexes using a flow cytometry-based FRET (fcFRET) approach and confocal microscopy. Furthermore, the formation of these specific heteroreceptor complexes is investigated *ex vivo*, in rat brain sections with the use of Proximity Ligation Assay (PLA) (Borroto-Escuela et al., 2017b). The OTR-5-HT_{2A} heteroreceptor complexes were identified in limbic regions (inter alia hippocampus, cingulate cortex area 1, and nucleus accumbens) associated with cognition and social-related behaviours. Finally, functional cellular-based assays such as; intracellular calcium mobilization, IP-One accumulation and ligand-mediated trafficking (Ramirez et al., 2018, Fuxe and Borroto-Escuela, 2016, Fuxe et al., 2014a, Gonzalez-Maeso et al., 2008, Kolasa et al., 2018, De la Mora et al., 2012, Borroto-Escuela et al., 2010a, Fuxe et al., 2014b, Borroto-Escuela et al., 2014b, Schellekens et al., 2013e) are used to demonstrate the significant changes in the Gαq-dependent signalling and trafficking of both receptors. Overall, these data show for the first time the formation of OTR-5-HT_{2A} functional heteroreceptor complexes *in vitro* in a heterologous expression system and *ex vivo* in rat brain sections, giving one possible molecular mechanism responsible for the interaction of oxytocin and 5-HT systems in the brain.

4.3 Materials and methods

4.3.1 Receptors ligands

Oxytocin (#O3251), carbetocin (#SML0748), 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). 3 mM stocks of compounds were prepared in H_2O (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.

4.3.2 Cell culture and stable transfection.

Plasmid containing the canonical sequence (transcript variant 1) of the human 5-HT_{2A} receptor (5-HT_{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 sub-cloned into the multicloning site of the pCMV-EGFP-N1 vector (Clontech #6085-1) to obtain the 5-HT_{2A} C-terminally tagged with EGFP. The obtained plasmid construct; pCMV-5-HT_{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% CO_2. HEK293A cells were transfected with the plasmid containing human 5-HT_{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 selection of cells with stably integrated pCMV-5-HT_{2A}-EGFP-N1 plasmid. The cells

with the highest expression of the receptor were selected using flow-assisted cell sorting (FACSAriaII, BD Biosciences), followed by clonal expansion in 96-well plates. Expression level of 5-HT_{2A} in generated monoclonal cell line was routinely monitored using an epifluorescence microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences).

4.3.3 Lentiviral transfection and transduction

The coding sequences of the OTR was sub-cloned into the multicloning site of the HIV-based, replication deficient, lentiviral expression vector; pHR-SIN-BX-tRFP. The construct containing the canonical sequence of the OTR (NM_000916.3), C-terminally tagged with red fluorescent protein (tRFP) was generated by inserting the coding sequence of the receptor lacking its stop codon from pCMV6-AC-OTR-GFP plasmid (#RG211797; OriGene, Rockville, MD) into the target vector (pHR-SIN-BX-tRFP) with the use of BamHI and XhoI restriction enzymes. The obtained pHR-SIN-BX-OTR-tRFP plasmid construct was validated by restriction analysis and DNA sequencing. HEK293A cells were then transiently transduced with the obtained lentiviral expression vector using a second generation packaging, gene delivery, viral vector production system, previously described by our group (Schellekens et al., 2013e). Briefly, HIV-based lentiviral particles containing the OTR sequence were produced using HEK293T-17 cells, by transient co-transfection of the expression construct; pHR-SIN-BX- OTR -tRFP, the packaging construct; pCMV ΔR8.91, and the envelope construct; pMD.G-VSV-G. Following this, HEK293A cells were transiently transduced with the OTR-tRFP expressing lentiviral particles diluted in transduction media, consisting of DMEM with 2% FBS, 1% NEAA, and 8 µg/ml polybrene (#H9268; Sigma). The efficiency of transduction was monitored with the use of an epifluorescence microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences) before each experiment.

4.3.4 Flow Cytometry Fluorescence Resonance Energy Transfer (fcFRET)

HEK293A cells stably expressing 5-HT_{2A} tagged with EGFP were transiently transduced with lentiviral OTR sequence tagged with tRFP. Following transduction, cells were washed with PBS and mechanically removed from the wells. Cell suspension was then centrifuged for 4 min at 200 x g, at room temperature. The

pellet of cells was re-suspended 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 tubes (#352054; Corning). The fcFRET analysis was performed on a FACS AriaII cytometer (BD Biosciences) according to the protocol optimized in our group (Chruścicka et al., 2018; Schellekens et al., 2015). Briefly, EGFP 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. FRET signal between EGFP 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 EGFP fluorescence and FRET emission from blue laser, a 505 Long Pass (LP) dichroic mirror (DM) was used. Wild-type HEK293A cells were used for initial instrument setup and to differentiate cells based on their size and granulation, according to forward and side scattering plot (FSC/SSC), which allowed to eliminate doublets, dead cells, and debris from further analysis. In the next step, cells expressing donor or acceptor construct only were used to fine tune PMT settings and to perform the proper compensation for spectral bleed through and cross-excitation, in particular for EGFP emission in the tRFP-fcFRET detector. The same number of cells (10^4) was recorded for each sample. Data was analysed using BD FACSDiva (BD Biosciences).

4.3.5 Colocalization with the use of fluorescent microscope

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

4.3.6 Receptor 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-HT_{2A}-EGFP and transduced with lentiviral OTR-tRFP, were seeded on 24-well plates (#83.3922.005; Sarstedt) at the density of 5 x 10^4 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-HT_{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. Quantification of the receptors trafficking was assessed by calculating the ratio between subcellular and membrane fluorescent intensity 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).

4.3.7 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.

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

To study the OTR-5-HT_{2A} heteroreceptor complexes the *in situ* proximity ligation assay (*in situ* PLA) was performed as described previously (Borroto-Escuela et al., 2018b). Adult age-matched male Sprague–Dawley rats (n=4) were anaesthetized and

perfused intracardially with 4% (wt/vol) formalin in saline. Brains were removed, post-fixed by immersion overnight in 4% formalin in PBS and coronal sections (30 um) 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. Then, after three PBS washes, were permeabilized with a permeabilization 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 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 slides twice, 5 min each time, with blocking solution at room temperature under gentle agitation and the sections were incubated with the hybridization-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 circularization or connector oligonucleotides (125-250 nM)) and incubated in a humidity chamber at 37°C for 30 min. The excess of connector oligonucleotides was 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 and the rolling circle amplification mixture was added to the slices and incubated in a humidity chamber at 37°C for 100 min. Then, 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 the 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 put on a microscope slide and a drop of appropriate mounting medium (e.g., VectaShield or Dako) 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 monoclonal anti-5-HT_{2A} (#SAB4301791, 1 µg/ml; Sigma-Aldrich, Stockholm, Sweden) and goat polyclonal anti-oxytocin receptor (#ab87312, 5µg/ml; Abcam, Stockholm, Sweden). Control experiments employed only one primary antibody. The PLA signal was visualized and quantified by using a Leica TCS-SL confocal microscope (Leica, USA) and the Duolink Image Tool software.

4.3.9 Intracellular calcium mobilization assay.

Receptor-mediated changes in intracellular calcium (Ca^{2+}) were monitored with the use of automatic fluorescent reader, FLIPR Tetra® (Molecular Devices, LLC Sunnyvale, CA) as previously described (Howick et al., 2018a, Ramirez et al., 2018). HEK293A cells with the expression of the receptors under investigation were seeded in black 96-well microtiter plates at a density of 3.0 - 4.0 x 10^4 cells/well and incubated overnight in standard culture conditions. 24 h prior to the experiment growth media was replaced with serum-free DMEM containing 1% NEAA. At the day of experiment cells were incubated for 90 min with 80 µl of the Ca5 dye diluted in assay buffer containing 1 x Hank's Balanced Salt Solution; HBSS (#14065049; Gibco Life Technologies, Gaithersburg, MD) and 20 mM HEPES (#H0887; Sigma-Aldrich) in the concentration recommended by the manufacturer's protocol (#R8186; Molecular Devices). The addition of receptor ligands (40 µl/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.

4.3.10 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 µL of cell suspension was pipetted to a flat bottom 96-well plate at the density of 3 x 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 µL of IP1-d2 conjugate and 15 µL 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 of relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained for non-stimulated cells. The specific signal is inversely proportional to the concentration of endogenous IP-One in the sample. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA).

4.3.11 Statistical analysis.

Data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA). The concentration-response curves of receptors ligands were generated using the nonlinear regression. The curves were fitted to a 3-parametric logistic equation, allowing for the determination of EC_{50} and E_{max} values. Statistical

comparison of the concentration-response curves parameters (EC_{50} and E_{max}) between cells co-expressing both receptors and cells solely expressing the corresponding receptor, were performed using the Student's test. Statistical comparison of each compound concentration used in calcium mobilization, IP-ONE accumulation, and ligand-mediated internalization assays between cells expressing the OTR, 5-HT_{2A} and cells co-expressing both receptors was performed using Two-way ANOVA with Bonferroni's multiple comparison tests. Statistical analysis of fcFRET was performed using One-way ANOVA with Bonferroni's multiple comparison tests. Statistical analysis of *in-situ* PLA was performed using One-way ANOVA followed by Tukey post-test. All data are presented as Mean ± SEM. The differences between groups were considered significant for $p<0.05$. The number of independent experiments performed is provided in figure legends.

4.4 Results and discussion

4.4.1 Flow cytometry based fluorescence resonance energy transfer demonstrates a physical interaction between the OTR and 5-HT_{2A}.

The interaction between the OTR and 5-HT_{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 granulation (Chruścicka et al., 2018). 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 (Schellekens et al., 2015a, Thyrock et al., 2010, Banning et al., 2010).

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 compared to cells expressing each tag separately or HEK293A cells without tags (data not shown). 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 ± 1.5%) when compared to cells expressing either the OTR tagged with tGFP (0%), or OTR tagged with tRFP (0.2 ± 0.2%) (Figure S4.1, 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 ± 0.4%) (Schellekens et al., 2015a). These results confirmed the ability of the OTR to form constitutive homodimers (Terrillon et al., 2003, Devost and Zingg, 2003) and showed the reliability of the experimental settings.

Next, analysis of fcFRET signal between the OTR and 5-HT_{2A} receptors was performed. A significant increase in the percentage of fcFRET positive cells was observed for cells co-expressing 5-HT_{2A}-EGFP and OTR-tRFP (HEK293A-5-HT_{2A}-EGFP-Lv-OTR-tRFP) (23.3 ± 3.1%) compared to cells solely expressing the donor construct (HEK293A-5-HT_{2A}-EGFP) (0.3 ± 0.03%) or acceptor construct (HEK293A-Lv-OTR-tRFP) (0.3 ± 0.03%) and compared to cells expressing the 5-HT_{2A}-EGFP with control-tRFP construct (3.2 ± 0.6%) (Figure 4.1A and 4.1C). Furthermore, fcFRET signal analysed as median fluorescence was also significantly higher in cells co-expressing the OTR/5-HT_{2A} pair (48 ± 1.6 RFU) compared to cells with the expression of the donor (15,3 ± 5.2 RFU) or acceptor (18,7 ± 10.3 RFU) construct only and compared to cells expressing the 5-HT_{2A}-EGFP with the control-tRFP construct (14.2 ± 6.9 RFU) (Figure 4.1B and 4.1D). Taking together, these results highlighted the constitutive and specific association between the OTR and 5-HT_{2A}, strongly indicating the formation of a heteroreceptor complex between these receptors *in vitro*, in intact HEK293A cells.

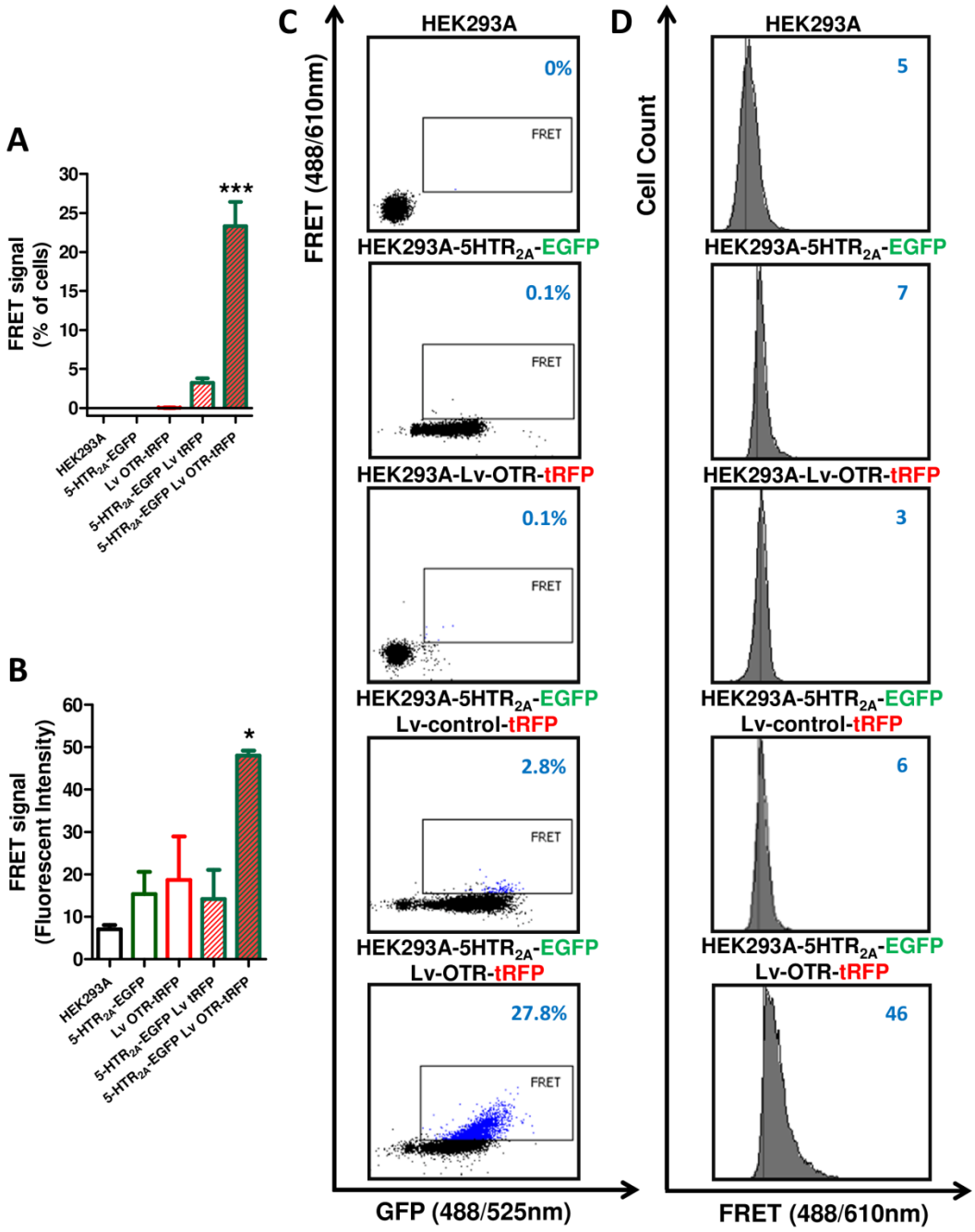

Figure 4.1. fcFRET between the OTR and 5-HT2A. *The fcFRET signal is presented as a percentage of cells (A,C) and as median fluorescence (B,D) in wild-type HEK293A cells, cells stably expressing the 5-HT2A tagged with EGFP (donor), cells transiently transduced with lentiviral vector expressing OTR tagged with tRFP (acceptor), cells expressing 5-HT2A tagged with EGFP and the control-tRFP, and cells co-expressing 5-HT2A tagged with EGFP and OTR tagged with tRFP. Graphs represent mean ± 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.*

4.4.2 The OTR and 5-HT_{2A} colocalize on the cell membrane.

Cellular localization of the receptors under investigation was investigated using a confocal microscopy in intact living HEK293A cells co-expressing the OTR and 5-HT_{2A}. The 5-HT_{2A} was mainly found within the cell membrane which was shown by the green fluorescence signal coming from 5-HT_{2A} fused with EGFP (Figure 4.2A). The red fluorescence signal coming from OTR fused with tRFP was also shown on the cell membrane, as well as in the intracellular space (Figure 4.2B). An overlap between green and red fluorescence as indicated by the yellow signal demonstrated colocalization of both receptors on the cell membrane (Figure 4.2C, merge picture). The colocalization within the same confocal plane of the OTR/5-HT_{2A} pair is evidence for the potential formation of OTR-5-HT_{2A} heteromers and reinforces the observed fcFRET signal (Figure 4.1). Interestingly, colocalization between the OTR and 5-HT_{2A} can also be observed in other locations within the cytoplasm of the cells. This may indicate possible co-trafficking of both receptors within the cell, which was previously observed in the case of other GPCRs heterodimer pairs (Schellekens et al., 2013e, Ward et al., 2011a).

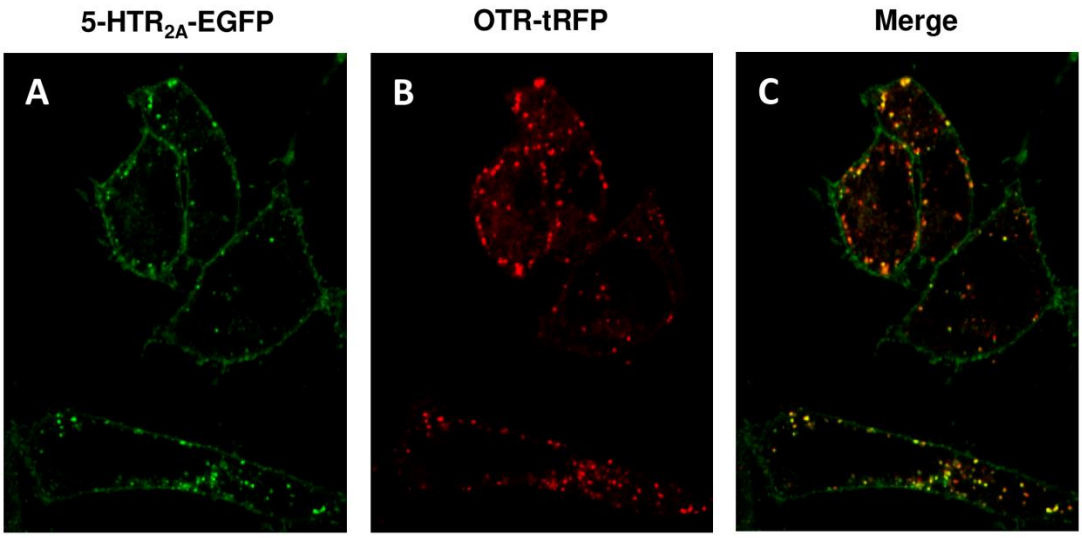

Figure 4.2. Cellular colocalization of the OTR/5-HT2A pair. *Images taken using 60x magnification show HEK293A cells stably expressing the 5-HT2A tagged with EGFP (green) (A) were transiently transduced with lentiviral vector expressing OTR tagged with tRFP (red) (B). Merged picture (yellow) shows colocalization of the two receptors within the cell (C).*

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

In the dorsal hippocampus of WT SD rats (Bregma -3.6 mm) a high density of PLA positive OTR-5-HT_{2A} heteroreceptor complexes clusters was found in the pyramidal cell layer of the CA2 and CA3 regions, while only a few were found in the stratum oriens and radiatum of these areas. This was similar to the background found in negative controls and the myelinated bundles of the crus cerebri (CC) (Figure 4.3A). A multiple z-scan (20) confocal microscopy photograph corresponding to the CA3 region with higher magnification of the high-density PLA positive clusters is shown in Figure 4.3B. The quantitative data present the number of PLA clusters (blobs) per nucleus per sampled field (30X30 µm). They range mainly from 8-13 PLA clusters in the CA2 and CA3 regions to 4-6 PLA clusters in the polymorphic layer of the dentate gyrus (PoDG) and it shows the high density in the pyramidal cell layer. A very low density of the PLA clusters is found in the granular cell layer of the dentate gyrus (gDG). The molecular cell layer of the dentate gyrus (mDG) contains densities similar to the densities and values found in negative controls.

In the cingulate cortex (Bregma 1.2 mm) a high-density of PLA positive OTR-5-HT_{2A} heteroreceptor complexes clusters is found in layer III, shown in low and high magnifications (Figure 4.3B), with a low to moderate number of PLA clusters in layer II. An overall high density of PLA positive clusters was also found in the nucleus accumbens shell (Bregma 1.2 mm) (Figure 4.3B) based on the average number clusters per nucleus per sample field. This was significantly increased compared to the values obtained in negative controls; the myelinated bundles of the crus cerebri (CC) and the anterior limb of the anterior commissure (aca). In the nucleus accumbens core a medium/low density of PLA positive OTR-5-HT_{2A} heteroreceptor complexes was found. In the dorsal striatum these receptor complexes could not be clearly observed.

A PLA positive signal validates the *in vitro* results and demonstrates the formation of OTR-5-HT_{2A} heteroreceptor complexes in rat brain sections under endogenous expression levels of both receptors. Moreover, specific distribution pattern of OTR-5-HT_{2A} heteroreceptor complexes clearly indicates their role in distinct cortical and subcortical limbic regions. The formation of these receptor complexes in the CA2 and

CA3 regions of the hippocampus and cingulate cortex may be involved in modulation of OTR dependent social recognition and memory (Raam et al., 2017a) as well as 5-HT_{2A} driven social exclusion processing (Preller et al., 2016). It is also tempting to hypothesize that OTR-5-HT_{2A} heteromers identified in nucleus accumbens can be partially responsible for the crosstalk between oxytocin and 5-HT neurotransmitters shown to be crucial for rewarding properties of social interactions (Dölen et al., 2013). In conclusion, the above results underlie the role of OTR-5-HT_{2A} heteroreceptor complexes in distinct limbic circuits relevant to social interactions.

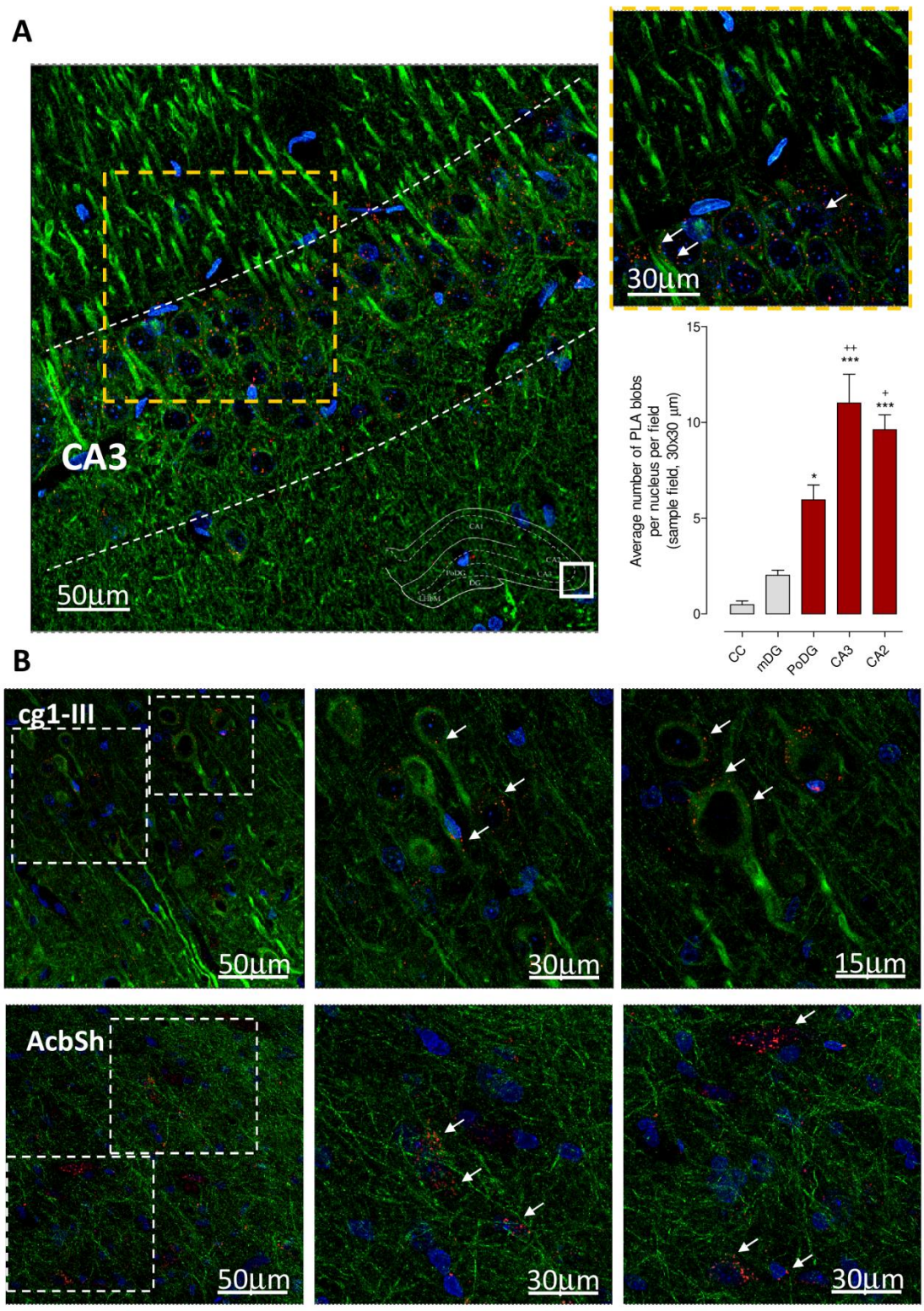

Figure 4.3. Illustration of the OTR-5-HT2A heteroreceptor complexes in the dorsal hippocampus and nucleus accumbens of rat brain. *Microphotographs from transverse sections of the rat dorsal hippocampus (Bregma level: -3.6 mm) show the distribution of the OTR-5-HT2A heteroreceptor complexes in CA3 using 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 (cornus ammonis). The nuclei are shown in blue by DAPI staining and the neuronal marker in green. In the higher right panel the PLA blobs are presented in higher magnification in the pyramidal cell layer. In the lower right part of the figure the density (per nucleus per sampled field) of the PLA positive complexes in PoDG (polymorph layer of the dentate gyrus), CA3, and CA2 are highly significantly different (***) from the density found in crus cerebri (CC) and the granular cell layer of the dentate gyrus (gDG). The density is also significantly higher in the CA2 (+) and CA3 (++) versus PoDG (Mean ± SEM, 4 rats per group) (A). The upper panel of B show representative examples of these PLA receptor complexes from transverse sections of the rat cingulate cortex, area 1 (Bregma level: 1.2 mm). They present the distribution of OTR-5-HT2A heteroreceptor complexes. They are shown as red PLA blobs (clusters) with high densities in layer III and low to moderate densities in layer II. Layer III represents the external pyramidal cell layer where large PLA positive clusters are found and appear to be located on the surface of many pyramidal cells. Higher magnifications of the two squares outlined in left panel are shown in the two right panels. The nerve cell bodies and apical dendrites are seen in green (neuronal marker). The lower panel in B is taken from nucleus accumbens shell (AcbSh). The neuronal markers here only show the neurite network. Discrete nerve cell bodies are associated with a high density of PLA positive blobs representing OTR-5-HT2A heteroreceptor complexes that may also have an intracellular location through trafficking. The outlined squares in the left panel are shown in higher magnifications in the two right panels (B).

4.4.4 Ligand-mediated internalization of the OTR and 5-HT_{2A}.

Desensitisation and subsequent internalization of GPCRs provides an important physiological mechanism that protects cells against overstimulation (Smith et al., 2006, Hasbi et al., 2004, Berrada et al., 2000). Most GPCRs, including the OTR and 5-HT_{2A} are rapidly internalized following agonist treatment and efficiently recycle to the cell surface after agonist removal (Smith et al., 2006, Hasbi et al., 2004, Conti et al., 2009, Hanley and Hensler, 2002). It has been documented that the formation of heteroreceptor complexes can affect basal and ligand-mediated internalization of the heterodimer protomers within the cell (Schellekens et al., 2013e, Ward et al., 2011a, Jordan et al., 2001). Thus, we investigated the effect of co-expression of the OTR and 5-HT_{2A} on their cellular trafficking under basal conditions and following treatment with their respective endogenous ligands, oxytocin (100 nM) and 5-HT (1 µM) (Figure 4.4). Significant oxytocin-mediated internalization of the OTR tagged with tRFP was observed in cells solely expressing the OTR (HEK293A-Lv-**OTR-tRFP**) (Figure 4.4A). Similarly, significant internalization of the 5-HT_{2A} tagged with EGFP was shown following 5-HT treatment in cells solely expressing the 5-HT_{2A} (HEK293A-**5-HT_{2A}-EGFP**) (Figure 4.4B). Interestingly, in cells co-expressing both receptors (HEK293A-5-HT_{2A} -EGFP-Lv-**OTR-tRFP**), a significant increase in basal internalization

of the OTR was observed. Further increase in oxytocin or 5-HT-mediated internalization compared to control conditions although small also was noted in these cells (Figure 4.4A, blue bars with stripes). Moreover, we observed that the basal internalization of the 5-HT_{2A} in cells co-expressing both receptors (HEK293A-**5-HT_{2A}-EGFP**-Lv-OTR-tRFP) was consistently increased compared to cells solely expressing the 5-HT_{2A} (Figure 4.4B). Interestingly, when both receptors were co-expressed (HEK293A-5-HT_{2A}-EGFP-Lv-OTR-tRFP) a small, albeit insignificant increase in 5-HT_{2A} internalization was consistently observed following oxytocin treatment compared to the control condition (untreated cells) (Figure 4.4B).

Changes in basal trafficking properties of the OTR and 5-HT_{2A} following their co-expression in mammalian cells may at least partially explain the colocalization of both receptors observed not only on the subcellular membrane but also intracellularly (Figure 4.2C). Moreover, these observations are similar to what was shown for the 5-HT_{2A} and the metabotropic glutamate receptor 2 (mGluR2) (Wischhof and Koch, 2016). These receptors demonstrated to form stable 5-HT_{2A}-mGluR2 heterodimers in HEK293 cells, which significantly increased their intracellular presence under basal conditions. This indicates that both receptors are assembled as heterocomplexes at an early stage, during maturation and trafficking to the cell membrane (Baki et al., 2016). Several other studies have shown that GPCRs are indeed secreted to the cell surface as oligomerized complexes (Terrillon et al., 2003, Lopez-Gimenez et al., 2008, Milligan, 2008). Thus, the significant intracellular presence of the OTR and 5-HT_{2A} following their co-expression in cells may suggest that OTR and 5-HT_{2A} also form constitutive heteromers during maturation and trafficking from endoplasmic reticulum to the cell membrane. Alternatively, the above results may indicate an increase in basal activity of both receptors and a subsequent higher internalization rate as previously shown for cannabinoid CB1 and orexin OX_1 receptor complexes (Ward et al., 2011a). Noteworthy, the increased internalization of the 5-HT_{2A} after treatment with oxytocin may support the hypothesis that the 5-HT_{2A} is co-internalized along with the OTR, from the cell membrane to membranes of the endosomal compartment as previously demonstrated for the 5-HT_{2A}-mGluR2 and the 5-HT_{2C}-GHSR1a heteromers (Schellekens et al., 2013e, Wischhof and Koch, 2016).

The OT-induced changes in cellular trafficking of the 5-HT_{2A} are also consistent with the formation of stable OTR-5-HT_{2A} heteromer complexes demonstrated in Figure 1.

Finally, the changes in basal and ligand-mediated cellular receptor trafficking may also lead to alterations in the downstream signalling pathways of each protomer. This may be particularly relevant for increased signalling over the β-arrestin pathway, which not only leads to an increased receptor internalization (Borroto-Escuela et al., 2011) but also directly affects the G protein-dependent downstream signalling pathways of GPCRs (Luttrell et al., 2018).

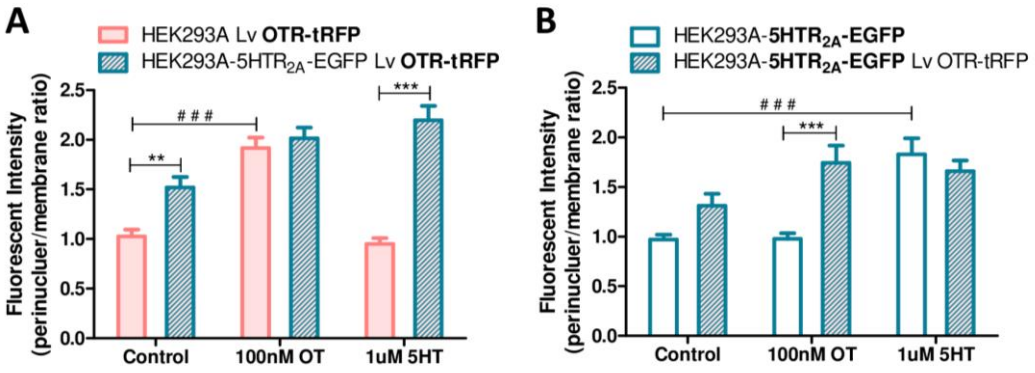

Figure 4.4. Cellular trafficking of the OTR and 5-HT2A. *Quantitative analysis of ligand-mediated internalization of OTR tagged with tRFP (A) and 5-HT2A tagged with EGFP (B) versus cells co-expressing both receptors. Graphs represents mean ± SEM from three independent experiments run in triplicate. Statistical 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 $p < 0.001$.*

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

Next, the downstream signalling consequences following co-expression of the OTR and 5-HT_{2A} were investigated. The OTR and 5-HT_{2A} are known to mainly signal through the Gαq subunit, where activation of the Gαq protein leads to generation of the second messenger, D-myoinositol 1,4,5-triphosphate (IP_3), causing subsequent intracellular calcium release from the endoplasmic reticulum into the cytoplasm (Gimpl et al., 2008, Gimpl and Fahrenholz, 2001a). Therefore, we assessed the ligand-mediated changes in intracellular calcium mobilization in HEK293A cells solely expressing the OTR or the 5-HT_{2A} and cells co-expressing both receptors. The cellular response was detected following the addition of endogenous receptor ligands,

oxytocin and 5-HT. The potency of oxytocin (EC_{50} = 0.12 ± 0.01 nM) in cells solely expressing the OTR (HEK293A Lv OTR) and potency of 5-HT (EC_{50} = 12.6 ± 0.7 nM) in cells solely expressing the 5-HT_{2A} (HEK293A-5-HT_{2A}) were consistent with literature data (Figure 4.5A and 4.5B), which confirms the functionality of the receptors expressed in the heterologous expression system (Smith et al., 2006, Albizu et al., 2007, Bonhaus et al., 1995). The intracellular calcium release following an increasing concentration of oxytocin was significantly reduced in cells co-expressing both the 5-HT_{2A} and OTR (HEK293A-5-HT_{2A} Lv OTR) compared to cells expressing only the OTR (Figure 4.5A). The concentration-response curve of oxytocin was characterized by a significantly lower potency (EC_{50} = 1.0 ± 0.4 nM) and efficacy (E_{max} = 82.7% ± 3.9) in cells co-expressing both receptors compared to cells solely expressing the OTR (EC_{50} = 0.1 ± 0.01 nM, E_{max} = 144.2% ± 18.5) (Figure 4.5A). Similarly, the concentration-response curve of 5-HT was characterised by a lower potency (EC_{50}= 67.5 ± 19.6 nM) but no significant changes in efficacy (E_{max} = 84.3% ± 3.2) in cells co-expressing both receptors compared to cells solely expressing the 5-HT_{2A} (EC_{50} = 12.6 ± 0.7 nM, E_{max} = 72.0% ± 6.1) (Figure 4.5B).

The transient release of calcium into the cytosol is also mediated by IP_3. Therefore, we evaluated the ligand-mediated changes in the production of this second messenger. The concentrations of oxytocin (10 nM and 1 nM) and 5-HT (100 nM and 10 nM) were chosen based on the calcium assay results (Figure 4.5A and 4.5B). As expected, OT-mediated IP-One (inositol monophosphate) accumulation was significantly decreased in cells co-expressing the 5-HT_{2A}/OTR pair compared to cells solely expressing the OTR, depicted as a significant increase in percentage IP-One values of control (Figure 4.5C). The analogous results were obtained for 5-HT-mediated IP-One accumulation in cells co-expressing both receptors (Figure 4.5D), validating the observed changes in Gαq-mediated signalling in calcium accumulation assay (Figure 4.5A and 4.5B).

In addition, the observed attenuation in ligand-mediated Gαq signalling in cells co-expressing the OTR and 5-HT_{2A} was shown to be independent of changes in the expression level of both receptors. Flow cytometry analysis of EGFP and tRFP assessed before each experiment showed no changes in the expression level of both

receptors between cell lines. The transient transduction with OTR-tRFP did not affect the 5-HT_{2A}-EGFP expression level in cells co-expressing both receptors (HEK293A-5-HT_{2A}-EGFP-Lv-OTR-tRFP) compared to non-transduced cells solely expressing the 5-HT_{2A}-EGFP. Similarly, the level of OTR-tRFP expression following transient transduction did not differ in cells co-expressing both receptors compared to cells solely expressing the OTR-tRFP (Figure S4.2, supplementary data). Moreover, control-experiments measuring the OTR-mediated calcium release between cells stably expressing the OTR-tGFP and cells transiently expressing the OTR-tRFP (following lentiviral transduction) demonstrated no significant differences (data not shown). This clearly shows no effect of the gene delivery mode (stable expression versus transient lentiviral transduction) or different fluorescent tags (EGFP or tGFP) on the OTR-mediated Gαq signalling. Finally, there were no changes in the OTR-mediated calcium response between cells solely expressing the OTR-tGFP and cells co-expressing the OTR-tGFP with OTR-tRFP (following lentiviral transduction), again showing no effect of the lentiviral transduction protocol, nor the OTR overexpression on Gαq-mediated signalling (Figure S4.3, supplementary data).

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

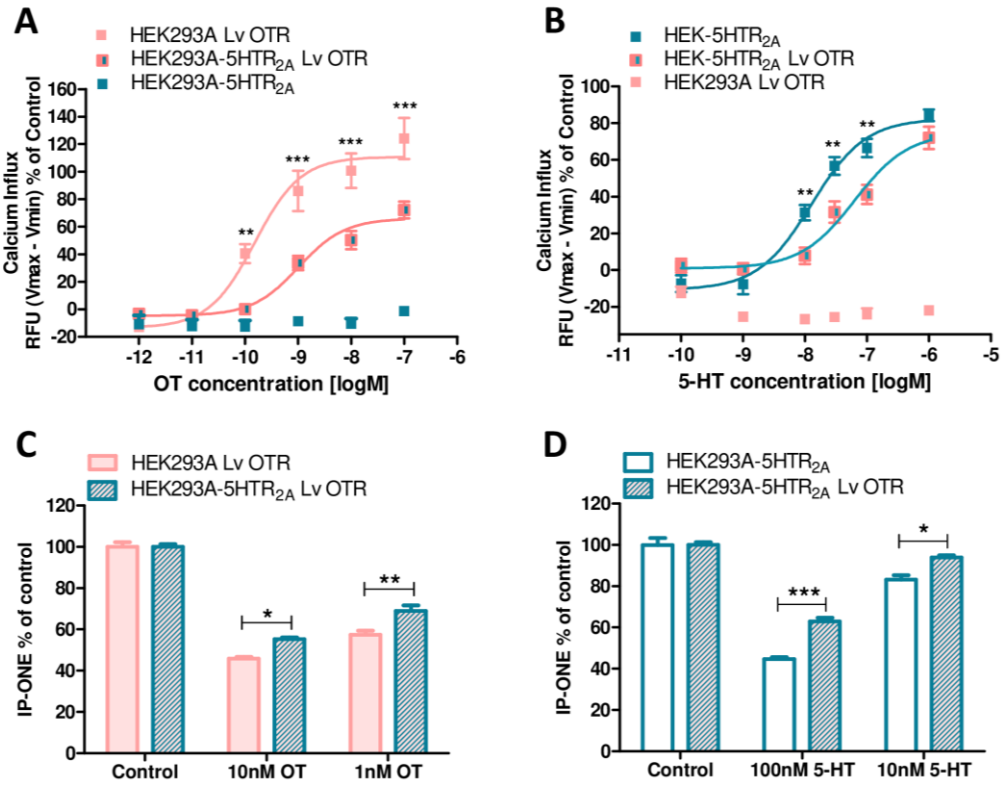

Figure 4.5. Co-expression of the OTR and 5-HT$_{2A}$ attenuates Gαq-dependent signalling of both receptors. *Intracellular calcium release induced by increasing concentration of oxytocin (A) and 5-HT (B) in HEK293A cells stably expressing the 5-HT$_{2A}$ tagged with EGFP, in cells transiently expressing 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 oxytocin (C), and 100 nM and 10 nM 5-HT (D) in HEK293A cells stably expressing 5-HT$_{2A}$ 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-HT$_{2A}$ could affect the observed attenuation in the Gαq-dependent downstream signalling pathway (Figure 4.6). Two antagonists of the OTR (Atosiban and L-371-257) and two antagonists of the 5-HT$_{2A}$ (M100907 and Eplivanserin) were used (Figure 4.6). As expected, both 5-HT$_{2A}$ antagonists used in 1 µM concentration were able to inhibit 5-HT-induced calcium mobilization in cells solely expressing the 5-HT$_{2A}$ and cells co-expressing both receptors (Figure 4.6B and 4.6D). Moreover, the lack of a non-specific interaction between oxytocin and 5-HT$_{2A}$ antagonists was

demonstrated in cells solely expressing the OTR (Figure 4.6A and 4.6C). Although a weak inhibition in oxytocin-induced calcium mobilization after co-administration with M100907 can be observed, it is not statistically significant (Figure 4.6A). The lack of non-specific interaction between these two ligands in cells solely expressing the OTR was also confirmed in additional experiments with various concentrations of oxytocin and M100907 (data not shown). Importantly, none of the 5-HT_{2A} antagonists significantly modulated oxytocin-mediated calcium release in cells co-expressing the OTR and 5-HT_{2A} (Figure 4.6A and 4.6C). These observations for 5-HT_{2A} antagonists were confirmed following their pre-treatment with cells co-expressing both receptors (Figure S4.4, supplementary data). Treatment with OTR antagonists (Atosiban and L-371-257) yielded similar results as observed for 5-HT_{2A} antagonists. Both, atosiban and L-371-257 used in 1 µM concentration inhibited OT-induced calcium mobilization in cells solely expressing this receptor (Figure 4.6F and 4.6H) but did not affect 5-HT-induced calcium signalling in cells co-expressing the OTR and 5-HT_{2A} (Figure 4.6E and 4.6G). Taking together, none of the antagonists used in the experiment were able to modulate the attenuation in Gαq-dependent signalling observed in HEK293A cells co-expressing the OTR-5-HT_{2A} pair (Figure 4.5).

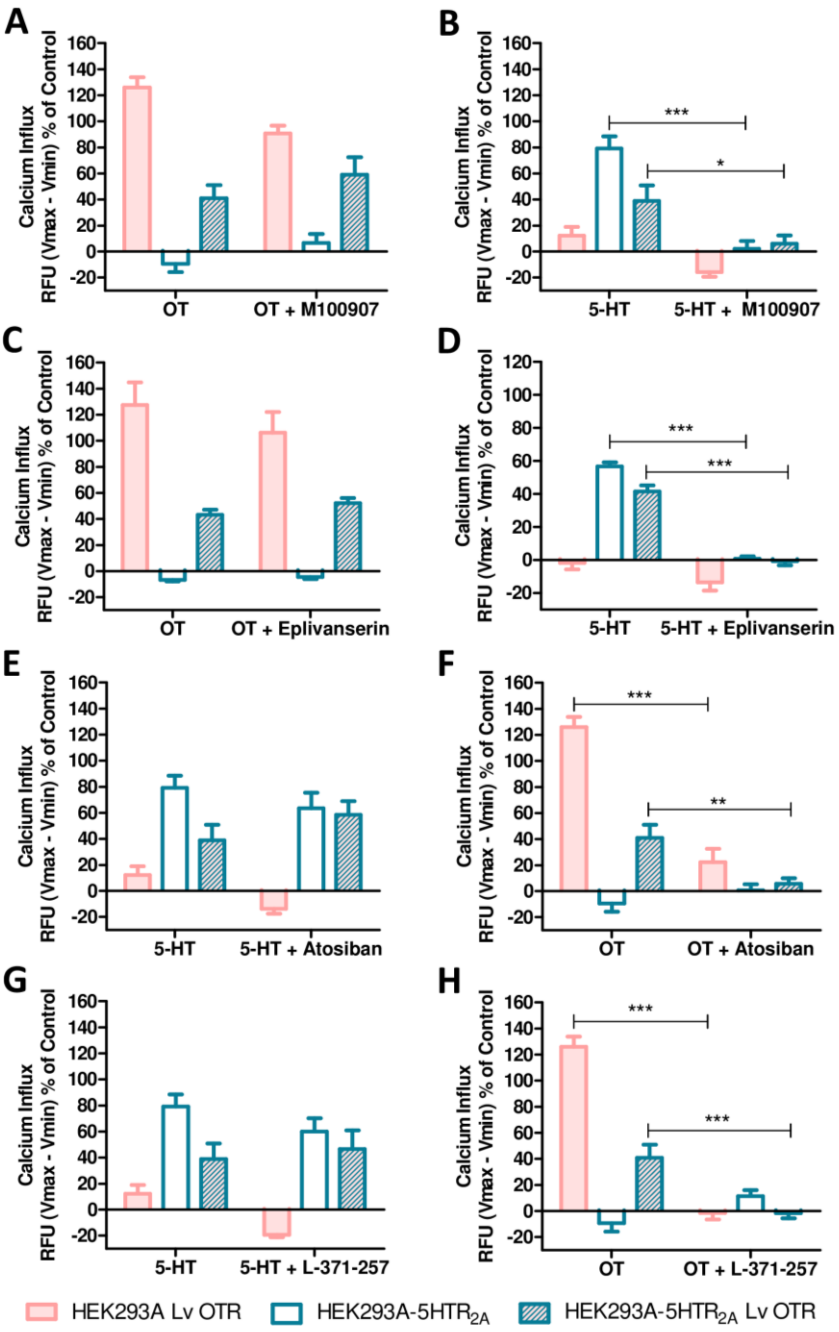

Figure 4.6. Pharmacological inhibition of the OTR or 5-HT2A does not affect the OTR-5-HT2A heterocomplex specific Gαq-dependent signalling. *Intracellular calcium release in cells solely expressing OTR, cells solely expressing 5-HT2A, and cells co-expressing both receptors induced by 10 nM oxytocin alone and in the presence of 1 µM 5-HT2A 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-HT2A 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 $p < 0.001$.*

To further investigate the modulation of Gαq-dependent signalling, cells with the co-expression of the OTR and 5-HT_{2A} were treated with different concentrations of two synthetic OTR agonists; carbetocin and WAY267464 (Ring et al., 2010, Chini and Manning, 2007). Similar to what we observed for oxytocin, the intracellular calcium release induced by increasing concentrations of carbetocin and WAY267464 was significantly reduced in cells co-expressing the 5-HT_{2A} and OTR compared to cells solely expressing the OTR (Figure 4.7). The potency and efficacy of carbetocin was significantly lower (EC_{50}= 9.4 ± 2.5 nM, E_{max} = 21.9% ± 4.1) in cells co-expressing both receptors compared to cells solely expressing the OTR (EC_{50} = 0.5 ± 0.3 nM, E_{max} = 86.0% ± 11.0) (Figure 4.7A). Intracellular calcium response induced by increasing concentrations of WAY267464 was completely abolished in cells co-expressing the OTR and 5-HT_{2A} (EC_{50}= nc, E_{max} = 2.7% ± 1.5) compared to cells solely expressing the OTR (EC_{50}= 11.6 nM, E_{max} = 61.2% ± 1.2) (Figure 4.7B). Nevertheless, pre-treatment of cells with 5-HT_{2A} antagonist (eplivanserin) did not affect carbetocin (Figure 4.7C) nor WAY267464 (Figure 4.7D) induced calcium response in cells co-expressing both receptors. In the same experimental setup, eplivanserin was able to inhibit 5-HT-mediated intracellular calcium accumulation in cells solely expressing the 5-HT_{2A} confirming compound specificity (data not shown).

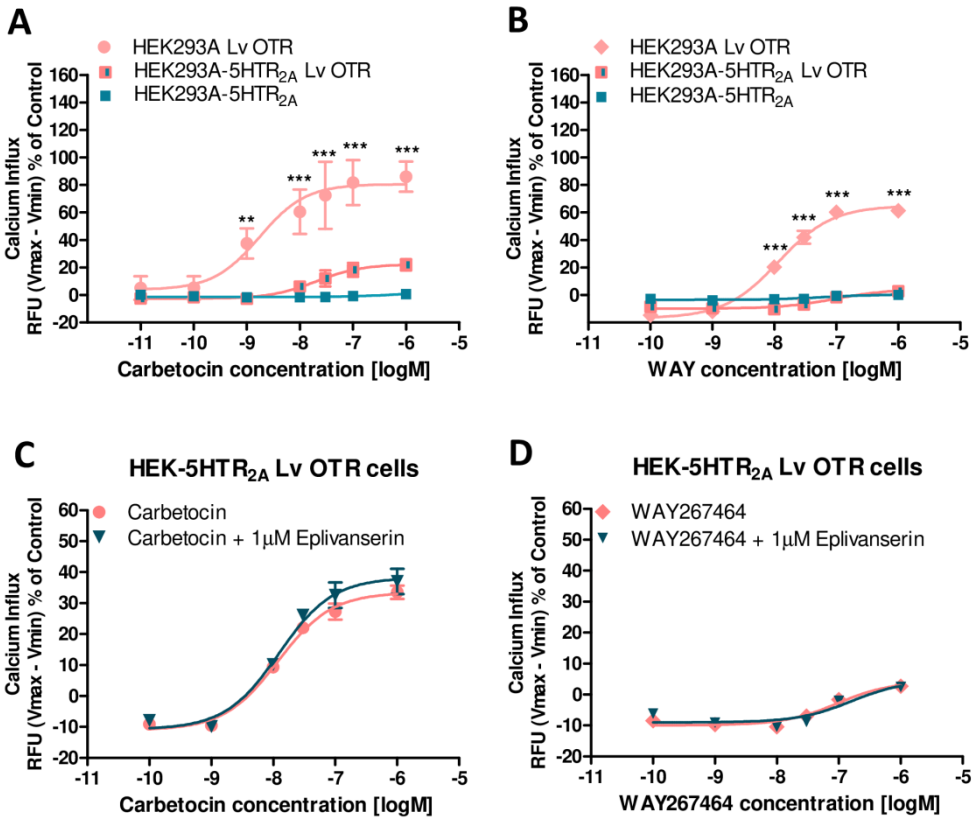

Figure 4.7. Synthetic OTR ligand-mediated attenuation of Gαq-dependent signalling in cells co-expressing the OTR and 5-HT2A*. Intracellular calcium release induced by increasing concentration of Carbetocin (A) and WAY265464 (B) in cells expressing OTR, in cells expressing 5-HT2A, and in cells co-expressing both receptors. Graphs present mean ± SEM from et 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-HT2A antagonist; Eplivanserin in cells co-expressing both receptors. Graphs present mean ± 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-HT_{2A} dependent signalling has been demonstrated upon co-expression with the OTR. The attenuated OTR-mediated signalling is even more evident compared to 5-HT_{2A}-mediated signalling changes. These interesting observations may be related to conformational rearrangements of one protomer when arranged in a heterocomplex, 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 (Figure 4.5A, 4.7A and 4.7B). This may suggest that

the potency of receptor ligands to activate Gαq signalling is lower for the OTR-5-HT_{2A} heteromer complexes than for the corresponding receptor homodimers. Current findings are in line with antagonistic interactions previously observed between the 5-HT_{2A} and mGluR2 which have been established *in vitro* in heterologous expression models and across multiple *ex vivo* and *in vivo* studies (Gonzalez-Maeso et al., 2008, Wischhof and Koch, 2016). The formation of 5-HT_{2A} heteromeric complexes with the DRD2 has also been demonstrated to result in DRD2-mediated Gαi signalling attenuation (Borroto-Escuela et al., 2010a). In contrast, concomitant activation of the DRD2 was shown to increase the 5-HT_{2A}-mediated Gαq signalling. Thus, the existence of a 5-HT_{2A}-mediated DRD2 trans-inhibition mechanism was suggested. Considering the above, the 5-HT_{2A} in heteroreceptor complexes with other GPCRs has a tendency to inhibit downstream signalling of the partner receptor. This further supports 5-HT_{2A}-mediated inhibition of the OTR-dependent signalling observed in the current study. Previously described heteroreceptor complexes of the 5-HT_{2A} with mGlu2 and DRD2, as well as formationn of heteromers between the OTR and DRD2, were shown to produce allosteric receptor-receptor interactions between the two protomers (de la Mora et al., 2016). Current results provide evidence that the formation of stable OTR-5-HT_{2A} heterocomplexes leads to bidirectional antagonistic receptor-receptor interactions with greater 5-HT_{2A} dominance. However, unlike previous studies, the antagonists of both OTR and 5-HT_{2A} did not affect the specific signalling driven by OTR-5-HT_{2A} heterocomplexes. Thus, one of the possible mechanisms of this specific receptor-receptor interaction is the physical binding between the two protomers and subsequent conformational changes without a steric hinderance of the binding pockets.

4.5 Conclusion

GPCRs comprise the largest family of cell surface receptors, which are major signalling mediators for many hormones and neurotransmitters involved in diverse physiological functions ranging from glucose metabolism to neurotransmission. GPCRs were originally thought to function as monomers. However, oligomerization of these receptors has now become accepted as a fundamental process in GPCR signalling. Oligomerization of specific GPCR protomers is characterized by an

increased receptor signalling diversity and exhibits unique functional and pharmacological properties adding a new dimension to GPCR pharmacology. Since mechanisms that lead to diverse brain pathologies such as social and cognition related disorders involve GPCR signalling, the distinct pharmacological profiles of GPCR assemblies may serve as novel mechanisms, important for the development of more specific pharmacological strategies to modulate cell response and regulate many physiological processes.

This study shows compelling evidence for a functionally relevant formation of a novel heteroreceptor complex between the OTR and 5-HT_{2A}. Both receptors have been shown to physically interact in living mammalian cells co-expressing both receptors (Figure 4.1). Confocal microscopy demonstrated that this specific interaction seems to appear at the cell membrane as well as intracellularly (Figure 4.2). Alterations in the trafficking of both receptors within the cell and their significant intracellular presence in basal conditions (Figure 4.4) are in line with the colocalized expression and strongly suggests changes in OTR and 5-HT_{2A} maturation and trafficking to the cell membrane. However, this phenomenon may also be a consequence of increased coupling to β-arrestin and subsequently higher basal activity of the two receptors. In any case both hypotheses support a physical interaction between the OTR and 5-HT_{2A} within the cell. Noteworthy, a significant attenuation was demonstrated primarily in OTR but also in 5-HT_{2A}-mediated Gαq-dependent signalling (Figure 4.5 and 4.7) indicating a functional relevant consequence of OTR/5-HT_{2A} interaction. In conclusion, the current study provides evidence that the OTR-5-HT_{2A} heterocomplex formation leads to bidirectional antagonistic receptor-receptor interactions *in vitro* in the heterologous system. As the antagonists of both receptors did not affect OTR-5-HT_{2A} heterocomplex specific signalling (Figure 4.6), it is likely that the physical binding between the two protomers serves as a mechanism for this specific receptor-receptor crosstalk.

Moreover, the formation of OTR-5-HT_{2A} heteroreceptor complexes were demonstrated *ex vivo* in rat brain sections using *in situ* PLA technique (Figure 4.3). OTR-5-HT_{2A} heteromers were preferentially observed in high densities on nerve cell bodies in the pyramidal cell layer of CA2-CA3 regions of the hippocampus, the layer

III of the cingulate cortex and in distinct nerve cell bodies of the nucleus accumbens shell. The proximal dendrites of these nerve cells had a reduced number of PLA blobs located close to them. This specific distribution pattern clearly indicates a role of OTR-5-HT_{2A} heteroreceptor complexes in distinct cortical and subcortical limbic regions. The formation of these receptor complexes may therefore have special role in distinct limbic circuits of relevance for social salience and memory, bearing in mind the importance of OTR in social interactions.

The existence of novel functional OTR-5-HT_{2A} heteroreceptor complexes constitutes one of the possible mechanisms for intriguing interactions between the oxytocin and 5-HT neurotransmitter systems. It also provides potential novel therapeutic strategies in the treatment of social and cognition-related diseases. Further in vivo studies exploring the physiological and behavioral nature of the specific interactions observed between the OTR and 5-HT_{2A} in limbic regions are now warranted.

4.6 Supplementary material

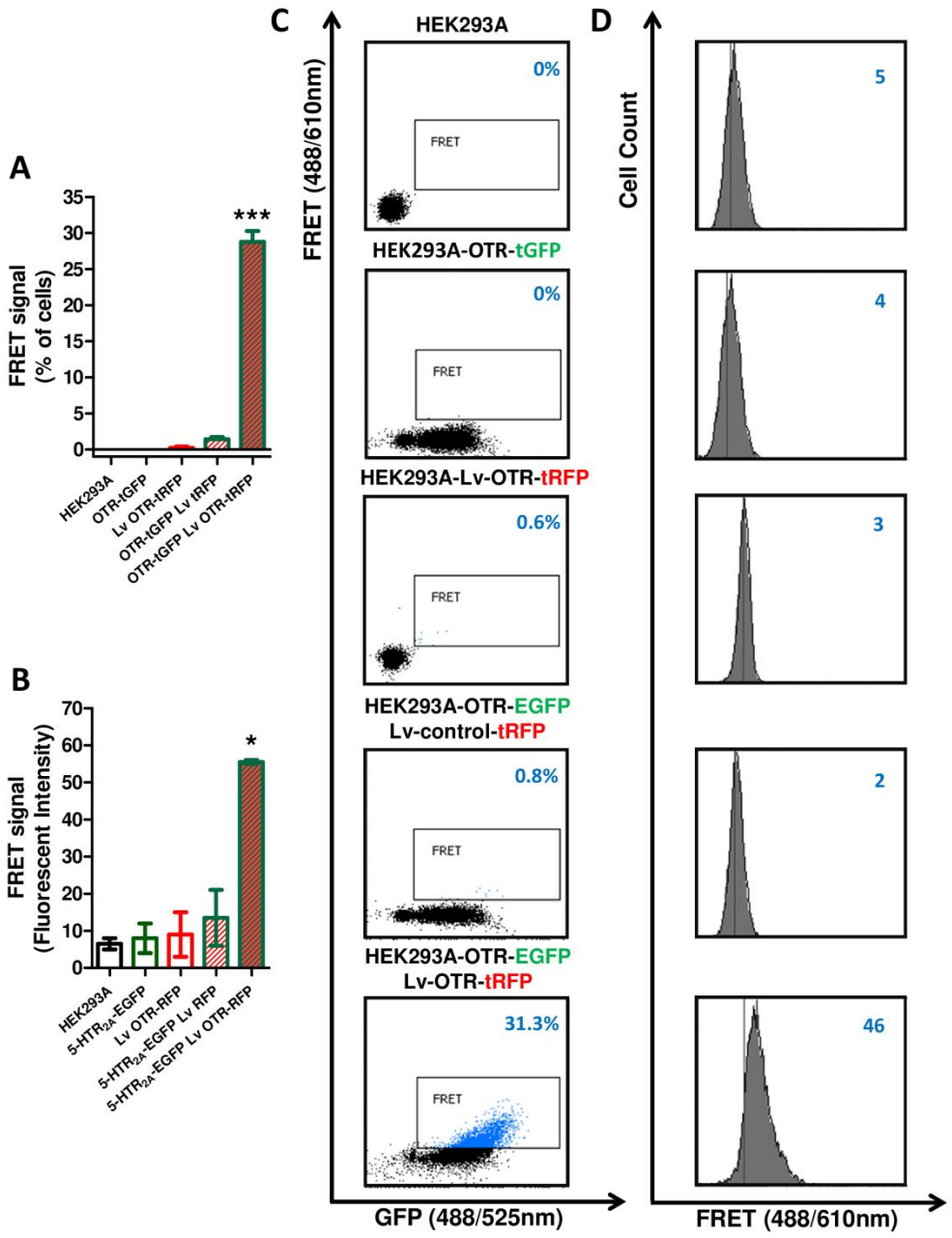

Figure S4.1. fcFRET between the OTR-tGFP and OTR-tRFP*. fcFRET signal presented as a percentage of cells (A,C) and median fluorescence (B,D) in wild-type HEK293A cells, cells stably expressing the OTR tagged with tGFP (donor), cells transiently transduced with lentiviral vector expressing OTR tagged with tRFP (acceptor), cells expressing OTR tagged with tGFP and the control-tRFP, and cells co-expressing OTR tagged with tGFP and OTR tagged with tRFP. Graphs represent mean ± SEM from two 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 tGFP plots), histograms (D) demonstrate median fluorescence of fcFRET signal (Cell count vs FRET signal). Dot plots and histograms are representative of two independent experiments.*

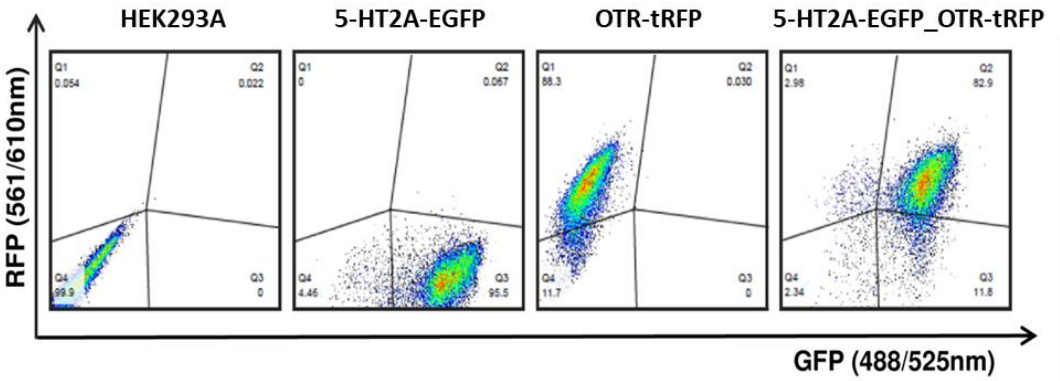

Figure S4.2. Flow cytometry analysis of EGFP and tRFP. *Dot plots demonstrate the percentage of EGFP or/and tRFP positive cells. Cells stably expressing the 5-HT2A tagged with EGFP showed 95.5% of EGFP positive cells. Wild-type HEK293A cells after transient transduction with the OTR tagged with tRFP showed 88.3% of tRFP positive cells. Cells stably expressing the 5-HT2A tagged with EGFP after transient transduction with the OTR tagged with tRFP showed 94.7% of EGFP and 85.8% of tRFP positive cells, where 82% of cells were EGFP and tRFP positive. Flow cytometry analysis was performed on cells used for all functional studies.*

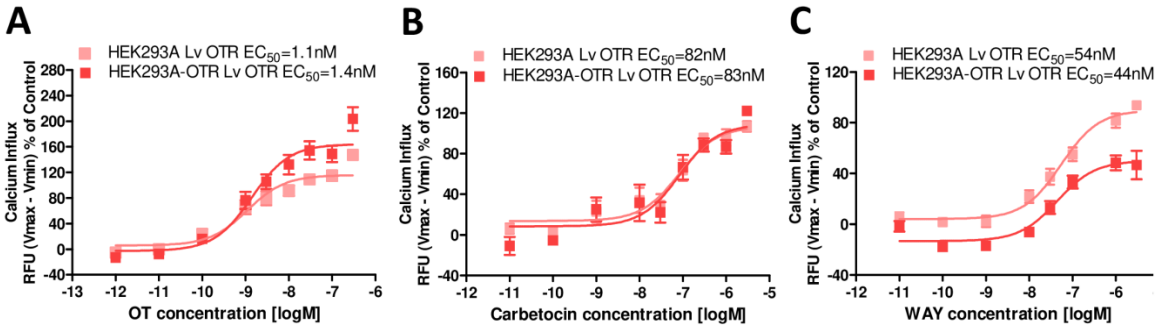

Figure S4.3. Transduction procedure doesn't affect Gαq-dependent signalling in cells co-expressing the OTR-tGFP and OTR-tRFP. *Intracellular calcium release induced by increasing concentration of oxytocin (A), Carbetocin (B) and WAY267464 (C) in HEK293A cells stably expressing the OTR tagged with tGFP and in cells stably expressing OTR-tGFP after transduction with OTR tagged with tRFP. Intracellular calcium mobilization is presented as a percentage of maximal calcium response elicited by the control (3% FBS). Graphs represent means ± SEM from three independent experiments run in triplicates.*

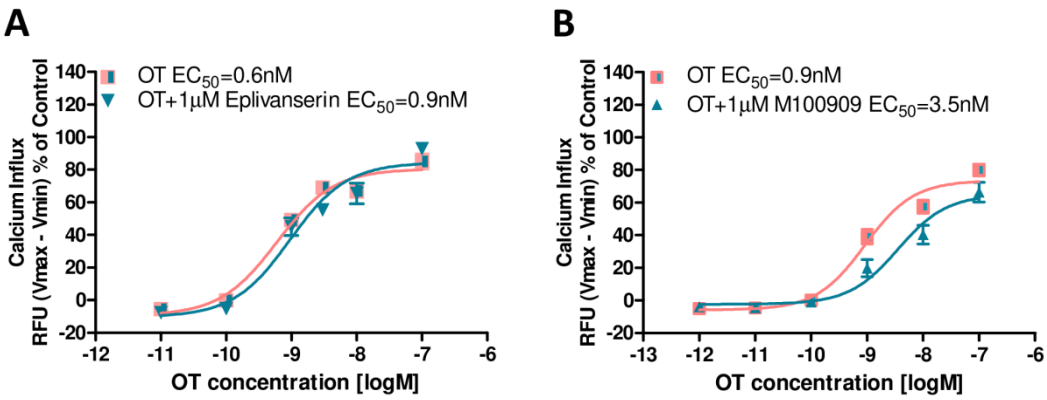

Figure S4.4. 5-HT2A antagonists don't affect the OTR-mediated Gαq-dependent signalling. *Intracellular calcium release induced by increasing concentration of oxytocin and oxytocin in the presence of 5-HT2A antagonists; Eplivanserin (A) and M100909 (B) in cells co-expressing OTR and 5-HT2A. Results are demonstrated as a percentage of maximum calcium response (3% FBS). Graphs represent means ± SEM from three independent experiments run in triplicates.*

Chapter 5

Novel crosstalk of the serotonin 2C and oxytocin receptor attenuates downstream Gαq signalling

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5.1 Abstract

The oxytocinergic and serotonergic systems have been long known to interact and crosstalk with one another. This crosstalk has been shown between the serotonin 2c receptor (5-HT_{2C}) and oxytocin or between the oxytocin receptor (OTR) and serotonin (5-HT), but interestingly an interaction between the OTR and 5-HT_{2C} receptors themselves has remained largely unexplored. Here we show a novel crosstalk and heterocomplex formation between the OTR and 5-HT_{2C} receptors. With the use of functional assays such as calcium mobilization, it is shown that co-expression with 5-HT_{2C} strongly influences OTR-mediated Gαq signalling, such signal being restored upon blockade of the 5-HT_{2C} receptor. Modulation of OTR-mediated downstream signalling subsequently corresponds with altered OTR trafficking following 5-HT_{2C} co-expression, with a further unexpected change to 5-HT_{2C} receptor trafficking also following co-expression, unexpected as there is no change seen to 5-HT_{2C} Gαq signalling. Physical interaction is observed using co-localization and flow cytometry fluorescence resonance energy transfer (fcFRET) experiments, strongly suggesting that the functional crosstalk following OTR and 5-HT_{2C} co-expression is due to the formation of an OTR-5-HT_{2C} heterocomplex. These data reveal an exciting new mechanism linking oxytocinergic and serotonergic systems, which may incur further research leading to much needed novel pharmacotherapies targeting such heterocomplex, due to their role in similar diseases such as autism spectrum disorder.

5.2 Introduction

The OTR and the 5-HT_{2C} receptors are both class A, rhodopsin-like G-protein coupled receptors, which are known to signal through the Gαq alpha subunit (Schellekens et al., 2015a, Takeuchi et al., 2014, Bakos et al., 2018). Upon activation, the Gαq subunit activates phospholipase C which cleaves inositol triphosphate (Billups et al., 2006) leading to the release of calcium from intracellular endoplasmic reticulum stores to the cytoplasm (Kania et al., 2017). In addition, β-arrestin, known to impact GPCR activation and receptor desensitization, has been shown to mediate receptor trafficking of the OTR and 5-HT_{2C} also (Bohn and Schmid, 2010, Grotegut et al., 2011).

Previous studies have shown a link between the oxytocinergic and serotoninergic systems; for example, oxytocin and 5-HT are known to interact to establish social reward and support oxytocin induced social reinforcement (Dolen et al., 2013). Interactions between these systems have been established whereby administration of oxytocin increased 5-HT_{1a} receptor expression in healthy individuals but not in subjects with autism spectrum disorder (ASD) (Lefevre et al., 2017). These results are corroborated by other oxytocin treatment studies in ASD and associated serotonergic alterations, specifically in terms on social behaviour (Hirosawa et al., 2017, Fukai et al., 2017). Likewise, 5-HT-induced social approach in C57BL/6 mice has been shown to be blocked upon OTR antagonism (Arakawa, 2017). Oxytocin is known to diminish stress through its anxiolytic effect (Peters et al., 2014). Antagonism of the 5-HT_{2C} receptor blocks the stress-associated increase in oxytocin secretion (Jorgensen et al., 2002), an effect which may be reversed by agonism of the 5-HT_{2C} receptor, resulting in increased circulating oxytocin levels (Jorgensen et al., 2003b, Jorgensen et al., 2003a).

Interestingly, the OTR and 5-HT_{2C} are both known to form functional GPCR heterocomplexes through physical interaction (Xue et al., 2018, Borroto-Escuela et al., 2014b). Dimerization of receptors can bring about an altered physiological response (Moreno et al., 2011, Schellekens et al., 2015a, Kern et al., 2012) through different mechanisms, namely via modifications of GPCR ligand binding (Goupil et al., 2013), alteration of receptor trafficking (Navarro et al., 2016), and changes associated with G-protein activation (Zhou et al., 2007b, Jiang et al., 2006).

Consequently,, central GPCR heterodimerization is now increasingly being appreciated and investigated for its impact across many centrally-regulated pathways including stress, reward, appetite and for its potential as a novel pharmaceutical target for psychiatric disorders (Borroto-Escuela et al., 2017a, Schellekens et al., 2013b, Moreno et al., 2013, Moreno et al., 2011).

It has been established that both the OTR and 5-HT_{2C} can form functional heterocomplexes with varying GPCRs. In this regard, 5-HT_{2C} dimerization with the ghrelin 1a receptor (GHSR) or melatonin type 2 receptor results in alteration to the Gαq signalling pathways of the heterodimerized pair, revealing 5-HT_{2C} to be regulated by, or *vice versa* be a regulator of, heterodimer-specific signalling (Schellekens et al., 2015a, Kamal et al., 2015). Similarly, the OTR is known to form functional heterodimers with receptors such as the β2-adrenergic receptor, dopamine D2 receptor and the vasopressin 1a & 2 receptors, leading to OTR-mediated signalling changes of secondary messengers ERK1/2, CREB and PLC (Wrzal et al., 2012, Romero-Fernandez et al., 2013, Terrillon et al., 2004, Devost and Zingg, 2004a). Together, this highlights the critical importance to identify and investigate novel heterocomplexes in the brain and their subsequent functional effect.

The established crosstalk between the oxytocin and serotonergic pathways, and the involvement of the OTR and 5-HT_{2C} in modulating similar centrally-regulated disorders (Valencia-Torres et al., 2017, Ott et al., 2013a, Thienel et al., 2016), lead us to hypotheses that the OTR and 5-HT_{2C} may form a functional GPCR heteromer. Such a discovery would lead to better understanding of the intricate signalling between the OTR and 5-HT_{2C}, aiding in the development of improved and more specific pharmacotherapies.

5.3 Materials and methods

5.3.1 Receptors ligands

Functional consequences of the OTR and 5-HT_{2C} co-expression were investigated using multiple receptor ligands. Receptor agonists included oxytocin (#O3251, Sigma-Aldrich), 5-hydroxytryptamine (5-HT) (#H9523, Sigma-Aldrich) and 5-HT_{2C} antagonists SB242084 (#2901, Tocris) and RS102221 (#1050 Tocris). For each a 3 mM stock of compounds were prepared per manufacturer instructions, subsequently compounds were further diluted to the required concentrations in assay buffer.

5.3.2 Cell culture and stable transfection

HEK293a transfected with an unedited 5-HT_{2C} -INI receptor expressing a C-terminally tagged enhanced green fluorescent protein were previously developed in-house as described (Schellekens et al., 2013e, Schellekens et al., 2015a). Cells were maintained in an environment of 5% CO_2 at 37^{o}C, using Dulbecco's modified eagle media (DMEM) (Sigma, D5796) with the addition of 10% fetal bovine serum (FBS) (Sigma, F7524), 1% Non-essential amino acids (NEAA) (Sigma, M7145). To maintain receptor expression 500ng/ml of G418 was added to each flask of cells (Sigma, G418-RO), expression level of OTR and 5-HT_{2C} receptors in were routinely monitored and quantified using flowcytometry (FACSCalibur, BD Biosciences).

5.3.3 Lentiviral transfection and transduction

The OTR sequence (RG211797, Origene) was cloned in to a replication deficient, lentiviral expression vector with a C-terminally tagged red fluorescent protein; pHR-SIN-BX-tRFP. The vector was cloned with the use of the original OTR plasmid lacking its stop codon from the pCMV6-AC-OXTR-GFP construct (RG211797, Origene) into the target lentiviral vector with the use of BamHI and XhoI restriction enzymes. The obtained pHR-SIN-BX-OTR-tRFP plasmid construct was validated by restriction analysis and DNA sequencing. The functional lentiviral construct was packaged through transfection of HEK293T-17 cells using a second-generation lentiviral packaging system as previously described in our lab (Schellekens et al., 2015a, Schellekens et al., 2013e). To co-express both receptors HEK-5-HT_{2C} -EGFP was transduced with the packaged lentiviral pHR-OTR-tRFP, by incubating the pHR-OTR-tRFP with HEK-5-HT_{2C} -EGFP in transduction media containing DMEM with 2% FBS,

1% NEAA, and 8 µg/ml polybrene (H9268, Sigma). The efficiency of transduction was using monitored flow cytometer (FACSCalibur, BD Biosciences) before each experiment to detect the % of cells expressing each tagged receptor.

5.3.4 Flow cytometry based fluorescence resonance energy transfer (fcFRET)

Following transduction cells were washed twice and mechanically removed from the wells. Cell suspension was then centrifuged for 4 min at 200 x g, at room temperature. The pellet of cells was re-suspended in 400µl of 2 nM EDTA (#E5134; Sigma) in phosphate buffered saline PBS. Prior to analysis cells were passed through a 100 µm nylon mesh cell strainer (#10199-658; VWR) and collected in a 5ml round bottom polystyrene tube (#352054; Corning). The fcFRET analysis was performed on a FACS AriaII cytometer (BD Biosciences) according to the published protocol (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. FRET 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 FRET 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 forward and side scattering plot (FSC/SSC). In the next step, cells expressing donor or acceptor construct only 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 (104) was recorded for each sample. Data was analysed using BD FACSDiva (BD Biosciences).

5.3.5 Co-localization

Cells were seeded on poly-L-lysine-coated (P4707; Sigma) 24 well plate containing borosilicate glass discs (631-0150; VWR) at the density of 5 x 105 cells per well. Cells were then fixed to borosilicate discs by incubating cells for 30 minutes with 4% paraformaldehyde. The borosilicate discs were then mounted onto microscope slides; subsequently, co-localization in cells was assessed using confocal microscopy

(FV 1000 Confocal System; Olympus) with 60 x objective lens and Olympus fluoview FV3000 software. Co-localization on a single confocal field was then analysed by overlaying cell images with the use of ImageJ software.

5.3.6 Ligand mediated receptor trafficking

To investigate ligand mediated trafficking of receptors cell were seeded at 5*10^4 cells/well in a 24 well plate (83.3922.005, Sarstedt) for 24 hours. Following this media was changed to serum free maintenance media for 24 hours incubated at 34^{o}C, 5% CO_2. Cells were then treated with corresponding ligands for 1hr followed by treatment with 4% paraformaldehyde to fix cells to the well. Fluorescent microscopy (IX71, Olympus) was used to analyse ligand mediated trafficking. Image J software was used to examine the ratio of perinuclear/ membrane fluorescent intensity, to do so Image J software was used to separately trace the subcellular region and membrane region of the cells. The subcellular region was measured as the fluorescence within the cytosol and perinuclear space, while the membrane region was measured as the outer area lining the cell. The fluorescent intensity values for GFP and for RFP in each area was obtained and used to calculate the ratio of subcellular/membrane fluorescent intensity. GraphPad (Prism 5.0; GraphPad Software Inc.) was used to graph results.

5.3.7 Calcium mobilization assays

Influx of calcium from intracellular stores was analysed using the FLIPR Tetra® automatic fluorescent reader (Molecular Devices) as previously described (Howick et al., 2018b, Howick et al., 2018a, Ramirez et al., 2018). In brief cells were seeded on a black 96-well microtiter plates at a density of 3.0 - 4.0 x 10^4 cells/well for 48hrs. Cells were then incubated for 1.5hrs in calcium 5 dye diluted in assay buffer containing 1 x Hank's Balanced Salt Solution; HBSS (14065049; Gibco Life Technologies) and 20 mM HEPES (H0887; Sigma-Aldrich), in the case of antagonist pre-treatment, antagonists were added to the wells with calcium dye for 1.5hrs. Ligands were then added to wells and calcium influx detected using the FLIPR Tetra® High-Throughput Cellular Screening System (Molecular Devices) . Fluorescent readings were taken for a total of 120 seconds with excitation wavelength of 485 nm and emission wavelength of 525 nm. Increases in intracellular calcium were calculated as the

difference between the maximum and baseline fluorescence where background fluorescence was recorded for non-stimulated cells and subtracted from RFU. The percentage of calcium influx was demonstrated as relative fluorescent units (RFU) normalized to maximum signal obtained for 3% FBS. Results were depicted using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.). 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 EC50 values.

5.3.8 Inositol monophosphate accumulation assay

Inositol monophosphate (IP-One) was detected using the homogeneous time-resolved fluorescence (HTRF) IP-One assay (#62IPAPEB; Cisbio). Ligands to be analysed for inositol monophosphate (IP-One) accumulation were prepared at a 2x concentration in assay buffer containing 50mM lithium chloride (LiCl). A total of 35µl were then added to the corresponding wells of a white, flat bottom, 96 well plate (675074, Greiner Bio-One). Cells of interest were prepared by removing media and washing with PBS, after which the cells were mechanically detached using PBS, centrifuged at 800 rpm and re-suspended in assay buffer (146 mM NaCl, 1 mM CaCl2, 10mM HEPES, 0.5 mM MgCl2, 4.2 mM KCl, 5.5 mM glucose) containing 50 mM LiCl. Cell were added at a concentration of $3.0x10^4$ cells/well (35µl) and incubated for 1hr at 37^oC. Next, 75µl of IP1-d2 acceptor and 75µl of anti- cryptate conjugate donor (62IPAPEB, Cisbio) were added to each well and then incubated for 1 hour at room temperature in the dark. Here IP1-d2 competes with native IP-ONE for binding with the anti- cryptate conjugate. The Flex-station II (Molecular Devices) was used to analyse the accumulation of IP-One, integration settings were adjusted to 400 µs with an integration delay of 50 µs. Excitation was set at 314nm and emission at 620nm and 665nM. The accumulation of IP-ONE was calculated as the HTRF ratio of 665/620*104 and expressed as a percentage of the control (untreated cells). Results were calculated using GraphPad Prism software (PRISM 5.0; GraphPad Software Inc).

5.3.9 Statistical analysis

Statistical analysis of Ca^{2+} mobilization, IP-ONE accumulation and ligand-mediated internalization assays was performed using Prism Software (GraphPad Prism 5.0). All

data is represented as the mean ± SEM. The number of independent experiments is described in each graph legend. D'Agostino & Pearson was used to test for normality. Data, which followed Gaussian distribution, was analysed using Two-way ANOVA with a Bonferroni post-hoc test. A non-parametric Mann Whitney was used for data which did not follow Gaussian distribution. Significance was defined as follows; * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.

5.4 Results

5.4.1 Gαq dependent signalling cross-talk between oxytocin and 5-HT_{2C} receptors

The OTR and 5-HT_{2C} receptors both couple to Gαq proteins following activation. Gαq protein activation generates second messenger D-myo-inositol 1,4,5-triphosphate (IP3), that triggers calcium release within the cell, through the activation of endoplasmic-gated calcium channels. Ligand mediated changes in the canonical signalling pathway of both receptors were assessed by measuring the level of intracellular calcium, with the use of fluorescent dye, in HEK293A cells expressing solely OTR and 5-HT_{2C}, and cells co-expressing both receptors. Here, intracellular calcium influx was measured following the addition of the endogenous receptor ligands, oxytocin and 5-HT, in cells expressing either the OTR, the 5-HT_{2C} or both co-expressed

Interestingly, intracellular calcium release induced by increasing concentration of oxytocin was dramatically and significantly reduced in cells co-expressing 5-HT_{2C}-EGFP with OTR-tRFP, compared to cells solely expressing OTR (Figure 5.1A, $F (12, 232) = 31.72$, $P < 0.0001$). In addition, no significant changes to 5-HT-mediated calcium release in cells co-expressing both receptors were observed, compared to cells solely expressing 5-HT_{2C} receptor (Figure5.1B, $P= 0.1427$).

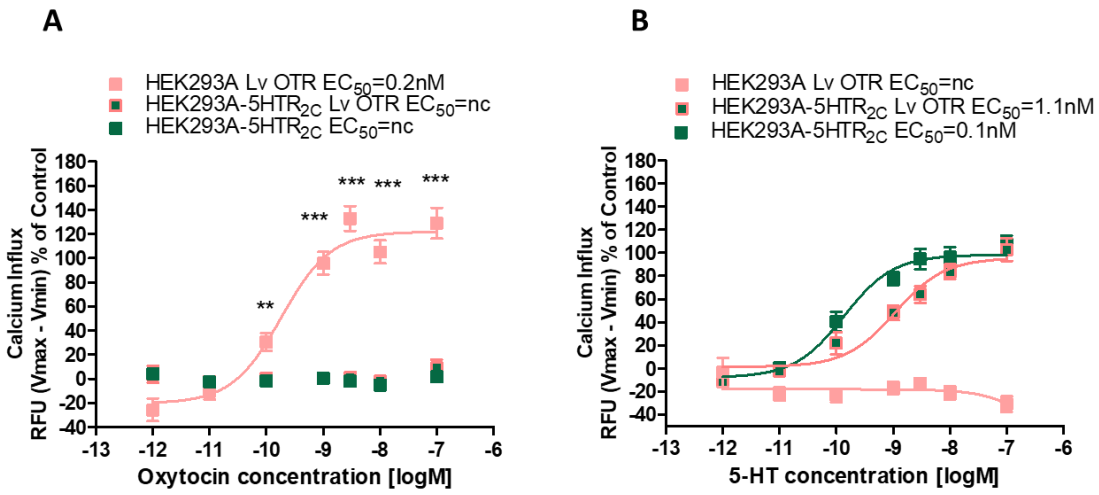

Figure 5.1. Co-expression of OTR and 5-HT$_{2C}$ receptors attenuates OTR-mediated Gαq dependent signalling. *Intracellular calcium release induced by increasing concentration of oxytocin **(A)** and 5-HT **(B)** in HEK293A cells stably expressing the 5-HT2C tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. The data are averages from six independent experiments run in duplicates. Intracellular calcium mobilization is presented as a percentage of maximal calcium response elicited by the control (3% FBS). Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed at each concentration, * indicating p < 0.05; ** indicating p < 0.01; or *** indicating p < 0.001.*

Further experiments were performed to confirm the modulation of downstream signalling when both receptors are co-expressed. Since the transient release of calcium into the cytosol is mediated by IP3 production, ligand mediated changes in the production of this second messenger were also assessed. To this end, homogeneous time resolved fluorescence (HTRF) based IP-One accumulation assay was used. As expected, the oxytocin-mediated IP-One accumulation was significantly decreased in cells that co-express 5-HT$_{2C}$ and OTR, compared to cells expressing only one of the receptors (Figure 5.2A, F (10, 62) = 2.650, P=0.0093). Similarly, to calcium mobilization there was no significant changes to 5-HT-mediated IP-One accumulation (Figure 5.2A, F (5, 28) = 0.07432, P= 0.9956), showing alterations to calcium mobilization are via Gαq subunit activation.

Next, modulation of Gαq dependent signalling was investigated in cells co-expressing the OTR-5-HT$_{2C}$ pair following treatment with 5-HT in combination with three

oxytocin concentrations (1nM, 3nM, or 10nM) (Figure 5.2B). As previously observed no calcium response was observed after treatment with oxytocin in cells co-expressing both receptors. Interestingly, there was no significant differences in calcium release induced by 1 nM 5-HT compared to response following treatment with 1 nM 5-HT in combination with all three concentrations of oxytocin in cells co-expressing both receptors.

For each experiment performed, the level of OTR and 5-HT_{2C} receptors expression in HEK293 cells has been assessed. Flow cytometry analysis of EGFP/tGFP and tRFP showed that transient transduction protocol did not significantly affect the level of 5-HT_{2C} expression in cells co-transduced with OTR compared to non-transduced cells, expressing only 5-HT_{2C} receptor, similar to that seen in Figure 5.4A. Also, the level of OTR-tRFP after transient transduction was the same in cells co-expressing both receptors compared to cells solely expressing OTR-tRFP. Thus, the observed attenuation in OTR mediated Gαq dependent signalling in cells co-expressing OTR and 5-HT_{2C} is not due to the changes in the OTR receptor expression level.

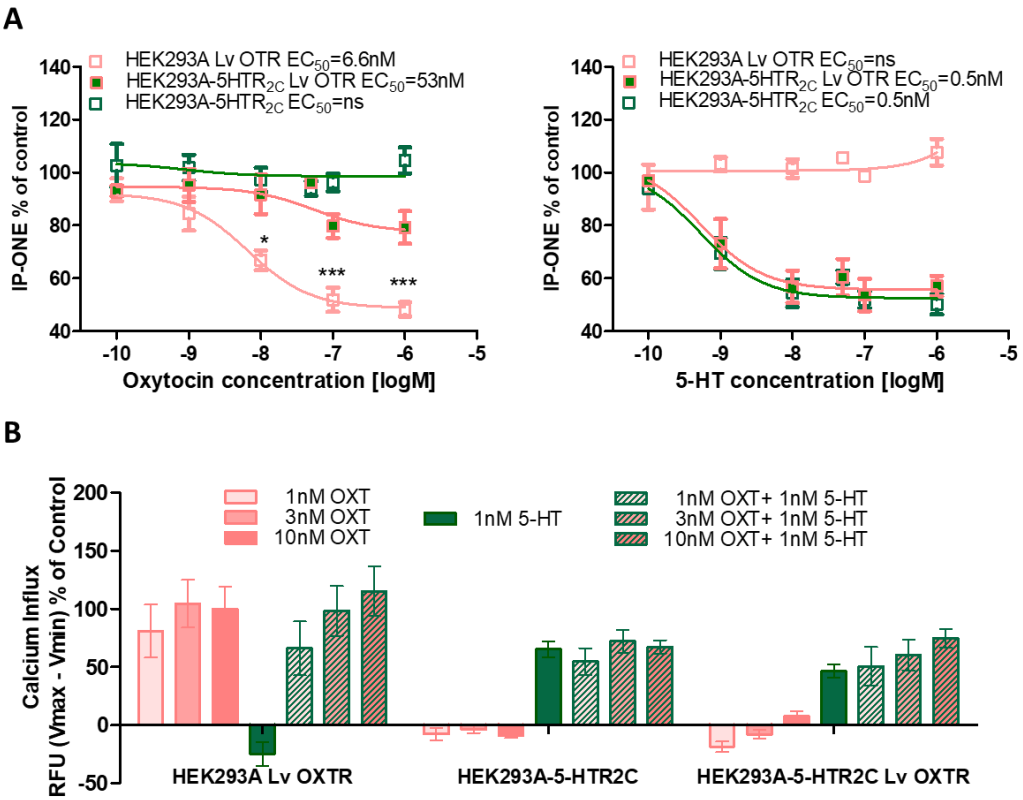

Figure 5.2. Confirmation of the OTR mediated attenuation of Gαq dependent signalling in cells co-expressing OTR and 5-HT$_{2C}$ receptors. ***(A)*** *IP-One production induced by increasing concentration of oxytocin (left graph) and 5-HT (right graph) in HEK293A cells stably expressing 5-HT$_{2C}$ tagged with EGFP, in cells transiently expressing OTR tagged with tRFP, and in cells co-expressing both receptors. Data are averages from two independent experiments run in duplicates presented as a percentage of maximum response.* ***(B)*** *Intracellular calcium release following treatment with 1 nM, 3 nM or 10 nM oxytocin with 1 nM 5-HT or with a combination of both ligands. The data are averages from three independent experiments run in triplicates demonstrated as percentage of maximum calcium response. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed at each concentration, * indicating p < 0.05; ** indicating p < 0.01; or *** indicating p < 0.001.*

Subsequent experiments were performed to investigate whether attenuation of OTR-mediated downstream signalling is dependent of the 5-HT$_{2C}$ receptor activation in cells that co-express 5-HT$_{2C}$ and OTR. To investigate this, oxytocin induced calcium response following blockage of 5-HT$_{2C}$ using its two specific antagonists; RS102221 and SB242084 was measured. As expected, pre-treatment with both antagonists

SB242084 ($F(6, 58) = 28.36$, $P < 0.0001$) and RS102221 ($F(5, 36) = 24.21$, $P < 0.0001$) in 1 µM concentration inhibited 5-HT induced calcium mobilization in cells solely expressing 5-HT_{2C} receptor (Figure 5.3B). However, the attenuation of OTR mediated calcium response in cells that co-express both receptors was restored, following pre-treatment with both 5-HT_{2C} receptor antagonists SB242084 ($F(6, 130) = 17.56$, $P < 0.0001$) and RS102221 ($F(6, 130) = 16.63$, $P < 0.0001$) (Figure 5.3A). The concentration response curves for oxytocin in the presence of SB242084 and RS102221 compounds were characterized by increased potency and fully restored efficacy (Figure 5.3A). Importantly, neither of the 5-HT_{2C} receptor antagonists SB242084 ($F(6, 106) = 0.3657$, $P= 0.8992$) or RS102221 ($F(6, 82) = 0.5110$, $P= 0.7984$) had an effect on oxytocin-dependent calcium response in cells solely expressing oxytocin receptor (Figure 5.3C).

These results strongly support the potential of 5-HT_{2C} receptor to attenuate OTR mediated Gαq dependent signalling, by direct interaction of both receptors co-expressed in cells.

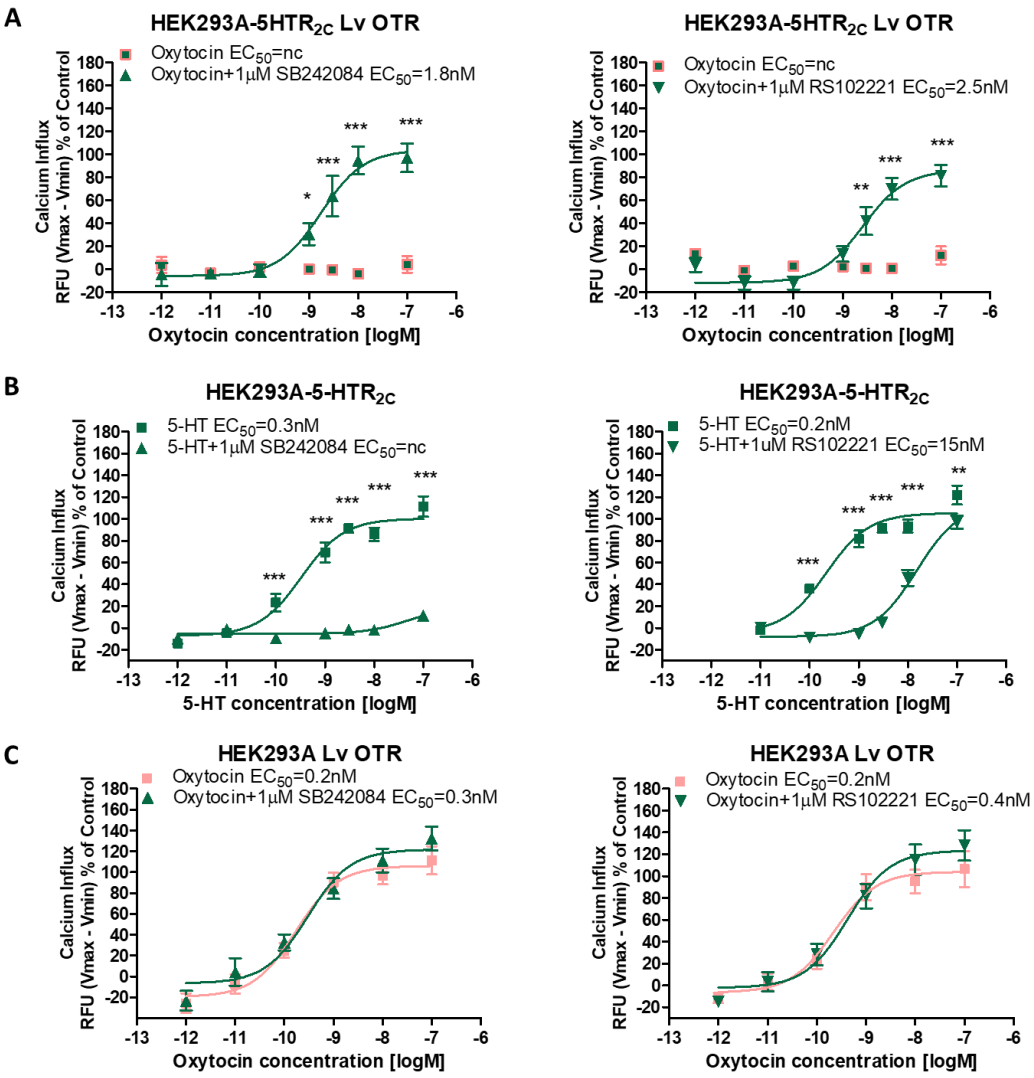

Figure 5.3. OTR mediated Gαq dependent signalling restored when 5-HT$_{2C}$ activity is blocked. ***(A)*** *Intracellular calcium release induced by increasing concentration of oxytocin alone and in the presence of 5-HT$_{2C}$ antagonists; SB242084 (left graph) and RS102221 (right graph) in cells co-expressing OTR and 5-HT$_{2C}$.* ***(B)*** *Intracellular calcium release induced by increasing concentration of 5-HT alone and in the presence of 5-HT$_{2C}$ antagonists in cells solely expressing 5-HT$_{2C}$.* ***(C)*** *Intracellular calcium release induced by increasing concentration of oxytocin alone and in the presence of 5-HT$_{2C}$ receptor antagonists in cells solely expressing OTR. The data are average from six independent experiments run in duplicates demonstrated as percentage of maximum calcium response. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed at each concentration, * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$.*

5.4.2 OTR and 5-HT_{2C} receptors form a constitutive heterocomplex

The interaction between oxytocin and 5-HT_{2C} receptors was assessed in HEK293A cells using flow cytometry-based FRET (fcFRET). Single cells expressing donor or acceptor construct, were used to perform the proper compensation for spectral bleed through and cross-excitation, in particular for EGFP emission in the tRFP-FRET detector and tRFP emission caused by excitation of tRFP from the blue laser. Cells with co-expression of the donor construct (5-HT_{2C}-EGFP) with the control acceptor construct (Lv-tRFP) were used for quantification of nonspecific FRET signal. FRET signal between 5-HT_{2C}-EGFP and OTR-tRFP (19.1%) highlighted constitutive and specific association between both receptors. The specificity of interaction was confirmed by weak FRET signal (2.7%) in cells co-expressing 5-HT2C-EGFP with control construct containing tRFP sequence (Lv-tRFP) (Figure 5.4B). Furthermore, FRET signal analysed as median fluorescence was significantly higher in cells co-expressing both receptors (118) compared to cells with the expression of donor, acceptor construct, and cells co-expressing 5-HT2C-EGFP with control construct (Lv-tRFP) (15) (Figure 5.4C).

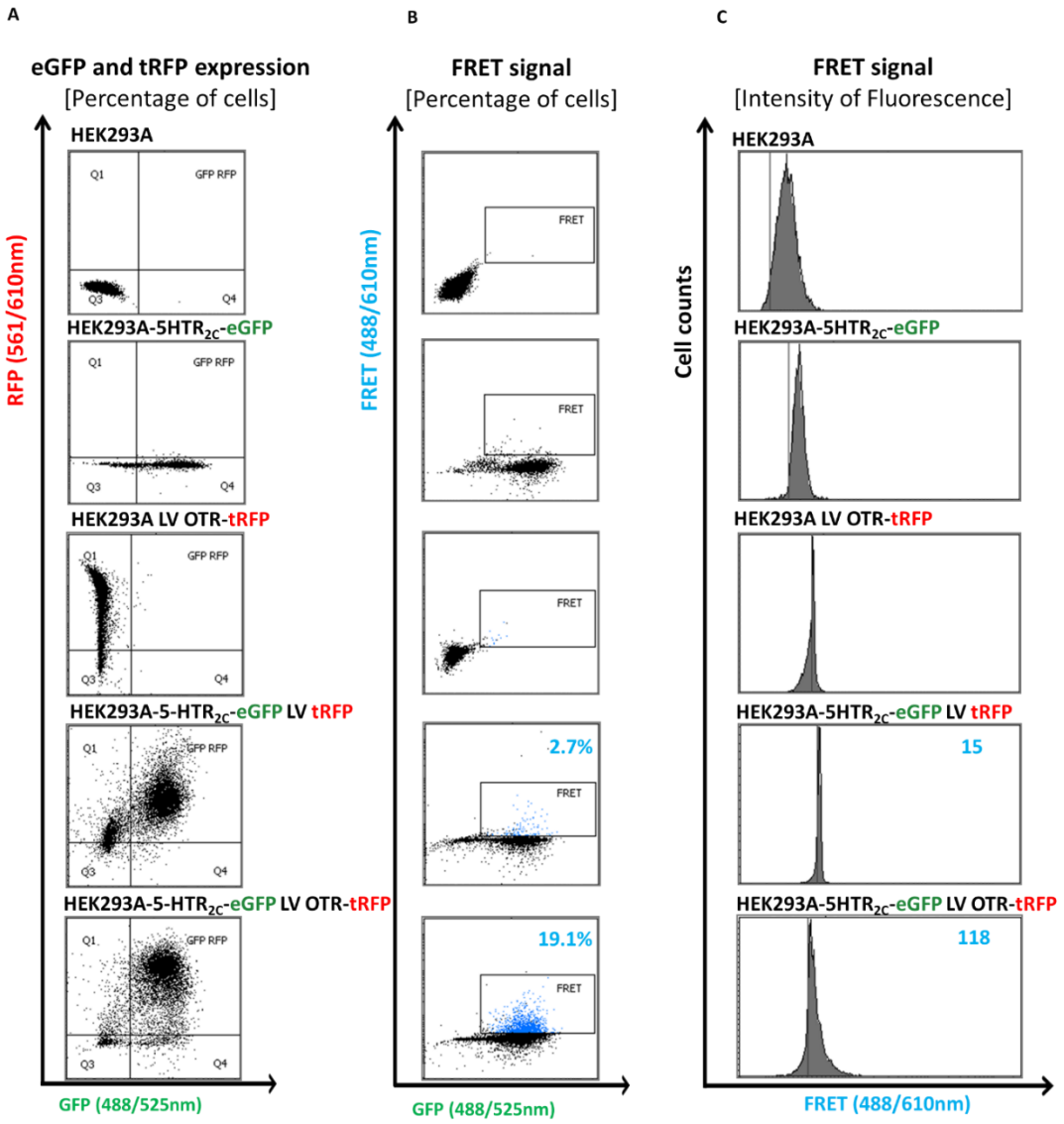

Figure 5.4. FRET between 5-HT$_{2C}$ and OTR. *HEK293A cells stably expressing the 5-HT$_{2C}$ receptor tagged with EGFP (donor) were transiently transduced with lentiviral vector expressing oxytocin receptor tagged with tRFP (acceptor) and with the control-tRFP vector. Dot plots and histograms are representative of three independent experiments. Panel A shows percentage of cells with tRFP expression (tRFP vs EGFP plots). Panel B demonstrates percentage of cells with FRET signal (FRET vs EGFP plots). Panel C demonstrates median fluorescence of FRET signal (FRET vs EGFP histogram).*

5.4.3 OTR and 5-HT_{2C} colocalize within the cell

Localization of heterodimers was shown using confocal microscope in HEK293A cells co-expressing OTR and 5-HT_{2C}. Both receptors were mainly found within the cell membrane, which was shown by the green fluorescence signal coming from 5-HT_{2C} fused with EGFP and the red fluorescence signal coming from OTR fused with tRFP (Figure 5.5). Overlap between green and red fluorescence is indicated as yellow signal (Figure 5.5, merge picture) and demonstrates colocalization of both receptors on the cell membrane within a single confocal plane, confirming also fcFRET results.

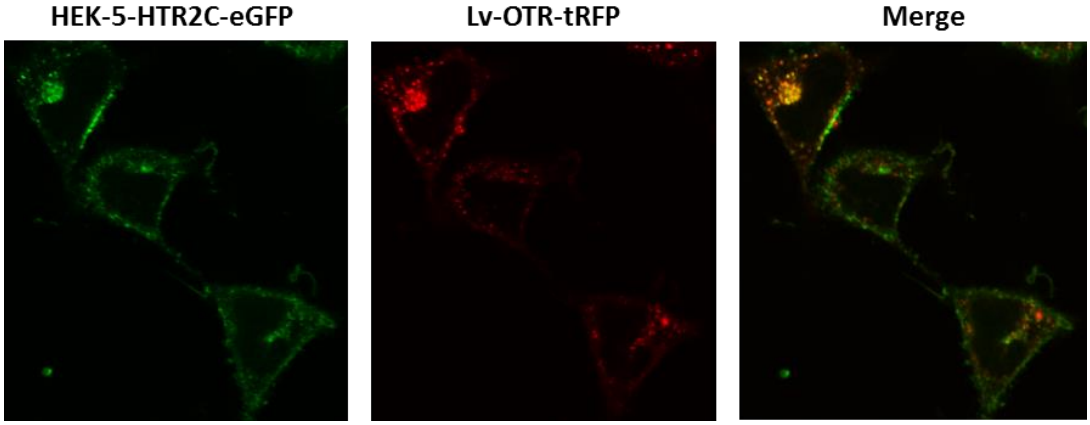

Figure 5.5. Colocalization between 5-HT_{2C} and OTR. Images taken using 60x magnification show *HEK293A cells stably expressing the 5-HT_{2C} receptor tagged with EGFP (green) were transiently transduced with lentiviral vector expressing OTR tagged with tRFP (red). Merge picture (yellow) show colocalization of the receptors mainly on the cell membrane.*

5.4.4 5-HT_{2C} receptor blocks ligand-mediated OTR trafficking

Next, the effects of the ligands on receptor trafficking and internalization into endosome, a characteristic of receptor desensitisation, was examined. Both, desensitisation and internalisation processes provide crucial physiological feedback mechanism that protect against overstimulation of receptors (Ritter and Hall, 2009). Increased basal activity of OTR was observed when both receptors were co-expressed, interestingly, this activity was restored to its monomeric activity upon treatment with 5-HT_{2C} receptor antagonist SB242084 (Figure 5. 6A, F (1, 416) = 13.04, < 0.0001). As anticipated 5-HT treatment mimicked the results seen in control conditions, meaning 5-HT had no effect on OTR trafficking alone (P<0.001), though pre-treatment with 5-HT_{2C} antagonist SB242084 began to restore OTR co-expressed

with 5-HT_{2C} receptor to its monomeric basal trafficking pattern (< 0.0001) (Figure 5. 6A). Indeed, this result corresponds with the Gαq signalling restoration seen in Figure 5. 3. Similarly, an increase in the basal activity of the 5-HT_{2C} receptor trafficking upon co-expression was seen across control and oxytocin treatment groups, indicating OTR co-expression has an effect on 5-HT_{2C} trafficking (Figure 5. 6B, F (7, 416) = 14.67, < 0.0001). Interestingly, 5-HT_{2C} receptor co-expressed with OTR resulted in reduced trafficking upon treatment with 5-HT compared to 5-HT_{2C} expressed alone (P<0.001) (Figure 5. 6B). There was a trend towards restoration of 5-HT_{2C} monomeric trafficking following SB242084 treatment with a significant decrease in activity observed upon 5-HT treatment (P > 0.05) (Figure 5. 6B).

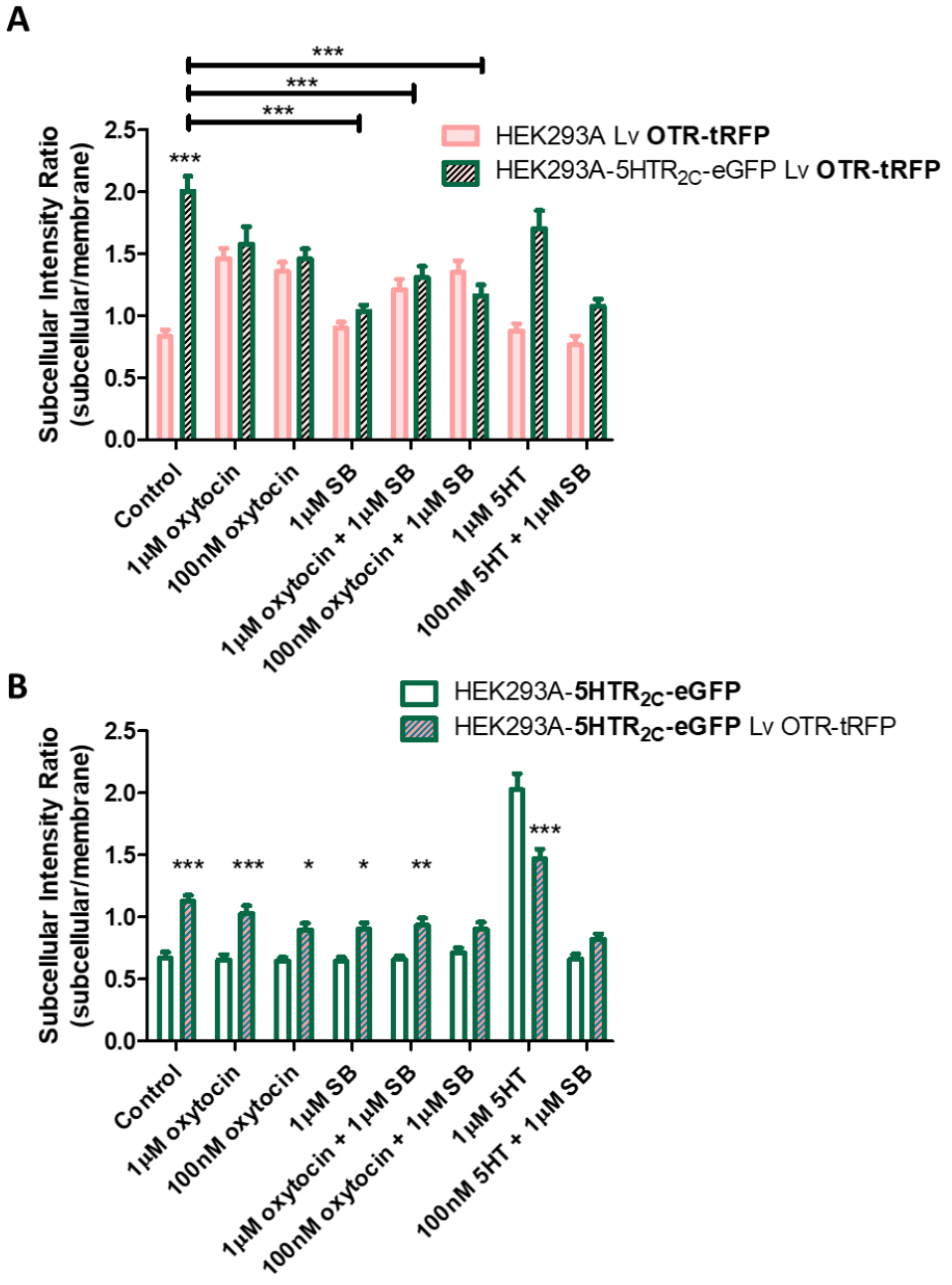

Figure 5.6: Receptor co-expression leads to altered OTR and 5-HT$_{2C}$ receptor trafficking. (A) *OTR alone versus co-expressed with 5-HT$_{2C}$ results increased basal OTR trafficking, with significant reduction following SB242084 treatment.* ***(B)*** *Following co-expression 5-HT$_{2C}$ basal activity increased in the presence of OTR, with a decrease is trafficking following 5-HT treatment. Data is representative of 3 independent experiments with each treatment in duplicate per experiment. Results are presented as ±SEM with statistical significance compared between cells with receptor alone versus receptors co-expressed or between treatment groups, * indicating $p < 0.05$; ** indicating $p < 0.01$; or *** indicating $p < 0.001$*

5.5 Discussion

It is well established that the formation of GPCR heterocomplexes can have drastic effects on the downstream signalling pathways of associated receptors (Scarlett et al., 2018, Siddiquee et al., 2013a, Schellekens et al., 2015a). The alterations to these downstream signalling pathways can have major physiological implications in the pathophysiology of many diseases, such as obesity and autism (Schellekens et al., 2013b, Bologna et al., 2017, Nagano et al., 2018) and, therefore, in their pharmacological treatment strategies. Therefore, these data identify a novel GPCR-GPCR heterocomplex between the OTR and 5-HT_{2C} receptors, with associated effect on their signalling pathways, which may have implications in such disease.

Here, a novel OTR-5-HT_{2C} heterocomplex has been identified with consequent effects to the Gαq signalling pathways of the OTR. It was observed, by the use of calcium mobilization assays, that oxytocin-mediated calcium influx was completely blunted upon co-expression with the 5-HT_{2C} receptor. This effect was solely seen for the OTR, as upon 5-HT treatment there was no observable alteration to 5-HT_{2C}-mediated calcium influx. As IP-ONE is a prerequisite to calcium influx (Tuteja, 2009a), subsequent IP-ONE assays were performed to ensure that this effect was mediated via the Gαq subunit. Again, a significant reduction in oxytocin-mediated IP-ONE accumulation was observed upon co-expression of OTR and 5-HT_{2C} receptor, with no corresponding alteration to 5-HT-mediated IP-ONE accumulation. Interestingly, this 5-HT_{2C} dominated effect in blunting GPCR Gαq signalling has been previously observed across 5-HT receptor subtypes where the 5-HT_{2C} receptor has been shown to blunt the calcium influx associated with 5-HT_{2A} and 5-HT_{2B} and fully attenuate GHSR signalling. (Moutkine et al., 2017, Schellekens et al., 2013e, Schellekens et al., 2015a). Due to the synergy sometimes observed between GPCR heterocomplexes (Ciruela et al., 2001, Gomes et al., 2000), the effect of co-administration of oxytocin and 5-HT was analysed for a synergistic effect. No change in Gαq signalling was observed upon treatment co-administration, indicating no synergistic effect between these receptors. The reduction in OTR-mediated Gαq signalling by the 5-HT_{2C} receptor can be contributed to the altered trafficking of these receptors observed in Figure 6A, namely an increase in basal OTR trafficking was observed, indicating

reduced expression of the OTR on the cell membrane, possibly contributing to the altered downstream signalling of the OTR. Contrastingly, 5-HT_{2C} receptor signalling was also increased under basal conditions but decreased in cells co-expressing OTR and 5-HT_{2C} receptor following 5-HT treatment. As no changes were seen in the Gαq signalling pathway of the 5-HT_{2C}, it can be inferred that prolonged Gαq signalling or biased signalling of 5-HT_{2C} is associated with OTR co-expression, both of which have been previously associated with GPCR trafficking (Pavlos and Friedman, 2017). Such a result warrants future investigation into further downstream functional implications associated with OTR-5-HT_{2C} co-expression.

The crosstalk between the OTR and 5-HT_{2C} receptor can be contributed to the formation of an OTR-5-HT_{2C} heterocomplex. Using confocal microscopy, co-localization of OTR and 5-HT_{2C} receptor can be seen on a single confocal plane of HEK293 cells; indicating the physical interaction of these receptors. Flow cytometry-based FRET further supported this interaction where, upon co-expression a 19.1% FRET signal was observed in cells co-expressing both receptors compared to the control condition which gave a signal of 2.7%. These data indicate that the reduced OTR-mediated Gαq signalling associated with OTR and 5-HT_{2C} co-expression is due to a heterocomplex of these receptors. It has been established that the frequency of formation of these GPCR complexes can be influenced by receptor ligands, and that ligands can affect the downstream signalling of the opposing receptor (Haack and McCarty, 2011, Hübner et al., 2016, Schelshorn et al., 2012). In addition, our lab as previously shown that antagonism of the 5-HT_{2C} reversed the attenuated signalling of GHSR associated with GHSR-5-HT_{2C} heterodimerization (Schellekens et al., 2015a). Therefore, in an attempt to restore OTR signalling, 5-HT_{2C} receptor antagonists were utilised and it was observed that blockade of the 5-HT_{2C} receptor restored OTR signalling associated with OTR-5-HT_{2C} co-expression. It can be hypothesised that this blockade resulted in an uncoupling of this OTR-5-HT_{2C} heterocomplex, consequential in the restoration of OTR to its monomeric state, though further experimentation would be required to prove this hypothesis.

These findings have the potential to be instrumental in the full understanding of OTR and 5-HT_{2C} signalling pathways due to the increased discovery of beneficial GPCR

bivalent ligands (Glass et al., 2016, Arnatt et al., 2016, Arnatt and Zhang, 2014) and the link of these receptors to disorders associated with mood, social behaviour and appetite. Fully understanding the extent of these pathways will lead to more selective, pharmacological strategies for the treatment of such disorders.

Chapter 6

General discussion and conclusion

6.1 Overall summary of findings

Understanding the full extent of GPCR signalling is vital for the design and function of pharmacotherapies that utilise GPCR signalling pathways to initiate their beneficial effect. Additionally, GPCR signalling pathways are known to be involved in a wide array of centrally-regulated disorders such as autism, depression and obesity (Wang et al., 2013, Guiard and Di Giovanni, 2015, Huang et al., 2018). While GPCRs are mainly described as functioning monomers, the formation of GPCR heterocomplexes is increasingly being recognised and has important consequences for the discovery and development of GPCR-based pharmaceutical targets as heteromerization is associated with altered GPCR signalling (Qian et al., 2018, Lensing et al., 2017). In this thesis we aim to uncover novel oxytocin receptor (OTR) heterocomplexes which may impact on the downstream signalling of the OTR.

In this thesis a multifunctional role of the OTR in GPCR signalling is presented, revealing the OTR as a key heteromerization hub of molecular connectivity within the GPCR network. We show novel heterocomplexes of the OTR with either the ghrelin receptor (GHSR) **(*Chapter 2*)**, glucagon like peptide 1 receptor (GLP-1R) **(*Chapter 3*)**, serotonin 2a receptor (5-HT_{2A}) **(*Chapter 4*)**, or serotonin 2c receptor (5-HT_{2C}) **(*Chapter 5*)**. Interestingly, each of the four OTR heterocomplexes studied resulted in unique signalling pathways as depicted in Figure 6.1. Using *in-vitro* co-localization and flow cytometry-based FRET it was shown that heterocomplexes formed from the OTR with either the ghrelin receptor (GHSR), glucagon like peptide 1 receptor (GLP-1R), serotonin 2a receptor (5-HT_{2A}), or serotonin 2c receptor (5-HT_{2C}) caused different cellular expression patterns, highlighting the variances between these dimer pairs. In addition, formation of a heterocomplex of the OTR with the GHSR and GLP-1R was shown in *ex-vivo* primary cultures of the hippocampus and hypothalamus from neonatal rat pups. Moreover, proximity ligation assays (PLA) showed that the OTR formed a heterocomplex with the 5-HT_{2A} receptors in areas of the hippocampus, cingulate cortex, nucleus accumbens and striatum. Each pair of heterodimers revealed novel functional consequences to receptor downstream signalling, which may be relevant to its physiological function throughout the brain.

Firstly, ***Chapter 2*** focused on the coupling and subsequent activation of Gαq and β-arrestin proteins as shown in Figure 6.1A, showing that the GHSR modulated the ability of OTR to activate the Gαq signalling pathway upon co-expression, reducing the potency and efficacy of oxytocin. Increased trafficking, revealed through ligand mediated co-trafficking assays, showed the OTR to be co-internalised upon GH treatment only when co-expressed with the GHSR. As reduced OTR was observed on the cell membrane, this may be a contributing factor towards the attenuation of OTR-mediated Gαq signalling.

Chapter 3 identified the role of the OTR and GLP-1r heterocomplex in the accumulation of cAMP, influx of calcium from intracellular stores, and receptor trafficking (Figure 6.1B). The OTR was shown to mediate GLP-1r specifically through the Gαq signalling pathway, blunting its ability to influence calcium influx, while no influence of the OTR-GLP-1r heterocomplex was seen on cAMP accumulation. Moreover, trafficking of the GLP-1r is reduced, in agreement with current literature associated with GLP-1r heterodimerization (Whitaker et al., 2012).

Formation of an OTR and 5-HT_{2A} receptor heterocomplex was shown in ***Chapter 4***, which revealed functional alterations to both the OTR and 5-HT_{2A} receptor Gαq signalling pathway (Figure 6.1C) Indeed, co-expression of the OTR/5-HT2A pair caused a significant attenuation of both calcium influx and IP-ONE accumulation. Resulting in significant reduction in potency of 5-HT for 5-HT_{2A} receptor and a significant attenuation of potency and efficacy of the OTR agonists, oxytocin, carbetocin and WAY. This reduction can be linked to the increased basal trafficking of both receptors, where expression of the receptors on the membrane is reduced when the OTR and 5-HT_{2A} receptor are co-expressed.

Finally, ***Chapter 5*** investigated the effect of an OTR and 5-HT_{2C} receptor heterocomplex on the Gαq signalling and β-arrestin pathways (Figure 6.1D). Co-expression of 5-HT_{2C} receptor and the OTR results in full blunting of OTR Gαq signalling, but intriguingly blockade of the 5-HT_{2C} receptor with its corresponding antagonist resulted in a full restoration of OTR Gαq signalling, indicating a ligand-mediated effect on this heterocomplex formation. Results from co-trafficking assays match those seen in calcium mobilization assays, where increases in receptor

trafficking corresponds to the blunting of the OTR Gαq signalling when 5-HT_{2C} receptor and OTR are co-expressed. Moreover, antagonism of the 5-HT_{2C} receptor restores OTR trafficking comparable to the OTR expressed alone, corresponding to the restoration seen in the OTR Gαq signalling pathway.

In conclusion, we have demonstrated compelling evidence for formation of novel OTR heteromers with significant downstream signalling consequences. The OTR receptor firmly places itself as a novel hub of molecular connectivity within the GPCR network and the data represented in this thesis, highlighting the functional diversity associated with GPCR heterocomplexes overall. Further investigations into the functional consequences of OTR heteromers are now warranted and the role of OTR heteromers in pathologies involving oxytocinergic signalling need to be considered as they are poised to significantly impact on the future development of novel OTR-based pharmacotherapies.

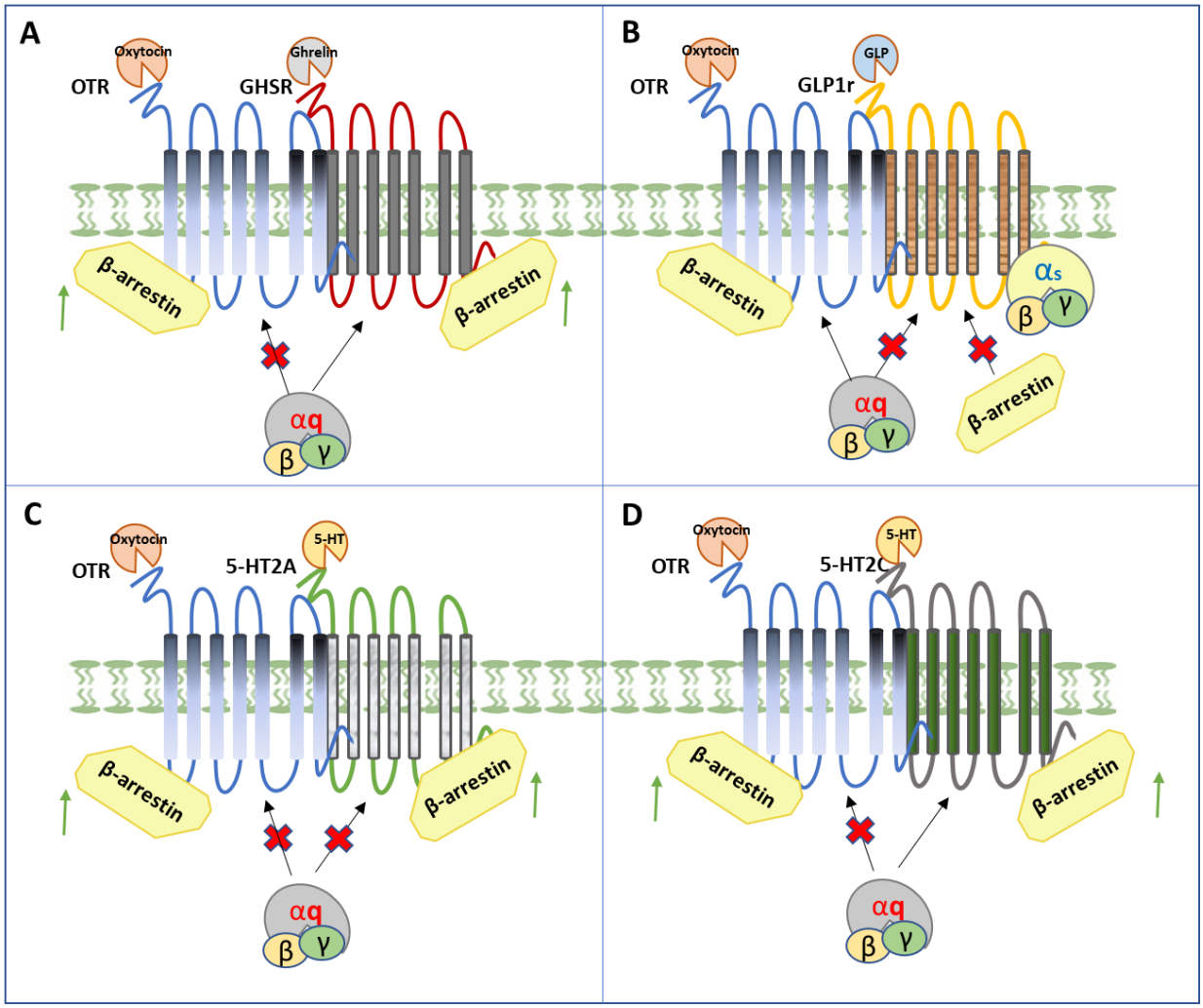

Figure 6.1: Schematic illustration highlighting the main results of Chapters 2 through 5. (A) *Physical interaction of the OTR and GHSR results in increased receptor trafficking and attenuation of OTR-mediated Gαq signalling.* ***(B)*** *OTR and GLP-1R co-expression leads to reduced GLP-1R ligand-mediated trafficking and reduction in GLP-1R-mediated Gαq signalling.* ***(C)*** *An OTR-5-HT$_{2A}$ heterocomplex results in attenuation of both OTR and 5-HT$_{2A}$ Gαq signalling, increasing basal receptor trafficking.* ***(D)*** *Co-expression of OTR and 5-HT$_{2C}$ results in increased basal trafficking of both receptor with subsequent blunting of OTR-mediated Gαq signalling.*

6.2 G-protein coupled receptor heterodimerization-functionally relevant or pharmacological epiphenomenon?

The concept of GPCR heterodimerization has been present for over two decades; with GPCR heterodimerization now being associated with many centrally-regulated disorders (Moreno et al., 2013, Borroto-Escuela et al., 2017a). Originally much debate was held to whether GPCR heterodimerization was indeed a functional feature of GPCRs or just a by-product of experimental design due to receptor over-expression in an *in-vitro* system. Due to the advances in GPCR heterocomplex methodology, it has now become clear that these functional alterations are undoubtedly due to the physical interaction and crosstalk between these receptors, with each complex resulting in unique alterations to GPCR signalling.

As discussed in section 6.1, each oxytocin receptor heterocomplex pair studied in this thesis results in novel signalling events only seen when receptors are co-expressed compared to receptors expressed alone. For example, in ***Chapter 2*** we showed an attenuation in OTR-mediated Gαq signalling with no alterations to the GHSR, accompanied by increased receptor trafficking of both the OTR and GHSR, indicating increased β-arrestin binding. It was shown that this effect is heterodimerization-specific for this pair as in ***Chapter 3*** this effect on OTR signalling is not observed. In its place we saw an attenuation of GLP-1R-mediated Gαq signalling. In comparison, the GLP-1R trafficking is significantly reduced with no significant changes to OTR trafficking. These findings are in line with many currently published GPCR heterodimer papers that reveal differential signalling between each GPCR dimer pair (Scarlett et al., 2018, Xue et al., 2018). For example, a histamine H3 and adenosine

A2A receptor heterodimer reduces both histamine H3 receptor Gαi signalling and A2A receptor ligand binding in the striatum when receptors are co-expressed (Marquez-Gomez et al., 2018). The angiotensin type 1A receptor and β2-adrenergic receptor heterodimer enhance β-arrestin recruitment and cAMP accumulation of the β2-adrenergic receptor upon ligand stimulation of both receptors (Toth et al., 2017). In contrast, co-stimulation of the orexin receptor 1 and cholecystokinin A receptor heterodimer results in reduced signalling of the Gα subunits including Gαq, Gi, Gα12 and Gα13 (Bai et al., 2017).

Although the many different GPCR heterocomplex pairs now discovered are known to signal in novel ways as described above, a trend in signalling alterations can often be observed when following the literature history of specific GPCRs in there heterocomplex forms. As previously described the GLP-1R results in reduced Gαq-dependent signalling with no accompanying alterations to OTR-mediated signalling. Interestingly, this Gαq-dependent decrease is observed when GLP-1R is heterodimerized with the gastric inhibitory protein receptor (GIPR), with no changes to the GIPR Gαq signalling (Schelshorn et al., 2012). Similarly, increased receptor membrane expression is shown when the GLP-1R is co-expressed with the glucose-dependent insulinotropic polypeptide receptor, an effect comparable to what is seen when the OTR and GLP-1R are co-expressed (Whitaker et al., 2012). Likewise, related characteristics are seen across 5-HT_{2C} receptor heterodimer studies. ***Chapter 5*** shows the dominating effect of the 5-HT_{2C} receptor and its ability to fully blunt OTR-mediated Gαq signalling. This dominant 5-HT_{2C} receptor effect is also seen upon its heterodimerization with the 5-HT_{2A}, 5-HT_{2B} receptors and GHSR (Moutkine et al., 2017, Schellekens et al., 2013e). Additionally, the ability to restore OTR signalling through antagonism of 5-HT_{2C} is again observed in the heterodimerization of 5-HT_{2C} receptor and GHSR where, upon 5-HT_{2C} receptor antagonism, the GHSR Gαq signal is restored (Schellekens et al., 2015a). Although these characteristics are not seen in all dimer pairs, such as heterodimerization of 5-HT_{2C} receptor and the melatonin MT2 receptor which results in increased Gαq signalling of both receptors with no effect of 5-HT_{2C} receptor antagonism (Kamal et al., 2015).

Again, this shows the differential signalling between each specific heterocomplex pair and its importance in GPCR signalling, further emphasising the gap in current knowledge and the requirement for further GPCR heterocomplex studies to allow for a fuller understanding of this GPCR pharmacology. This thesis has contributed to expanding our current knowledge of the OTR signalling and its implications in GPCR heterocomplex formation, hopefully aiding in the development of more specific pharmacotherapies.

6.3 GPCR heterocomplexes as a new pharmacological target

The concept of GPCR dimerization adds another layer to the ability of GPCRs to regulate physiological function. This is evident through the changes in downstream signalling associated with GPCR dimerization as described above which may aid in identifying potential future pharmacotherapies. Dimerization has already been implicated in disorders and diseases associated with the periphery, such as the heterodimerization of C-X-C chemokine receptor type 4 and cannabinoid receptor type 2 and its resulting effect on tumour migration associated with altered changes to the Gα13/RhoA signalling pathway (Scarlett et al., 2018). Heterodimerization of beta-adrenergic receptor subtypes 1 & 2 is implicated in correct functioning of the beta-adrenergic pathway in cardiac contractility (Zhu et al., 2005).

The knowledge of the central physiological impacts of GPCR dimerization, such as its effect in neuropsychiatric disorders, is now on the rise (Moreno et al., 2013). Interestingly, clozapine and haloperidol, antipsychotics used in the treatment of schizophrenia are seen to significantly increase and decrease the levels of dopamine D2 receptor-5-HT1a and 5-HT1a-5-HT_{2A} receptor heterodimerization respectively in the mouse cortex (Szlachta et al., 2018). These variations may explain the increased side effects associated with haloperidol treatment in comparison to clozapine (Rosenheck et al., 1997). Moreover, heterodimerization of the OTR and dopamine D2 receptor modulates OTR anxiolytic effects, whereby dopamine D2 receptor antagonism blocks OTR-mediated response (de la Mora et al., 2016). Similarly, GHSR and 5-HT_{2C} receptor heterodimerization blocks the GHSR-mediated orexigenic effect, whereby blockade and uncoupling of the GHSR-5-HT_{2C} complex increases food intake in mice (Schellekens et al., 2015a).

The expression patterns of such heterocomplexes in different brain regions can also be associated with many central functions, such as the association of increased 5-HT1a and CB1 receptor heterodimerization with cognitive decline following cannabis use (Galindo et al., 2018). Similarly, in post mortem samples from schizophrenic patients with the mGluR2-5-HT_{2A} psychosis-associated heterodimer is reduced relative to healthy controls, implicating it in the onset of psychosis in schizophrenia (Moreno et al., 2013, Gonzalez-Maeso et al., 2008). This study revealed GPCR heterodimerization to be a viable target when treating such disorders as antagonism of the mGlu2 receptor resulted in reduction of 5-HT_{2A} receptor-associated psychosis (Gonzalez-Maeso et al., 2008). Additionally, antagonism of the 5-HT_{2C} receptor is associated with an increase in GHSR activity and subsequent food intake (Schellekens et al., 2015a). Thus, by targeting this heterocomplex a viable treatment for cachexia may be generated. Moreover, it may further explain the weight gain associated with many antipsychotic treatments. However, much further in vivo validation of this would be required to confirm such hypotheses.

Such data highlights the importance of the current heterocomplex findings of this thesis. The activity of these GPCRs across multiple brain regions, behaviours and disorders, suggests future *in-vivo* studies would be worthwhile to further elucidate their function. Co-localization of these GPCR heterocomplexes shown *ex-vivo* reveals the possible physiological implication of such heterocomplex pairs. The OTR-GHSR heterocomplex co-localization within hypothalamic and hippocampal primary culture shown ***Chapter 2*** is an example of such a possible function where reduced OTR signalling within areas of the hippocampus may impact on OTR-mediated hippocampal neurogenesis (Lin et al., 2017b). Furthermore, dysfunction to OTR signalling within the hypothalamus can increase the likelihood of obesity (Blevins et al., 2016). Similarly, the OTR-GLP-1R heterocomplex, which results in decreased GLP-1R mediated Gαq signalling as seen in ***Chapter 3***, can have major implications in areas such as hippocampal-driven memory and learning (Hansen et al., 2015) and hypothalamic implications such as type 2 diabetes (Ten Kulve et al., 2016). **Chapter 4**, revealed expression of the 5-HT2A-OTR heterocomplex in the CA2 and CA3 regions

of the hippocampus areas involved in OTR dependent social recognition and memory (Raam et al., 2017a).

Therefore, the vast expression of these heterodimer pairs across different brain regions further highlights the complexity of GPCR heterocomplex formation and its cross-functional role within centrally-regulated physiological functions.

6.4 OTR heterocomplexes as pharmaceutical targets- what's next?

Although a vast amount of data has been shown throughout this thesis, expanding our knowledge in the different signalling pathways of the OTR, it leads to many still unanswered questions.

Functional changes

The initial step would be to identify other functional alterations associated with the formation of these heterocomplex pairs. Other studies of the GHSR have highlighted that it can be highly biased in its signalling, with different agonist treatments resulting in the activation of different G-proteins (Ramirez et al., 2018). This bias is similarly seen with GHSR heterodimerization where a GHSR-ghrelin 1B receptor heterodimer increases the GHSR ability to bind to the Gαi alpha subunit, but the GHSR-orexin 1 receptor heterodimer results in increased coupling of the Gαs subunit (Leung et al., 2007, Xue et al., 2018). This may indicate the possible reduction in receptor signalling seen throughout this thesis (i.e. OTR-GHSR heterodimer whereby a reduction in the OTR Gαq signalling was observed) suggesting possible enhanced binding of a secondary biased G protein such as the Gαi alpha subunit which the OTR is also known to signal through (Zhou et al., 2007b). Although trafficking results such as the enhanced OTR internalization seen upon OTR-GHSR heterodimerization (***Chapter 2***) may indicate favoured binding of β-arrestin.

Ligand binding and heterocomplex conformation

Another area of future investigation is changes to ligand binding, a common feature of GPCR heterodimerization which can result in alterations to downstream signalling or regulate the formation of the heterodimer itself (Siddiquee et al., 2013a, Schellekens et al., 2015a). Although the main obstacle to such studies is the use of

radioactive ligands (Zhang and Xie, 2012) which require special licences which many universities and institutions no longer hold. Moreover, current commercial assays for non-radioactive GPCR ligand binding assays are not intended to investigate more than one receptor and are therefore not suitable for heterodimerization studies. Finally, for functionality another potential study is investigation of higher order complexes formed by these receptors. As has been shown in this thesis, the OTR forms functional dimers with the GHSR and 5-HT_{2C} receptor (***Chapter 2 & Chapter 5***) and it has previously been shown in our lab that the GHSR and 5-HT_{2C} form a functional heterodimer (Schellekens et al., 2015a, Schellekens et al., 2013e). It can, therefore, be hypothesised that the OTR, GHSR and 5-HT_{2C} receptor may form a functional GPCR trimer.

Physiological implications

A gap in the physiological relevance of many receptors remains, with a limited number of *in-vivo* heterodimerization studies published to date compared to the number of identified heterocomplexes. Most *in-vivo* studies are conducted on heterodimer pairs where a mechanism for restoration of receptors to their monomeric signalling state has been identified (Schellekens et al., 2015a, de la Mora et al., 2016, Gonzalez-Maeso et al., 2008). Such mechanisms aid identification and comparison of a heterodimeric state vs. a monomeric state *in-vivo*, allowing for an indication of a heterodimer's physiological response. This approach could be used to identify the physiological response induced by the formation of an OTR-5-HT_{2C} receptor heterodimer due to the ability of 5-HT_{2C} receptor antagonism to restore OTR Gαq signalling to its monomeric state (as seen in ***Chapter 5***).

A second approach to *in-vivo* analysis of these receptor pairs would be via receptor knockout models, particularly conditional knockout models (Milligan, 2009). An example of this is the dopamine D2 (DRD2) and cannabinoid 1 receptor (CB1), it was shown that DRD2 can induce bias signalling of the CB1 Gα subunit binding, subsequently modulating its downstream signalling (Jarrahian et al., 2004). A follow up study investigated the in-vivo implication of this signalling bias, interestingly CB1 knockout resulted in reduced ethanol-induced conditioned place preference, a output which was linked to increased DRD2 receptor expression in the striatum, such

result indicating the role of the reward system in ethanol consumption and indicated a role of CB1-DRD2 dimerization on DRD2 synthesis (Houchi et al., 2004). Therefore, knockout models may be a feasible option to study the in-vivo implication of GHSR-OTR, 5-HT_{2A}-OTR and GLP-1r-OTR heterodimers, though precautions need to be taken to avoid knockout effects on other dimer pairs, which may result in a false behavioural output.

Due to the wide variety of behaviours involving the signalling of OTR, GHSR, GLP-1r, 5-HT_{2A} and 5-HT_{2C} many in-vivo behavioural approaches can be taken. Although through the identification of these heterocomplex expression patterns throughout different regions of the brain, the possible effect of these dimer pairs can be narrowed to target specific behaviours. In ***Chapter 2***, OTR-GHSR were shown to be co-expressed within the hippocampus, where attenuation or deletion of the OTR is shown to reduce social discrimination and memory, therefore it could be hypothesised that uncoupling of the OTR-GHSR heterocomplex may enhance these behaviours in such tests as the three chamber social approach assay (Lin et al., 2018, Raam et al., 2017a). Furthermore, in ***Chapter 3***, co-localization of OTR and GLP-1r receptors are visualised in the hypothalamus where GLP-1r signalling is implicated in food intake, receptor knockdown shown to increase food intake, hedonic feeding and overall bodyweight (Lopez-Ferreras et al., 2018). Therefore, the uncoupling of the OTR-GLP-1r heterocomplex may be a viable option in obesity treatment as separation may lead to enhanced GLP-1r signalling and subsequently reduced food intake. ***Chapter 4***, reveals co-localization of the OTR and 5-HT2A receptors in areas of the hippocampus, as mentioned above OTR signalling within the hippocampus is implicated in social discrimination and long term social memory (Lin et al., 2018, Raam et al., 2017a). Moreover, 5-HT_{2A} expression and signalling in the hippocampus is linked to memory and learning (Zhang and Stackman, 2015, Zhang et al., 2016). Due to the OTR and 5-HT_{2A} receptor involvement in memory it can be hypothesised that separation of these receptors may lead to increased social memory in tasks such as social place preference, a test previously shown to be impacted by oxytocin administration (Kent et al., 2013).

When identifying the physiological role of OTR heteroreceptor complexes *in-vivo*, a major consideration is the differences in OTR expression between sexes. Due to oxytocin's role in reproduction, labour and lactation females tend to have higher levels of circulating oxytocin compared to males (Veening et al., 2015). Due to this the largest effect of current oxytocin treatments have been shown in males, where similar oxytocin treatments have little to no effect in woman (Dumais et al., 2013, Dumais and Veenema, 2016, Gao et al., 2016). For example, in one study social interaction investigated by administration of intranasal oxytocin using the Prisoner Dilemma task resulted in oxytocin having little to no effect on female counterparts when examining behavioural responses in comparison to males (Rilling et al., 2014). This was further elucidated on during neuroimaging displaying significantly higher brain region activation in males in comparison to females (Rilling et al., 2014). Adding yet another layer of complexity required to be unravelled before OTR heteromerization can be fully understood, as the level of OTR dimers may differ between sexes resulting in different functional outputs.

Similarly, to differences in receptor expression between sexes, another area which must be alluded to, to discover the true physiological function of these dimer pairs is the expression of each receptor across neurodevelopment. In rat pups it has been identified that there a three major shifts in the central expression of the OTR following birth, throughout embryonic development and up to post-natal day 10 (PN10) OTR levels are seen to increase. This then begins to shift and decline following PN13-PN18, then upon weaning a final shift was observed the "adult pattern" of the OTR which is summarized in table 6.1 (Grinevich et al., 2015).

Expression pattern	Structure
Appearance in prenatal and/or early postnatal life and permanence to adult life	Dorsal nucleus of the vagus nerve Anterior olfactory nucleus Amygdaloid complex Nucleus accumbens° Dorsal peduncular cortex Lateral septum CA1 subfield of the hippocampus Ventral tegmental area Bed nucleus of the stria terminalis* Hypothalamic ventromedial nucleus* Ventral subiculum
Transient expression in prenatal and/or early postnatal life	Parietal cortex Cingulate cortex Retrosplenial cerebral cortex Mammillary complex Dorsal subiculum Caudate putamen Anterior and paraventricular thalamic nuclei Reticular nucleus Substantia gelatinosa of the V nerve Nucleus of the hypoglossus
Expression in late postnatal life	Olfactory tuberculum (Calleja islands) Ventral pallidum

° In the adult, binding in the accumbens has been reported to almost completely disappear (Tribollet et al., 1989) or to be greatly reduced as compared to its pick at PN20 (Shapiro and Insel, 1989).

**The appearance of OTR in the hypothalamic ventromedial nucleus has been reported to appear at PN1 (Tribollet et al., 1989) or to emerge only in the adult brain (Shapiro and Insel, 1989).*

Table 6.1: Brain structures associated with changes in OTR expression during development (Grinevich et al., 2015).

In females, oxytocin and OTR expression is again changed during pregnancy, revealing shifts in central expression across four time points, proestrus, during pregnancy, labour and postpartum (Meddle et al., 2007). Again, revealing the dynamic nature of the oxytocin signalling pathway. Therefore, when investigating the physiological response associated with these dimer pairs, the animal or humans' stage of development is a vital consideration, as reduced receptor expression may result in little or null physiological response. To examine this immunohistochemistry may be performed on brain slices of the animal of interest at different developmental time points to investigate the level of dimer expression, which will aid in choosing the correct developmental age to perform behavioural testing.

Thus, the above highlight the wide variety of future experiments which can be used to further elucidate the physiological response of these heterocomplexes, the vital considerations such as sex differences and neurodevelopment changes to receptor expression levels. and how prior ex-vivo co-localization assays can aid in identifying possible behavioural targets for such receptor pairs.

Bivalent ligands

Due to the inability of antagonism to restore signalling across the OTR-GHSR, OTR-GLP-1R and OTR-5-HT_{2A} receptor heterodimers, and that knockout models can often be unspecific, another approach can be taken to enable the identification of the effect of these dimers *in-vivo.* The development and synthesis of bivalent ligands to target GPCR heterodimers is becoming one of the largest drug designs for dimers, this is the joining of two pharmacophores with a spacer molecule, which are responsible for the pharmacological interaction between the molecule and GPCR. This leads to the ability of the linked molecule to bind to two receptors at once and produce two pharmacological responses (Hübner et al., 2016, Soriano et al., 2009, Qian et al., 2018, Berque-Bestel et al., 2008). Some suggesting the ability of these bivalent ligands to promote heterodimerization, suggesting it may be possible to reverse this and reduce the formation of heterodimers (Portoghese et al., 2017). If unable to uncouple the dimer pair, bivalent ligands have the additional advantage of enhancing ligand binding of heterodimerized GPCRs and subsequent GPCR signalling, which may allow for a substantial effect *in-vivo* allowing comparison of monovalent and bivalent treatment (Lensing et al., 2017, Qian et al., 2018). The advantage of bivalent ligands targeting OTR homodimers has already been shown, where bivalent treatment enhances OTR signalling, with the novel OTR bivalent ligand later shown to enhance social behaviours at lower concentration then its monovalent counterpart (Busnelli et al., 2016). Bivalent ligands targeting the 5-HT receptors in particular 5-HT_{2A} and 5-HT_{2C} have also been identified, with novel ligand profiles effecting receptor calcium and $ERK_{1/2}$ signalling (M. Hartley et al., 2015). With the known ability of bivalent ligands to effect OTR, 5-HT_{2A} and 5-HT_{2C} receptor signalling it may be possible to target the OTR-GHSR, OTR-GLP-1r, OTR- 5-HT_{2A} and OTR-5-HT_{2C} dimer pairs with such ligands. The development of new bivalent ligands targeting

these heterocomplex pairs could aid identification of the physiological relevance of these heterodimers, moreover, enabling identification of new therapeutic targets as previously seen (Lensing et al., 2016).

Drug discovery

When the physiological impact of the GPCR dimers is understood, it's link to different diseases and disorders can be inferred. If the impact to health is significant due the alteration in GPCR signalling associated with dimerization, then the next step would be discovery of drugs which restore the GPCR signalling to its healthy state. One of the most common drug discovery methods used is high throughput screening (HTS), this is where thousands of high affinity compounds are screened in quick succession to find drug "hits", these are drugs which produce the desired result whether it be a attenuation or enhancement in receptor signalling, HTS is often used in conjunction with other methods such as nuclear magnetic resonance to fully identify these "hits" (Szymanski et al., 2012). Fragment based drug discovery (FBDD) is an alternative option to HTS although similar FBDD deals with a library of low molecular weight molecules, FBDD is often used for challenging targets, such as a dimeric structure (Price et al., 2017). FBDD molecules tend to bind with low affinity, therefore such techniques as protein crystallography can be used to image and better understand the drug/target interaction, which will aid in synthetically enhancing the molecules affinity (Caliandro et al., 2013). Another method which is utilised to reduce the amount of physical screening required is de-novo computer aided drug design, this can encompass many computational approaches but each are based on a good understanding of the biological function and physical structure of the target proteins and there binding sites, from here a drug can be computationally designed upon its interaction with the binding site, thus identifying a possible "hit" molecule which can be synthetically designed (Yu and MacKerell, 2017). Due to its ability to predict interaction and function, *in-silico* computational drug design may be the best option when trying to design and predict the function of a possible bivalent ligand that will target the heterocomplex of interest. Once a drug has been identified and its function validated *in-vitro*, the study can then progress to pre-clinical trials, where its physiological efficacy and safety is tested (Andrade et al., 2016).

6.5 Conclusions

This thesis highlights the ability of the OTR to form heterocomplexes with a number of other GPCRs associated with mood, appetite and social behaviour, implicating these complexes in the ability of the OTR to regulate such behaviours. Interestingly, each pair resulted in different alterations to the downstream signalling of each receptor, revealing the novelty of these heterocomplex pairs. The alterations to the downstream biochemical signalling of these pairs highlights the complexity of signalling associated with GPCRs and reveals the limited knowledge regarding the full extent of GPCR signalling that is currently known, and the continued research required into GPCR dimerization. Upon full understanding of GPCR signalling the interlink between these pathways can be uncovered which may help in identifying the undiscovered pathologies of some disorders associated with GPCR signalling.

Further functional studies are required to identify the full extent of these heterocomplex pairs on downstream signalling, and additional *in-vivo* studies would be required to understand the physiological relevance of these receptors in mood, appetite and social behaviour. It can be hypothesised that the novel signalling of the OTR-GHSR, OTR-GLP-1R, OTR-5-HT_{2C} and OTR-5-HT_{2A} heterodimers may play critical roles in the dysfunctional GPCR signalling associated with many-centrally-regulated disorders of mood, appetite and social behaviour due to the link between these receptors and behaviour. Thus, this thesis emphasises the future challenges which may come to light in future GPCR dimerization studies yet understanding the functioning of these dimer pairs will aid in the development of much needed pharmacotherapies that are more potent and selective, with reduced associated side effects. This thesis highlights the exciting and novel signalling of the OTR which can be utilised in the discovery of these pharmacotherapies.

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Appendix

Detection and Quantitative Analysis of Dynamic G-Protein Coupled Receptors Interactions using Flow Cytometry-based FRET.

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Abstract

Heterodimerization of specific G-protein coupled receptors (GPCR) protomers is associated with increased receptor signalling diversity and exhibits unique biochemical, functional and pharmacological properties. Evidence for the formation of heteroreceptor complexes has been demonstrated *in vitro* using cellular models and biochemical assays and *ex vivo* using brain slices and primary cell cultures. Since mechanisms that lead to brain pathologies such as depression, anxiety, addiction and schizophrenia involve GPCR signalling, the distinct pharmacological profiles of GPCR assemblies may serve as new target for the development of novel therapeutic strategies with enhanced specificity. Therefore, development and standardization of novel methods for detection and analysis of dimer pairs in both recombinant systems and in native tissue is warranted. This chapter describes a step-by-step protocol for detecting and quantifying dynamic receptor-receptor interactions in living cells using flow cytometry-based fluorescence (Förster) resonance energy transfer (fcFRET). This method has significant potential to identify novel GPCR dimers within the central nervous system while simultaneously allowing analysis of the dynamic nature of these receptor interactions, which is poised to contribute significantly to the field of GPCR neuropsychopharmacology across brain diseases.

Key words Fluorescent resonance energy transfer, Flow Cytometry, G protein-coupled receptors, Heteroreceptor complexes, Receptor-receptor interactions, Dimerization

1. Introduction

G-protein coupled receptors (GPCRs) comprise a large superfamily of seven-transmembrane (7TM) domain proteins, which are major signalling mediators for a variety of endogenous hormones and neurotransmitters involved in diverse physiological functions ranging from glucose metabolism and appetite regulation to immune response and neurotransmission [1, 2]. GPCRs mediate their downstream signalling, following binding of their respective endogenous ligands, which leads to a changes in receptor conformation and further intracellular signalling through interaction with intracellular signalling molecules such as heterotrimeric G proteins, GPCR kinases and arrestins. Subsequently modulation of downstream enzymes, such

as adenylate cyclase, phospholipase C, or extracellular-signal-regulated kinases leads to specific cellular responses [3–5].

Initially GPCRs were thought to exist and function exclusively as monomeric signalling units [6]. However, oligomerization of GPCRs, in which protomers of the same or different families combine to generate homo- or heterodimers, as well as higher-order multimers, is becoming increasingly accepted as a fundamental process in GPCR signalling [7–10]. These higher order complexes have been suggested to exhibit unique biochemical and functional properties, compared to their monomeric counterparts [11]. Heterodimerization of specific protomers has been shown to affect ligand binding to the receptors [12, 13], alter G-protein subunit coupling, and influence intracellular signalling [14], as well as ligand mediated internalization of the receptors [15, 16]. Interestingly, GPCR heterodimers are able to activate intracellular signalling cascades that cannot be triggered by each of the individual receptors alone [17]. Furthermore, GPCRs heterodimers can potentiate or attenuate their downstream signalling by modulating the expression, maturation, and trafficking of the protomers from the endoplasmic reticulum (ER) to the cell membrane, changing their subcellular localization [18]. Since GPCRs are considered to be major drug targets, the distinct pharmacological profiles of GPCR assemblies are likely to serve as novel mechanisms, important for the development of more specific pharmacological strategies to modulate cell response, and regulate a plethora of physiological processes [2]. Formation of heteroreceptor complexes and altered functionality has also been demonstrated in the brain [12, 19–21]. What is more important is that dysfunctions of heteroreceptor complexes may be involved in many brain pathologies [22, 23]. For instance modulation of neuronal signalling via dopamine 2 receptor (DRD2) heterocomplexes with adenosine 2A, serotonin 2A, NMDA and metabotropic glutamate 5 receptors found in striatum, have been implicated in the pathophysiology of schizophrenia, and may provide new possibilities for the treatment of positive, negative, and cognitive symptoms of schizophrenia [7, 24, 25]. Moreover, heterodimers of DRD2 with adenosine receptor are implicated in cocaine associated addiction [26], while more recently established positive allosteric interactions between DRD2 and oxytocin receptor seem to be essential for social behaviour [27]. The formation and functioning of serotonin 1A

heteroreceptor complexes with galanin 2, serotonin 7 and 2A receptors, located mainly in the raphe-hippocampal system, may serve as a new targets for drug development for conditions such as major depression and anxiety [28, 29].

Due to the physiological importance of GPCR receptor interactions in the central nervous system, development of novel methods for the identification, detection and analysis of dimer pairs has seen a surge in research interest. Physical receptor-receptor interactions have been investigated using a plethora of *in vitro* and *in vivo* approaches [30]. Co-immunoprecipitation followed by western blotting has been considered for years as the reference method to demonstrate a physical interaction. Nevertheless, this technique requires the use of non-physiological buffers and detergents to lyse and solubilize tissue, which causes disruption to the natural cells environment, and in consequence preclude the analysis of subcellular localization rising to artefacts. In addition, biophysical proximity assays, based on resonance energy transfer (RET) between two chromophores, allow investigations into physical interaction in intact living cells. Bioluminescence resonance energy transfer (BRET) compared to fluorescence (Förster) based RET (FRET) is characterised by a more specific signal that is easier to quantify. However, this method doesn't allow for microscopic observation and analysis of protein-protein interactions at the subcellular level [31, 32]. The advantage of FRET-based techniques (FRET, TR-FRET, TIRF-FRET, FRET spectroscopy) is that they provide this type of additional information concerning the physiology of receptor-receptor interactions in living cells. In this chapter, we describe the protocol for the quantitative analysis of dynamic receptor-receptor interactions through FRET measurements of fluorescently-tagged GPCR pairs using flow cytometry (fcFRET).

2. The principle of fcFRET methodology

FRET is a physical phenomenon known since 1946 [33], and from then onwards has become a powerful tool to study protein-protein interactions [34–36]. FRET is based on a non-radiative transfer of energy from an excited fluorophore (donor) to another fluorophore (acceptor), leading to a reduced emission of energy from the donor (donor quenching) and an increased emission energy from the acceptor (FRET signal) [37]. The efficiency of energy transfer depends on the distance between donor and

acceptor, and occurs only within the range of 1-10nm (10-100Å). Therefore, FRET-based biosensors can work as spectroscopic rulers, used for monitoring the proximity changes at the molecular level (fig.1) [38]. High performance of FRET requires an emission spectrum of the donor that overlaps significantly with the excitation spectrum of the acceptor [39]. Three main types of FRET biosensors are small organic dyes, quantum dots and fluorescent proteins (FPs). However, only FPs have the ability to label sensing domains without the need for antibodies (compared to quantum dots), and allow the analysis of FRET in intact living cells (compared to organic dyes) (Note 1). The choice of FRET-pair depends, beyond their spectroscopic features, on the instrument used for FRET measurements. FRET can be detected by all instruments capable of recording fluorescence emission, like spectrofluorometers, microscopes and flow cytometers. In contrast to microscopy-based FRET, flow cytometry analysis allows the evaluation of interactions in large population of cells in a short period of time, providing statistically more robust and reliable data. Compared to spectrofluorometers, flow cytometry makes it possible to classify the population of cells and elucidate the difference in FRET efficiency with other cellular parameters [40, 41]. On top of that, fcFRET allows for quantitative analysis of ligand-mediated changes of FRET signal in heterologous expression systems (*in vitro*) and in cells endogenously expressing receptors of interest (*ex vivo*) (Note 2). In conclusion, the fcFRET-based proximity assay provides a unique opportunity to combine the quantitative measurement of physical GPCR interactions with the ability to analyse dynamic changes following exposure to ligands and novel neurotherapeutics.

3. Materials

3.1. Generation of human GPCRs expressing cell lines

Cells, cell culture media and buffers

1. Cell lines: Human Embryonic Kidney (HEK) cell lines 293A and 293T-17 were purchased from Invitrogen and American Type Culture Collection (ATCC), respectively. Alternatively, any cells that can be prepared as a suspension can be used for fcFRET.
2. Complete culture media: Dulbecco's Modified Eagle's Medium (DMEM) - high glucose (Sigma, #D5796-500ML) supplemented with 1 % MEM Non-Essential

Amino Acids solution (NEAA) (Gibco, #11140035) and 10 % heat-inactivated Fetal Bovine Serum (FBS) (Sigma, #F7524) is stored at 4 ºC and pre-warmed to 37 ºC before use.

3. Cell dissociation reagent: 0.25 % Trypsin-EDTA solution (Sigma, #T4049) is stored at 4 ºC, pre-warmed before use.
4. Washing buffer: Phosphate-buffered saline (PBS) without calcium and magnesium, pH = 7.4, (Gibco, #10010015) is stored in room temperature (RT).

Development of a stable cell line expressing GPCR-A tagged with eGFP

1. Expression vector: Plasmids containing the open reading frame (ORF) coding for the receptor of interest fused with eGFP (e.g. pCMV-GPCR-A-eGFP) and resistance markers (e.g. G-418) are commercially available (OriGene, GeneCopoeia, cDNA Resource Center) or can be cloned.
2. Transfection reagent: Lipofectamine LTX Reagent with PLUS Reagent (Invitrogen, #15338100) is stored at 4 ºC and pre-warmed to RT before transfection.
3. Selection antibiotic: G-418 (Caldiochem, #345812) is stored at 4 ºC.
4. Fluorescence enrichment: Flow Associated Cell Sorter (FACS) is used to sort cells expressing GPCR-A-eGFP (FACSAriaII, BD Biosciences). An epifluorescence microscope (Olympus IX70) and a flow cytometer (FACSCalibur, BD Biosciences) are used to monitor expression level of GPCR-A-eGFP in cells.

Lentiviral transduction of cells to transiently co-express GPCR-B tagged with RFP

1. Viral plasmids: Lentiviral expression vector containing sequence of the receptor under investigation fused with RFP (pHR-GPCR-B-tagRFP), packaging plasmid (e.g. pCMV ΔR8.91), envelope plasmid (pMD.D-VSV.G) [16].
2. Transfection reagent: Transfection reagent prepared prior to use contains 2.5 mM calcium chloride (Sigma, #C1016) and N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid sodium salt solution (BES). BES solution is prepared as a 2X concentrated stock by adding 50 mM BES (Sigma, #B2891), 280 mM NaCl, and 1.5 mM Na2HPO4. Solution is adjusted to pH = 7.0 and stored in 4 ºC.
3. Packaging media: DMEM supplemented with 1 % NEAA and 2 % heat-inactivated FBS.
4. Packaged virus harvesting: 0.45 um filters (Sartorius, #16555).

5. Transduction Media: DMEM supplemented with 1 % NEAA, 2 % heat-inactivated FBS and 8 ug/ml of polybrene (Sigma, #H9268).

3.2. Sample preparation for fcFRET

1. Washing buffer: PBS without calcium and magnesium, pH = 7.4 (Gibco, #10010015).
2. Suspension buffer: Suspension buffer is prepared prior to use by adding 20X concentrated EDTA stock (Sigma, #E5134) to the washing buffer. 40mM EDTA stock is dissolved in H_20 and stored at 4 ºC.
3. Single-cell suspension: 100 µm nylon mesh cell strainers (VWR, #10199-658).
4. Flow cytometry tubes: 5 ml round-bottom polystyrene tubes (Corning, #352054).

3.3. Flow cytometer and software configuration for fcFRET analysis

1. The measurement of cell-by-cell FRET can be performed with the use of any flow cytometer equipped with two lasers to allow for separate excitation of donor and acceptor biosensors. fcFRET analysis between eGFP (GPCR-A-eGFP) as a donor and tagRFP (GPCR-B-tagRFP) as an acceptor is performed with the use of blue (488nm) and yellow/green (561nm) lasers (fig.2). eGFP is excited at 488 nm from blue laser and detected with a 525/50 nm bandpass filter, whereas TagRFP is excited at 561 nm from yellow/green laser and detected with a 610/20 nm bandpass filter. FRET signal between eGFP and TagRFP is measured by excitation at 488 nm from blue laser and detection with a 610/20 nm bandpass filter located on the same laser (excitation of the donor and measurement of the acceptor emission). For the proper separation of eGFP fluorescence and FRET emission from blue laser, a 505 Long Pass (LP) dichroic mirror (DM) should be used [42, 43].
2. To obtain the proper analysis of cell-by-cell FRET signal, data should be analysed with flow cytometric data analysis programs such as FlowJo (FlowJo, LLC), BD FACSDiva (BD Biosciences) or FCS 6 Express Cytometry (De Novo Software).

4. Methods

4.1. Generation of cells co-expressing fluorescently-tagged GPCR pairs

Cell Culture

HEK293A and HEK293T-17 cells are cultured in complete culture media and maintained at 37 ºC and 5 % CO_2 in a humidified atmosphere to a confluence of >85 %, after which the cells are washed with the use of washing buffer, dissociated with cell dissociation reagent, and passaged to a lower density. Cells should be passaged according to a strict schedule in order to ensure reproducible behaviour, optimal health and GPCR expression levels.

Generation of stable human GPCR-A expressing cell line

HEK293A cells are stably transfected with the plasmid construct of the receptor A (GPCR-A) C-terminally tagged with eGFP using transfection protocol, according to manufacturer's instructions. Cells with the highest expression of the GPCR-A-eGFP fusion protein are further selected using FACS, following by monoclonal expansion on 96-well plates. The monoclonal HEK293A-GPCR-A-eGFP cell line is then maintained in complete media supplemented with 400 ng/µl G-418 (selection media). Stability of the GPCR-A-eGFP fusion protein expression is monitored with the use of epifluorescent microscopy and flow cytometry. Functionality of GPCR-A is validated by measuring agonist-mediated signalling in a cellular-based assay.

Obtaining cells with the transient co-expression of GPCR-B using lentiviral transduction

The DNA fragment containing the sequence of GPCR-B is cloned into a HIV-based, replication deficient, lentiviral expression plasmid pHR-SIN-BX-tagRFP, suitable for the second generation lentiviral packaging system. HIV-based lentiviral particles are produced using HEK293T-17 cell line, by transient transfection of the cloned expression construct, pHR-GPCR-B-tagRFP; the packaging construct, pCMV ΔR8.91; and the envelope construct, pMD.G-VSV-G at a concentration of 1.9ug, 1.5ug and 1ug, respectively. Transfection reagent is used to increase efficiency of plasmid packaging. 24-30 h post transfection media is changed to packaging media. 48 h post media change the supernatant containing pHR-GPCR-B-tagRFP virus is removed and filtered. HEK293A cells with the stable expression of GPCR-A-eGFP can now be transduced with the pHR-GPCR-B-tagRFP packaged virus diluted in transduction media. The dilution of virus for transduction, as well as incubation time are optimized for each cloned expression construct. The efficiency of transduction is monitored with the use of fluorescent microscope and flow cytometry (Note 3). Functionality of

the transiently expressed GPCR-B is validated by measuring agonist-mediated signalling in a cellular-based assay.

4.2. Flow cytometry measurements

Sample preparation

Media is aspirated and cells are washed twice with washing buffer. To avoid possible effects of the trypsin and/or EDTA on distribution and functionality of the receptors, we recommend to suspend cells mechanically with the use of washing buffer. Cell suspension is then centrifuged for 4 min at 200 x g, at room temperature. Supernatant is discarded and cell pellet is suspended in 400 µl of suspension buffer. Prior to analysis, cells should be passed through a cell strainer with 100 µm nylon mesh and collected in a flow cytometry tubes.

In order to assess FRET between stably expressed GPCR-A-eGFP and transiently co-expressed GPCR-B-tagRFP receptors using flow cytometry, several control samples must be included in the experimental protocol:

Sample 1: HEK293A (control for fluorescence background correction)

Sample 2: HEK293A-GPCR-A-eGFP (control for compensation)

Sample 3: HEK293A-Lv-GPCR-B-tagRFP (control for compensation)

Sample 4: HEK293A-GPCR-A-eGFP-Lv-tagRFP (nonspecific FRET signal)

Sample 5: HEK293A-GPCR-A-eGFP-Lv-GPCR-B-tagRFP (FRET efficiency)

As a positive control, the same set of samples with known molecular interaction pair of receptors can be used. If it's known that one of the receptors under investigation forms homodimers (GPCR-A), the positive control sample set can be as follow (sample 1, 2 and 4 are the same as above):

Sample 3': HEK293A-Lv-GPCR-A-tagRFP (control for compensation)

Sample 5': HEK293A-GPCR-A-eGFP-Lv-GPCR-A-tagRFP (FRET efficiency)

Flow cytometer settings and data acquisition

Non-transfected HEK293A cells (sample 1) are firstly used for initial instrument setup. Forward Scatter versus Side Scatter (FSC vs SSC) plot is used to identify cells of interest based on their size and granularity. Sample 1 is also used for setting up photomultiplier tubes (PMT) voltage in order to correct eGFP and tagRFP fluorescence background. In the next step, cells expressing donor or acceptor construct only (sample 2 and 3, respectively) are used to fine tune PMT settings.

Sample 2 and 3 are also necessary to perform the proper compensation for spectral bleed through and cross-excitation, in particular for eGFP emission in the tagRFP-FRET detector and tagRFP emission caused by excitation of tagRFP from the blue laser [42, 44]. Next, cells co-expressing the donor construct with the empty acceptor construct (sample 4) are analysed for nonspecific FRET signal, following by analysis of cells with the expression of the two receptors under investigation (sample 5). At least 10^4 cells for each sample need to be recorded.

Analysis of FRET signal

Non-transfected cells (sample 1) are first used to differentiate cells based on their size and granulation, according to forward and side scattering plot (FSC/SSC). This step allows to eliminate doublets, dead cells and debris from further analysis. Additionally, the viability dye can be used to definitely eliminate dead cells. Next, two-dimensional dot-plot of eGFP fluorescence against tagRFP are constructed to gate cell populations with co-expression of both GPCRs under investigation (samples 1, 2 and 3 are used for the proper gate placement). Those cells are next used to create two-dimensional dot-plot of eGFP fluorescence against FRET signal. The gate for FRET positive signal is further corrected by using cells co-expressing donor with control acceptor constructs (sample 4) (Note 4).

An example of fcFRET analysis between the 5-HT2C-VSV and GHS-R1a receptors is demonstrated in figure 3. Following lentiviral transduction of HEK293A WT and HEK293A-5-HT2C-VSV cells with the lentiviral GHS-R1a-tagRFP vector, 61.6% and 52.2% of cells were analyzed as positive for tagRFP expression (sample 3 and 5, respectively). HEK293A-5-HT2C-VSV cells transduced with the control-tagRFP vector gave equal high transduction efficiency (61.5%, sample 4). Subsequent analysis of FRET signal was performed on the gated population of the successfully transduced cells and showed an increase in FRET signal from 1.9 % in control sample (sample 4) to 30.26 % in cells co-expressing both receptors (sample 5) (FRET vs eGFP plots). No tagRFP or FRET signal in HEK293A WT or HEK293A-5-HT2C-VSV cells was observed (sample 1 and 2).

5. Notes

1. The choice of fluorescent protein pair

Choice of optimal FRET pair should be considered not only based on spectroscopic features of individual FP, but also depending on the biological question and the compatibility of the fluorescent pair's with the selected FRET signal measurement method. Selected and validated FP pairs for flow cytometry-based FRET are depicted in table 1.

Tailored FPs are now possible due to improved optical properties that have resulted in highly sensitive FRET biosensor pairs and improved FRET efficiency. One of the most common biosensor pairs used for fcFRET are GFPs and red RFPs, in particular their brighter derivatives (eGFP and tagRFP), which have been used successfully to demonstrate GPCR interactions [43]. The eGFP/tagRFP combination demonstrate greater excitation wavelengths, larger emission peak separation, reduced auto-fluorescence and phototoxicity, compared to CFPs-YFPs pairs [45]. While the eGFP/tagRFP pair has been validated extensively and has many advantages, additional FPs with better optic properties, greater dynamic range and photostability are warranted to allow the fcFRET method to reach its full potential [46].

2. Antibodies as an alternative for tagged fluorescent proteins

FPs fused with receptors of interest are commonly used to investigate receptor-receptor interactions. However, in some cases binding of FPs to the N- or C-terminus of the receptors can influence their maturation, structure, subcellular localization and subsequently cause changes in function. Moreover, detection of receptors tagged with FPs requires heterogeneous expression systems and is not specific for membrane-bound GPCRs. This can be overcome by using fluorescence-conjugated antibodies targeting receptors of interest in cells with endogenous GPCRs expression and used to confirm the occurrence of membrane receptor-receptor interactions (without permeabilization)[47]. Furthermore, dynamic changes in membrane receptor-receptor interactions induced by specific treatments can be studied after sample fixation. In this case, staining and fixation have to be optimized for each antibody. Also, the relative expression of the donor and the acceptor receptors have to be determined in order to use the appropriate concentration of respective antibody (i.e. antibody concentration must be high enough to label all receptor binding sites available at the cell membrane). If fluorescence-conjugated antibodies are not commercially available, a multiple step staining must be performed. In this

case higher spatial separation of two fluorophores is observed, which rise the risk of false negative results. Even though fixed samples can be stored at 4 ºC until measurement, fluorescence intensity decreases over time. Also, time required for labelling needs to be considered. Another major limitations of antibody labelling for receptors interaction analysis is the consequence of increasing protein structure size (with a single antibody adding 10 nm, this being doubled with a secondary antibody), although nanobodies are being developed to tackle such issues [48].

3. Stable or transient receptor expression

Monoclonal cell lines with stable receptors expression have the advantage over transient expression because of the same or very similar receptor expression level maintained over time, leading to a consistency in FRET signal between experiments [49]. However, the establishment of a monoclonal stable cell line is time consuming, while lentiviral transduction-mediated transient expression allows analysis of a wide range of receptors in a much shorter time frame. Furthermore, lentiviral expression is a highly efficient method for transient expression of genes of interest in neuronal cell [50]. This allows for the analysis of dynamic GPCR interactions in more physiologically relevant cell models.

4. Alternative gating strategy

In this book chapter, cells with the expression of donor construct (GPCR-A-eGFP) versus acceptor construct (GPCR-B-tagRFP) are used to determine the gating parameters of the population of cells with co-expression of both GPCRs and create a two-dimensional dot-plot for analysis of FRET signal. Gating of FRET signal is also corrected using cells co-expressing donor construct with control acceptor construct (GPCR-A-eGFP & control-tagRFP). Alternatively, cells expressing only the donor FP (eGFP) or acceptor FP (tagRFP), without target receptors, can be used to determine the gating parameters of cells co-expressing both FPs. Gating for FRET positive signal is then corrected using cells co-expressing donor with acceptor FP constructs (eGFP & tagRFP). This gating strategy can be implemented especially for the initial high throughput screening of new protein-protein interactions [51].

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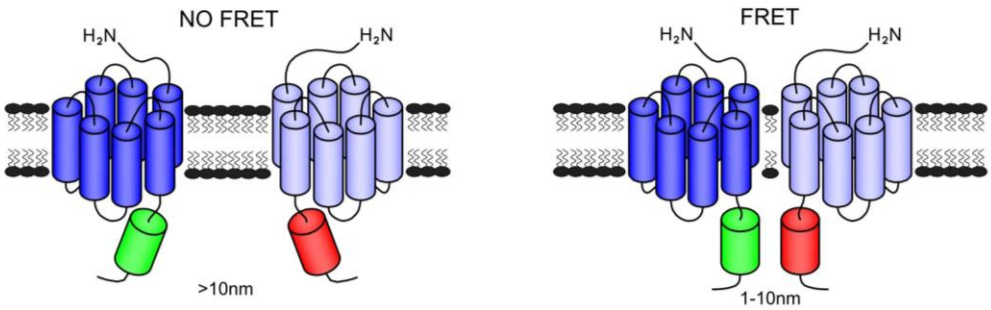

Figure 1. Principle of fluorescence resonance energy transfer (FRET). FRET between fluorescent proteins (FPs) attached to the carboxy-terminus of two interacting GPCRs. Upon excitation with light at 488 nm the eGFP emits light at 525 nm (left panel). When the distance between two FPs is between 1-10 nm, the excited eGFP (donor) transfers energy to the RFP (acceptor) via a dipole-dipole resonance energy transfer mechanism, causing the RFP to emit light at 610 nm, which is measured as the FRET signal (right panel).

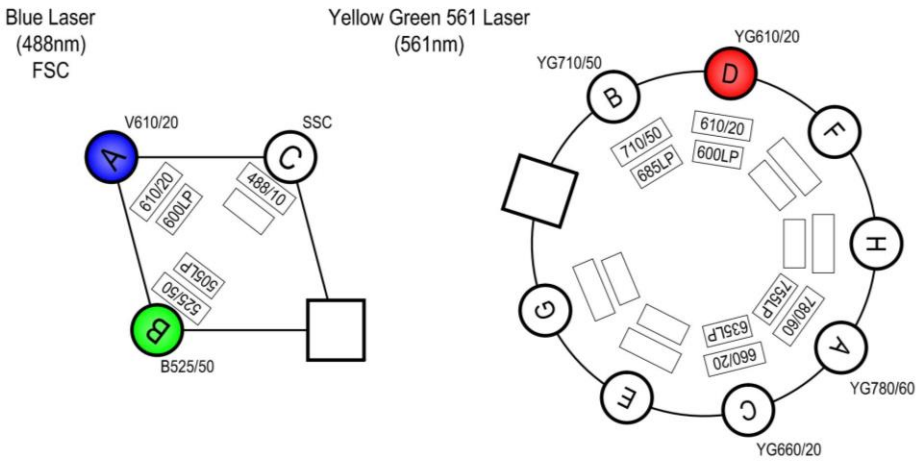

Figure 2. LSR II configuration used for measurement of FRET between eGFP as a donor and tagRFP as an acceptor. eGFP is excited at 488 nm from blue laser and detected with a 525/50 nm bandpass filter (B), whereas tagRFP is excited at 561 nm from yellow/green laser and detected with a 610/20 nm bandpass filter (D). FRET signal between eGFP and tagRFP is measured by excitation at 488 nm from blue laser and detection with a 610/20 nm bandpass filter (A).

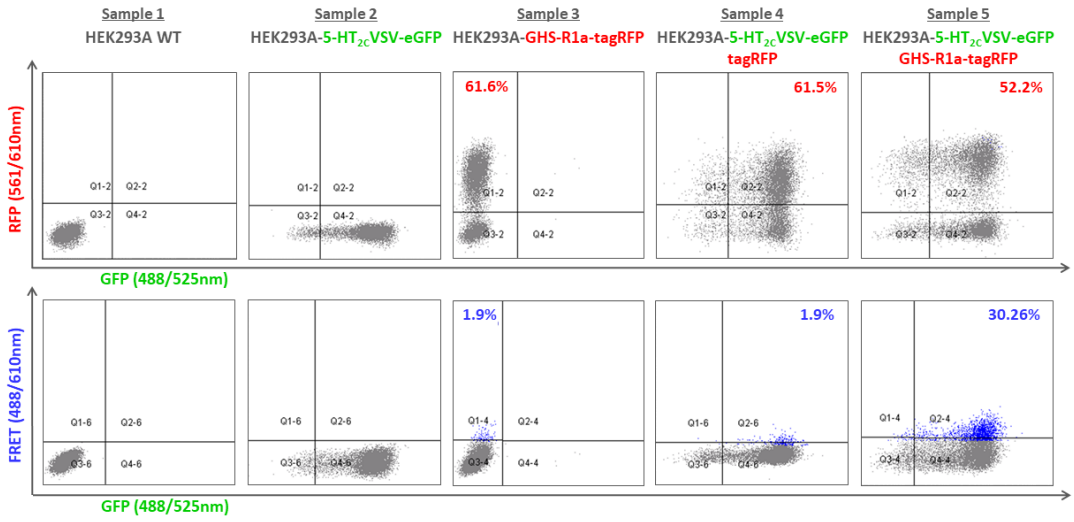

Figure 3. fcFRET between 5-HT2C-VSV/GHS-R1a receptor pair is analysed using the LSRII flow cytometer. HEK293A cells stably expressing the partially edited 5-HT2C isoform were transiently transduced with lentiviral vectors expressing control-tagRFP or GHS-R1a-tagRFP. Percentages indicate levels of tagRFP expression (tagRFP vs eGFP

plots) or FRET levels (FRET vs eGFP plots). Adapted with permission from Schellekens, et. al., 2015. “Ghrelin’s Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction.” ACS Chemical Neuroscience 6 (7): 1186–97. doi: 10.1021/cn500318q. Copyright, 2015, American Chemical Society.

Table 1. Fluorophore pairs suitable for fcFRET analysis.

Donor $\lambda_{Ex}/\lambda_{Em}$ [nm]	**Acceptor** $\lambda_{Ex}/\lambda_{Em}$ [nm]	**Biological Process/Cellular Parameter**	**Ref**
CFP (435/485)	**YFP** (514/527)	molecule-molecule interaction protein aggregation	[52, 51, 53]
	mCitrin (516/529)	conformational changes	[54]
GFP (488/510)	**RFP** (555/584)	receptor-receptor interaction intramolecular conformational changes	[43, 55]
	Alexa-555 (555/580)	enzymatic activity	[56]
	mOrange (548/562)	molecule-molecule interaction	[57]
Alexa-555 (555/580)	**Alexa-647** (650/665)	subunit receptor spatial arrangement	[58]
mCerulean (433/475)	**mVenus** (515/528)	molecule-molecule interaction	[59]
	mCitrin (516/529)	enzymatic activity	[60]
Alexa-488 (490/525)	**Alexa-594** (590/617)	molecule-molecule interaction	[61]
	Alexa-546 (556/573)	molecule-molecule interaction receptor-receptor interaction	[62, 63]
PE (488/578)	**Cy5** (650/670)	molecule-molecule interaction receptor-receptor interaction	[64, 65]
	Alexa-647 (650/665)	receptor variant clusters	[66]

$\lambda_{Ex}/\lambda_{Em}$ - values for maximum excitation/emission of fluorescent biosensor

Additional receptor pairs investigated for co-localisation using fluorescent microscopy

During the original investigation of OTR heteromerization, apart from co-expression of the GHSR, GLP-1R, 5-HT2C and 5-HT2A with OTR, co-expression with AVPR1a, DRD1 and MCR3 was also explored. As can be seen in figure 3, OTR and AVPR1a co-localises throughout the cell, this was not selected going forward as dimerization of the OTR and AVPR1a receptors has already been shown in previous studies (Terrillon et al., 2003). As can be seen in figure 3B little co-localization can be seen between the OTR and DRD1 receptors, where the insignificant overlap observed may be due to receptors overlapping but on different planes of the cell. Figure 3C shows both cell co-localizing OTR and MCR3 and cells revealing independent expression, upon review of current studies, little evidence was found to link MCR3 and OTR signalling although some have linked MCR4 and oxytocin signalling in social behaviour (Modi et al., 2015, Mastinu et al., 2018). Due to level of co-localization and previous literature linking their signalling pathways OTR co-expression with GHSR, GLP-1R, 5-HT2C and 5-HT2A receptors was further investigated.

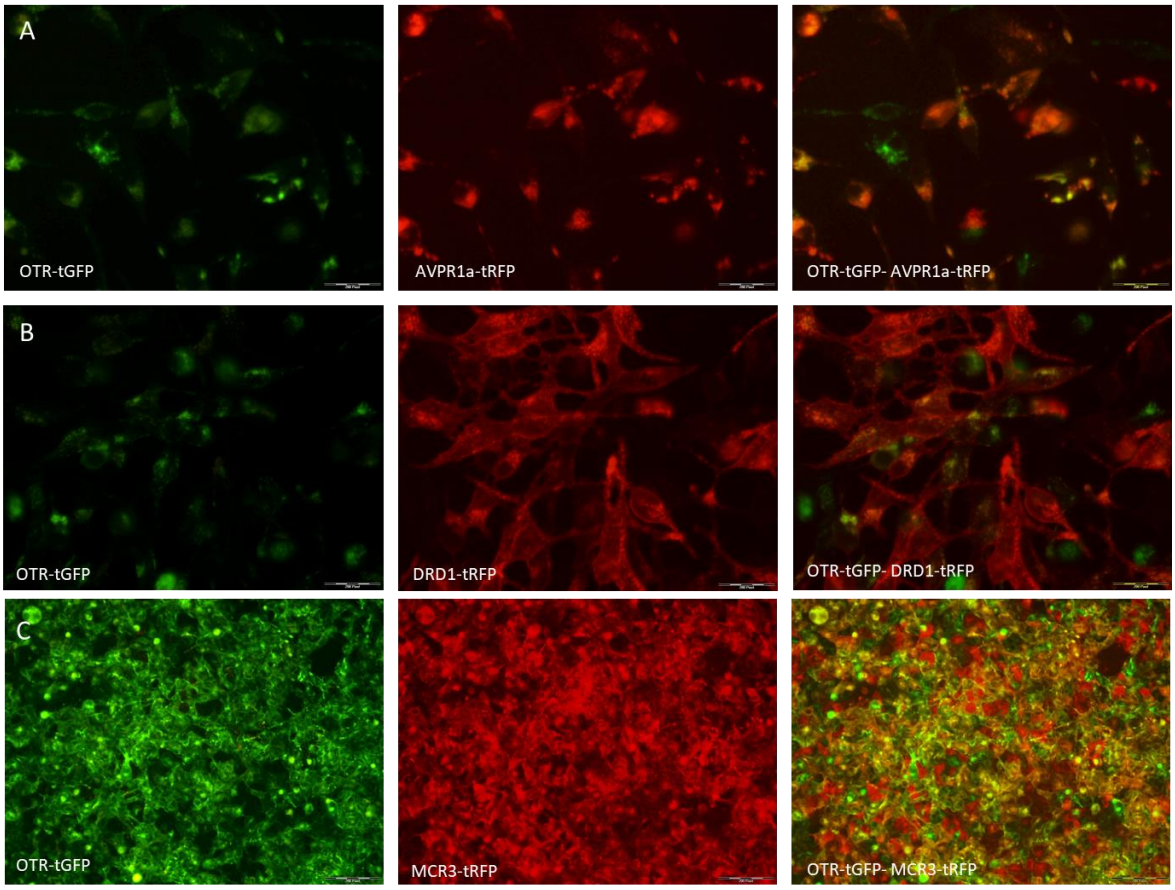

Figure 4: (A) The vasopressin 1a receptor (AVPR1a), **(B)** the dopamine D1 receptor (DRD1) and **(C)** the melanocortin 3 receptor (MCR3) co-expressed with the OTR in HEK293A cells.

Ligand information and references

Compound	Company	Mode	Associated Receptor	Model	Reference
Oxytocin	O3251, Sigma-Aldrich 1910/1, Tocris	Full agonist	OTR	*In-vivo and ex-vivo*: Humans and rhesus macaques *Ex-vivo*: Mouse	(Dal Monte et al., 2017, Xiao et al., 2018)
Carbetocin	SML0748, Sigma	Partial agonist	OTR	*In-vivo:* Human	(Gawecka and Rosseland, 2014)
Ghrelin	SP-GHRL-1, Innovagen	Full agonist	GHSR	In-vitro: HEK292	(Howick et al., 2018b, Schellekens et al., 2015a)
Glucagon-like peptide 1 (7-37)	Tocris, 5374	Full agonist	GLP-1R	*In-vivo:* Human	(Vahl et al., 2003)
Exendin-4	1933, Tocris	Full agonist	GLP-1R	C57BL6 mice, Humans	(Suissa et al., 2013, Lutter et al., 2017)

5-hydroxytryptamine	H9523, Sigma-Aldrich	Full agonist	5-HT_{2C}, 5-HT_{2A}	*In-vitro:* Human EC celss *Ex-vivo:* Rat	(Westerberg et al., 2018, Das et al., 2008)
Exendin-3	2081, Tocris	Competitive antagonist	GLP-1R	*In-vivo:* Sprague Dawley rat	(Maniscalco et al., 2015)
L-371-257	2410, Tocris	Competitive antagonist	OTR	In-vitro: Human glioblastoma U-87MG cells *In-vivo*: Male Wistar rats	(Ataka et al., 2012, Lestanova et al., 2017)
Atosiban	A3480, Sigma-Aldrich	Competitive antagonist	OTR	*Ex-vivo*: Human	(Pohl et al., 2018)
JMV 2959	345888, Merck	Competitive antagonist	GHSR	*In-vitro:* HEK293	(Pastor-Cavada et al., 2016)
M100907	M3324, Sigma-Aldrich	Competitive antagonist	5-HT_{2A}	*In-vivo:* Sprague Dawley rat	(Agnoli and Carli, 2012)
WAY267464	3933, Tocris	Competitive antagonist	OTR	*In-vitro:* CHO-cells	(Ring et al., 2010)

Eplivanserin hemifumarate	4958, Tocris	Competitive antagonist	5-HT_{2A}	*In-vivo:* Sprague Dawley rat	(Ball and Rebec, 2005)
SB242084	2901, Tocris	Competitive antagonist	5-HT_{2C}	*In-vitro:* T-RExTM 293 cell lines	(Ward et al., 2015)
RS102221	1050, Tocris	Competitive antagonist	5-HT_{2C}	*In-vitro:* T-RExTM 293 cell lines	(Ward et al., 2015)