Dimer-dependent allosteric modulation within GPCR signalling complexes can influence signalling diversity

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ABSTRACT (ENGLISH)

G protein-coupled receptors (GPCRs) comprise the largest group of cell surface receptors, translating environmental signals into cellular responses via cognate G protein partners. Contrary to our initial understanding, most GPCRs do not function in living cells as monomers, but most likely dimers, or even larger arrays of receptors. Standard drug design approaches rely on the notion that drugs binding the two receptors in a given dimer likely function independently of one another. However, this view has been challenged by recent work showing that ligand binding at both receptors can modulate dimeric receptors via allosteric communication. While one receptor may actually be needed to drive signalling, the other acts to control or modulate these signals, without a direct signalling outcome itself. Based on the notion of allosteric modulation within homo- and heterodimers, I tested and compared changes in signalling downstream as well as at the level of the receptor-G protein-effector (RGE) complex in response to different combinations of ligands at each protomer. Using a combination of calcium, cyclic adenosine monophosphate, and mitogen-activated protein kinase signalling assays, I have demonstrated functional interactions for a putative D2 dopamine receptor, oxytocin receptor heterodimer (D2R/OTR), in HEK 293 cells. Immunoprecipitation, bioluminescence resonance energy transfer (BRET) and confocal microscopy experiments reveal D2R and OTR do in fact form a heterodimer *in vitro,* which may explain the nature of these potential allosteric functional interactions. Using BRET, I assessed the RGE complex conformational dynamics in HEK 293 cells for two other heterodimers, β_2-adrenergic receptor

with cannabinoid CB1 receptor (β_2AR/CB1R) and β_2AR/OTR, in order to determine how they manifest in parallel to signalling events themselves. These studies reveal functional interactions can occur in terms of signalling complex conformation. Thus GPCR signalling can be modulated by its partner receptor at the level of downstream effector signalling or at the level of the signalling complex itself. With that said, putative heterodimers need to be reanalyzed *in vivo* for their allosteric properties, which may explain some of the side effects of so many drugs, and may have implications in drug design.

ABSTRACT (FRENCH)

Les récepteurs couplés aux protéines G (RCPG) constituent le plus grand groupe de récepteurs de la surface cellulaire, qui traduisent les signaux environnementaux en réponses cellulaires via leurs protéines G associées. Contrairement à notre compréhension initiale, la majorité des RCPG ne fonctionnent pas en tant que monomères, mais possiblement en tant que dimères ou même oligomères. Les approches actuelles de conception de médicament estiment que lors de la liaison d'un médicament aux deux récepteurs d'un dimère quelconque, ces derniers fonctionnent potentiellement indépendamment l'un de l'autre. Cependant, cette notion a été reconsidérée par une étude récente montrant que la liaison d'un ligand aux deux récepteurs peut les altérer par voie de communication allostérique. Alors qu'un premier récepteur peut être requis pour initialiser la signalisation, un second peut contrôler ou modifier ces signaux, n'ayant pas nécessairement une signalisation directe comme résultante. Dans l'étude suivante, basée sur la notion de modulation allostérique au sein d'homodimère et d'hétérodimère, les changements de signalisation en aval ainsi qu'au niveau du complexe récepteur/protéine G/effecteur (RGE) ont été étudiés et comparés en réponse à différentes combinaisons de ligands pour chaque protomère. En utilisant une combinaison d'essais de signalisation de calcium, d'adénosine monophosphate cyclique (cAMP) et de protéine kinase activée par des agents mitogènes (MAPK), une interaction fonctionnelle entre le récepteur dopaminergique D2 et le récepteur de l'ocytocine (D2R/OTR) a été démontrée

dans les cellules HEK 293. Des expériences d'immunoprécipitation, de transfert d'énergie de résonance par bioluminescence (BRET) et de microscopie confocale ont révélé la présence d'hétérodimère entre le D2R et l'OTR *in vitro*, ce qui pourrait expliquer la nature des interactions fonctionnelles allostériques. En utilisant la technique de BRET, la dynamique fonctionnelle du complexe RGE dans les cellules HEK 293 a été examinée chez deux autres hétérodimères, soit celui composé du récepteur adrénergique β_2 et du récepteur cannabinoïde CB1 (β_2AR/CB1R) et l'hétérodimère β_2AR/OTR, afin de déterminer comment ils traduisent les évènements de signalisation. Ces études démontrent donc qu'une interaction fonctionnelle peut survenir sur le plan de la conformation du complexe de signalisation. Par conséquent, la signalisation d'un RCPG peut être modulée par son récepteur partenaire au niveau des effecteurs ou au niveau du complexe de signalisation lui-même. Pour cette raison, il serait impératif de réanalyser *in vivo* les propriétés allostériques d'hétérodimères putatifs, ce qui pourrait expliquer certains effets secondaires d'une multitude de médicaments et ce qui pourrait impliquer des changements majeurs dans la façon de concevoir de nouveaux médicaments.

LIST OF ABBREVIATIONS

A2AR	adenosine A2A receptor
AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
AVPR1	vasopressin receptor
β_2AR	β_2-adrenergic receptor
B/P	blocking and permeabilizing
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid CB1 receptor
CCK	cholecystokinin
CREB	cyclic AMP response element binding protein
CRH	corticotropin-releasing hormone
D1R	dopamine D1 receptor
D2R	dopamine D2 receptor
DA	dopamine
DDM	*n*-dodecyl-β-D-maltoside
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycoltetraacetic acid
EPAC	exchange protein activated by cAMP
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
FRT	flippase recognition target
G protein	guanine nucleotide binding protein
$GABA_BR$	γ-aminobutyric acid, type B receptor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GHSR1a	growth hormone secretagogue/ghrelin receptor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HEK	human embryonic kidney
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPA	hypothalamic-pituitary-adrenal

HRP	horseradish peroxidase
HTM	human trabecular meshwork
IOP	intraocular pressure
Iso	isoproterenol
MAPK	mitogen activated protein kinase
MLCK	calcium/calmodulin-dependent myosin light chain kinase
NU-IUPHAR	International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification
OT	oxytocin
OTA	[1-D(CH2)5,Tyr(ME)2,Thr4,Tyr-NH2(9)] ornithine vasotocin
OTR	oxytocin receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDL	poly-D-lysine
PEI	polyethylenimine
PHDA	periventricular hypophyseal dopaminergic
PKA	protein kinase A
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PRL	prolactin
PrRP	prolactin releasing peptide
P/S	penicillin/streptomycin
PTX	pertussis toxin

PVN	paraventricular nucleus
RGE	receptor/G protein/effector
RLuc	*Renilla* luciferase
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFD2s	signal peptide Flag-tagged dopamine receptor D2 short
SNP	single nucleotide polymorphism
SON	supraoptic nucleus
TBS	tris-buffered saline
TBST	tris-buffered saline tween
THDA	tuberohypophyseal dopaminergic
TIDA	tuberoinfundibular dopaminergic
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
WT	wild type
WIN	WIN55,212-2

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INTRODUCTION

Overview – The Textbook View of GPCRs, Old and Revised.

Constituting one to two percent of the entire human genome, G protein-coupled receptors (GPCRs) represent the largest family of eukaryotic cell surface receptors. On this basis alone, they make for attractive drug targets and dominate therapeutic research in large measure. These seven α-helical transmembrane domain-containing proteins transduce extracellular signals from a vast array of environmental cues including hormones, neurotransmitters, peptides, lipids and photons, into cellular responses via their cognate intracellular guanine nucleotide-binding protein (G protein) partners. G proteins are heterotrimeric proteins made up of various combinations of different Gα, Gβ and Gγ isoforms, however all are composed of one Gα subunit and an obligate Gβγ dimer. Gα can be further divided into four main classes, Gαs, Gαi/o, Gαq/11 and Gα12, which differ not only in sequence, but in their functional coupling to different receptors and their downstream signalling properties as well. G proteins regulate the specificity of cellular responses to different signals [1]. In a canonical view, endogenous ligand binding to the GPCR activates initial receptor signalling events, which include stimulating its guanine nucleotide exchange factor (GEF) function. This facilitates the release of the Gα-associated guanosine diphosphate (GDP) and subsequent binding of the more prevalent guanosine triphosphate (GTP), ultimately resulting in dissociation of Gα and Gβγ subunits. Thereafter, G protein subunits act on various target effectors such as stimulation or inhibition of adenylyl cyclase (AC)

by Gαs or Gαi, respectively, activation of phospholipase C (PLC) by Gαq, and Gβγ binding to ion channels or G protein-coupled receptor kinases (GRKs), the latter resulting in recruitment of β-arrestin to GPCRs. Gα subunits possess an intrinsic guanosine triphosphatase (GTPase) activity that terminates signalling via GTP hydrolysis to GDP, and the GDP-bound Gα preferentially binds Gβγ with greater affinity over effector, which allows for the heterotrimer to re-assume its initial conformation.

Beyond this textbook explanation, GPCR signalling is considerably more complicated at every level. For instance, receptors can be modulated at multiple sites beyond the orthosteric ligand binding site. Thus, ligand occupation has been distinguished as orthosteric or allosteric depending whether a ligand occupies the endogenous ligand binding site, or another noncompetitive site, respectively. This second site (of which there can be several on a given receptor) can independently activate or inactivate the receptor, acting as allosteric ligands, or modulate the orthosteric signal, acting as an allosteric modulator, depending on the particular ligand. Binding an allosteric modulator can alter the affinity, potency, or resulting efficacy of the orthosteric ligand positively or negatively, and vice versa. The mutual interaction between the two ligands is termed allostery, since the ligands themselves are distinct, and they bind distinct sites on the receptor. Typically, allosteric sites are associated with synthetic chemical ligands, however they may bind endogenous ligands as well [2]. Despite much recent research, it is still not fully understood how GPCRs actually activate G proteins following ligand binding. Recent structural analyses show that receptor undergoes a

conformational change that facilitates guanine exchange on Gα. This conformational change is related to the G protein which contacts the receptor via its carboxy-terminus and releases GDP from its amino-terminus allowing for Gα and Gβγ dissociation after GTP binding and consequent signalling events [3]. Adding to this complexity, GPCRs have been reported to activate signalling pathways independent of the G protein altogether, or at least after G protein-dependent responses have desensitized – possibly via activation through an allosteric site [4, 5]. Lastly, GPCR signalling can involve multiple receptor equivalents acting in dimers or even higher order multimers (for a comprehensive review on GPCRs and G proteins, see [6-8]).

GPCR Oligomerization, Allostery and Asymmetry

Until recently, GPCRs were thought to signal exclusively as monomeric entities based on long accepted observations for visual signal transduction via the class A receptor, rhodopsin and its cognate G protein, transducin [9, 10]. GPCRs can signal as monomers as demonstrated for both rhodopsin as well as the β_2-adrenergic receptor (β_2AR), however, now we know a majority of GPCRs likely exist in the living cell as dimers, not monomers [11]. In other words, they probably function as protomers in both homodimers or heterodimers that assemble in the endoplasmic reticulum (ER, see [12, 13] for review).

Referring back to the notion of allostery, conceivable ligand interactions now become more complicated and numerous in the context of receptor oligomers. Equivalent ligand occupation of the same site on two different protomers within a

homodimer may involve positive or negative cooperativity, whereas ligand binding at a different site on either receptor, i.e. orthosteric and allosteric sites, represents a distinct type of allostery. For a heterodimer, orthosteric ligands at either protomer can interact allosterically with each other, and with allosteric ligands acting on either protomer (Figure 1) [2].

In most cases, dimers along with their associated G proteins, likely make up only a minimal, functional signalling unit. Ligand cooperativity studies indicate that GPCRs can actually form tetramers (reviewed in [6]). For simplicity, the term, dimer, referred to from this point on, does not exclude the possibility for oligomers, but implies at the very least more than one receptor as the basic signalling unit. The best characterized example for which dimerization is necessary for receptor function remains the class C GPCR, γ-aminobutyric acid, type B receptor ($GABA_BR$). This heterodimeric receptor is made up of two independent receptor subtypes. $GABA_BR$-2 is required for $GABA_BR$-1 to make it out to the cell surface, and both receptors are needed to signal [14-16]. Thus GPCRs should no longer be considered as simply lone signalling receptors, but as sophisticated signalling machines – sensitive to multiple allosteric inputs. The $GABA_BR$ also demonstrated asymmetry for the first time in the G protein coupling of heterodimeric receptors, both in how the protomers physically interact with G proteins, as well as to their roles in signalling. That is to say, $GABA_BR$-1 binds the ligand and transactivates $GABA_BR$-2, and $GABA_BR$-2 activates the G protein (Figure 2) [17, 18]. Thus, each protomer has a different role with respect to signalling.

More recently, the notion of transactivation in a receptor dimer led to an experimental strategy developed by Dr. Jonathan Javitch, to test as much in homodimers (Figure 3) [19]. The strategy exploits dopamine D2 receptor (D2R) constructs fused to a mutated Gαi, Gqi5, which switches typical D2R-coupled Gαi signalling to that of Gαq and calcium signalling in order to characterize the role of individual signalling units within a class A GPCR homodimer. These studies revealed that not only is the *homo*dimer physically asymmetrical with respect to the structural components of either protomer that contact the G protein, but that the two receptors within the dimer play distinct roles with regard to signalling. They noted that different combinations of ligands could bind both protomers of the homodimer simultaneously and consequently produce distinct signalling outputs, such that occupation of one receptor's orthosteric binding site allosterically modulated signalling through the other. Essentially, occupation of the unfused receptor equivalent, protomer A, is required to activate signalling, while ligand binding of the other Gqi5-fused counterpart, protomer B, allosterically modulates this signal, without signalling itself. Experiments with the D2R homodimer show that binding of both receptors by agonist resulted in a reduced signalling output (negative cooperativity) relative to an agonist at protomer A, with a non-ligand binding mutant protomer B (positive cooperativity). Asymmetry between protomers became clearer when an agonist at protomer A in combination with an inverse agonist at the protomer B produced an optimal signal (positive allostery, Figure 3). On the other hand, sole ligand occupation at protomer B resulted in no signal. Thus, one receptor, protomer A, can be thought

of as the primary signalling receptor and the other fused to Gqi5, as a silent, allosteric modulator. This phenomenon is likely to be of far greater importance in heterodimers because they can potentially organize in at least two different arrangements with respect to the G protein, which could invert the role each receptor adopts in regard to signalling *in vivo*. On the one hand, a dimer may exist in which, receptor A is allosterically modulated by a silent receptor B, and on the other hand, another dimer may exist in which, receptor B is allosterically controlled by a silent receptor A. Theoretically, these dimers no longer exhibit the same signalling properties as each other or either receptor alone. The power of the Javitch system allows the dimer to be locked in one of the two arrangements depending which receptor is fused to the G protein and be studied in isolation, which formed the grounds of my first objective.

Cells use multiple G protein-mediated signalling pathways, which may be organized around various combinations of GPCR complexes containing Gα and Gβγ subunits, effectors and multiple distinct receptors. These complexes likely establish the efficacy, kinetics and specificity of signalling in a cell-specific way [20]. Furthermore, certain ligands reportedly activate only a select number of GPCR-mediated pathways out of the total attributed to any one type of GPCR. Essentially, these ligands promote one or some signalling pathway(s) over the other(s), and are termed biased ligands (see review by [21]). The concept of intrinsic efficacy relies on the notion that one unitary outcome is possible per ligand, which involves activation of all cellular responses induced by ligand binding in the case of an agonist (or lesser efficacy in the case of a partial agonist),

or inhibition of all these agonist-mediated responses by antagonist through no intrinsic activity of its own [22, 23]. However, this notion has been debated over the years through the emergence of biased ligands based on reports demonstrating agonists can differentially activate distinct signalling pathways and that antagonists can possess intrinsic activity [24-31]. For example, two D2R agonists were recently shown to promote β-arrestin recruitment over Gαi signalling [22]. Thus, biased ligands can be described in their capacity to expand the concept of intrinsic efficacy.

The possibility for different arrangements of receptor complexes based on the same molecular components, adds another level of signalling diversity and organization. This could mean that each receptor could have distinct roles beyond signalling *per se* and can perform multiple tasks, allowing for the assembly of very diverse signalling systems that do not require additional GPCRs. The step at which it is determined whether the receptor will function as the primary signalling receptor or the silent allosteric modulator serves as another level of organization. This could be a cell-type specific phenomenon. More specifically, it may be that such decisions are regulated by the timing of receptor transcription, translation and assembly and/or Gβγ subunits, which have been implicated as potential GPCR chaperones (as reviewed in [32, 33]). In short, heterodimers are more intrinsically complicated than their homodimer counterparts because they can generate functionally different signalling complexes depending not only on the receptor they pair with, but the role each protomer adopts with respect to signalling. Furthermore, heterodimers can exhibit altered trafficking, ligand

binding and internalization patterns, some of which may be regulated by receptor allosterism within the dimer complex [13]. For these reasons, I became interested in studying allosterism and asymmetric signalling in the context of heterodimers. My project centres around three rhodopsin-like, class A GPCR heterodimers in particular, the D2R with the oxytocin receptor (OTR), β_2AR with the cannabinoid CB1 receptor (CB1R), and β_2AR with OTR, with a greater focus on the first. Subsequent discussion of these receptors will be in the context of these putative heterodimers.

Prolactin – at the heart of potential D2R and OTR functional interactions

Prolactin (PRL) is a polypeptide hormone synthesized in and secreted from specialized cells within the anterior pituitary gland, named lactotrophs [34]. PRL regulates upwards of 300 different physiological processes ranging from stress to reproduction, including that for which it was named, stimulation of lactation and mammary gland development (for a comprehensive review, see [34, 35]). The most important physiological stimuli of PRL secretion are suckling, mating, stress and ovarian steroids, namely estrogen [36-41]. These stimuli are transduced via the hypothalamus, which produces a host of PRL-releasing and -inhibiting factors.

Pituitary lactotrophs constitutively release PRL, and therefore hypothalamic control is primarily inhibitory over pituitary PRL secretion in mammals [42, 43]. Mechanisms must exist to control and downregulate PRL levels, and they include endocrine, paracrine and autocrine regulation of

lactotrophs [34, 38, 44-47]. Dopamine, a monoamine neurotransmitter, is the primary regulatory factor and a potent inhibitor of PRL expression and secretion from the anterior pituitary gland via activation of D2R localized on lactotrophs [48-52]. Dopamine activation of D2R inhibits AC and suppresses levels of intracellular cyclic adenosine monophosphate (cAMP), which leads to inactivation of protein kinase A (PKA)/cyclic AMP response element binding protein (CREB) response elements that otherwise upregulate PRL expression [53]. Dopamine is discharged tonically, via its coordinated release from three different dopaminergic neuronal populations arising from the mediobasal hypothalamus: 1) tuberoinfundibular dopaminergic (TIDA) neurons of the dorsal medial arcuate nucleus are considered the primary dopaminergic regulator of PRL secretion and project into the median eminence where hypophysial portal blood vessels carry dopamine to the anterior pituitary, and both 2) tuberohypophyseal dopaminergic (THDA) neurons of the rostral arcuate nucleus, and 3) periventricular hypophyseal dopaminergic (PHDA) neurons of the periventricular nucleus (PVN) project through the internal zone of the median eminence, to short portal vessels in the intermediate lobe of the pituitary, which draws dopamine into the anterior pituitary [34, 52, 54-58]. This falls in line with dopamine-responsive lactotrophs being more abundant in the inner rather than outer zone of the anterior pituitary, which displays the functional heterogeneity of lactotrophs [59]. The tonic release of dopamine coincides with tonic inhibition of PRL secretion.

There is a coordinated interplay of both excitatory and inhibitory factors controlling PRL secretion. Dopamine action is counteracted primarily by TIDA

neuron inhibition or disinhibition of dopaminergic signalling, as well as pituitary-derived PRL-releasing factors. However, absence or excessive inhibition of D2R signalling can lead to pituitary adenomas in humans [60]. They are primarily treated with D2R agonists to effectively decrease serum PRL and reduce tumour size [61-63]. Further physiological evidence for the inhibitory role of D2R on PRL secretion are genetic disruption of the D2R encoding gene, which eventually leads to hyperprolactinemia and progressive lactotroph hyperplasia, and D2R deficient mice, which eventually develop pituitary lactotroph adenomas [64-67]. Under normal physiological conditions, relief of dopamine signalling does not sufficiently yield the full PRL surge induced by the suckling stimulus [68]. Thus, an interest in PRL-releasing factors grew including thyrotrophin-releasing hormone, vasoactive intestinal peptide, vasopressin, serotonin, angiotensin II, and notably oxytocin [37, 69-85].

Oxytocin is a nonapeptide classically recognized for its role in lactation and parturition. The peptide is synthesized in the PVN and supraoptic nucleus (SON) of the hypothalamus from which, it is transported to and stored in the posterior lobe of the pituitary via the median eminence [34, 38]. Oxytocin is released into the long portal vessels within the median eminence leading to the anterior pituitary as well as into short portal vessels directing oxytocin from the posterior lobe to the inner zone of the anterior pituitary [86, 87]. Oxytocin stimulates PRL secretion through binding the high affinity OTR present in the anterior lobe, which resemble uterine OTRs [34, 88-90]. Furthermore, oxytocin administration to lactotrophs *in vitro* induces PRL release following an increase in

intracellular calcium, which suggests oxytocin-mediated PRL secretion is a calcium-dependent mechanism [91]. This finding is not all unexpected since OTR couples to Gαq, which activates PLC and downstream calcium release intracellularly. Oxytocin stimulates PRL secretion only under certain physiological states such as the proestrous surge of PRL, the endogenous stimulatory rhythm and mating [92-96]. Following mating stimuli, oxytocin neurons within the PVN and SON are activated, resulting in a surge of oxytocin release and subsequent rhythmic PRL secretion in rats [97-99]. Blocking peripheral OTR prevents the PRL surge, whereas relieving the block, reestablishes the PRL rhythm, further substantiating oxytocin as a PRL releasing factor [93, 100].

PRL, dopamine and oxytocin are closely linked in regulating PRL levels. Not only does an oxytocin antagonist block rhythmic PRL secretion, it downregulates the tonic dopamine release from THDA neurons, due to the consequentially lowered PRL concentration [100]. PRL regulates its own levels by stimulating dopamine signalling in a time-delayed, negative feedback loop and promoting oxytocin signalling in a positive feedback loop (Figure 4) [101]. PRL activates prolactin receptors (PRLRs) on dopaminergic neurons in the hypothalamus, resulting in increased hypothalamic dopamine synthesis and release [102-109]. The dopamine-PRL feedback loop is prevented with a PRL receptor antagonist [100]. Therefore this dopaminergic release does not occur without an increase in PRL, likely since there is no need to negatively feedback and downregulate PRL levels [38]. However, TIDA neuronal activity still remains

high prior to the PRL surge, suggesting differential regulation of PRL secretion by the neuroendocrine dopaminergic neuronal populations [110, 111]. Oxytocin-mediated PRL secretion accompanies a decrease in dopamine release, which suggests the oxytocin-PRL positive feedback loop can overcome the inhibition by dopamine [112, 113]. Indeed, the dopamine-PRL negative feedback mechanism is uncoupled for example, during lactation, wherein PRL binds PRLRs on oxytocinergic neurons in the PVN to initiate oxytocin secretion, which further upregulates PRL levels and together mutually increase milk production [114-117]. Once PRL levels reach threshold, the PRL-dopaminergic negative feedback takes over again [38]. This pattern may vary with different physiological states, but the basic mechanism remains the same in order to maintain the quiescence of lactotrophs [38].

Essentially, D2R and OTR are co-expressed in lactotroph cells of the pituitary, where they have opposing actions on PRL secretion. Beyond their shared target and potential physical association, the two receptors share grounds for other physiological interactions as well. Interestingly, D2R plays a role in olfactory-stimulated PRL secretion during pregnancy. There exists a phenomenon in mice, known as the Bruce effect, whereby newly mated females revert to the oestrus stage if exposed to unfamiliar males within seventy-two hours of initial mating [118]. In accordance with this effect, pheromones secreted by a strange male to a pregnant female, induces premature loss of pregnancy in mice, and this involves the suppression of PRL secretion by dopamine released from TIDA neurons [119-121]. Replacement of PRL reverses this abortive effect [122, 123].

OTR is largely implicated in pregnancy and parturition, which merits their functional interaction in reproduction, where D2R is destructive, and OTR is protective. The three receptors are also all associated with stress, namely restraint stress, which impacts greatly on PRL secretion. A dopamine receptor antagonist, pimozide, inhibits the restraint stress-mediated decrease in the estrogen-induced afternoon proestrus-like surge of PRL [124]. Furthermore, higher TIDA neuronal activity is seen in estrogen-treated rats exhibiting restraint stress [125]. While D2R activity is a mediator of stress, OTR and PRLR action is a consequence and attenuator of stress. The anxiolytic actions of oxytocin and PRL are supported by many studies that showed oxytocin- and PRL-mediated attenuation of the hypothalamic-pituitary-adrenal (HPA) axis, reduction of depression like behaviour, and lowered blood pressure in mice and rats [126-133]. In contrast, an OTR antagonist facilitates stress-induced activation of the HPA axis, increases anxiety-related behaviour during pregnancy or lactation, and impairs anxiolytic effects of mating in rats [128]. Human studies have shown nasal application of oxytocin reduces cortisol release following stressful stimuli, including public speaking and couple conflict [134, 135].

Restraint stress specifically, is thought to induce oxytocin secretion from the pituitary gland by activating noradrenergic neurons, which also contain prolactin-releasing peptide (PrRP) [136, 137]. PrRP-containing noradrenergic neurons may also be involved in stimulating oxytocin release following food intake, which potentially puts the PrRP-oxytocin system right at the crossroads of stress and food intake, which are closely tied in obesity, for example [138].

Cholecystokinin (CCK), an octapeptide, is a peripheral satiety signal released following food intake, and is known to increase plasma oxytocin levels in rats [139, 140]. Peripheral administration of CCK induces the release of noradrenaline within the hypothalamic SON through activation of catecholaminergic neurons in the medulla oblongata projecting to the SON, and CCK-induced activation of oxytocinergic neurons is blocked by an adrenergic receptor antagonist [141-145]. Food intake and CCK administration both also activate PrRP-expressing neurons in the medulla oblongata [146, 147]. Furthermore, oxytocin is released following food intake, whereas mRNA levels actually decrease in neurons of PVN following a two week restricted diet in rats [139, 140, 148]. Oxytocin exhibits anorexigenic actions following its release induced by food intake. Intracerebroventricular and peripheral administration of oxytocin suppresses feeding in rodents, which is reversed by OTR antagonists [149-151]. As for D2R, dopamine seems to both increase and decrease food intake depending on the context. Apart from its pro food intake function in the reward pathway, dopamine also inhibits feeding, dependent on the physical and functional association of D2R with the growth hormone secretagogue receptor, i.e. the ghrelin receptor (GHSR1a) [152]. Furthermore, D2R is expressed in adipocytes of human adipose tissue, where it inhibits PRL secretion, similar to the pituitary [53]. These cells are key players in the regulation of weight, energy expenditure and food intake.

Lastly, although the aforementioned suggests that dopamine and oxytocin signalling exhibits PRL-dependent functional interactions, they may also be involved together in a more “social” interaction, perhaps independently of PRL. It

is proposed that oxytocinergic neurons are activated after social interaction [153]. For example, plasma oxytocin levels have been shown to mirror parental behaviours in humans and increase after performing a task requiring intimate trust such as secret sharing [154, 155]. The neural pathways involved in activating oxytocinergic neurons in response to social interactions are still unclear. However, patients with dopamine-dependent behavioural disorders experience disruption of central oxytocin signalling, suggesting dopamine signalling might be important for activation of oxytocinergic neurons after social interaction [138, 156].

Insight into interactions between β_2AR and CB1R

β_2AR and CB1R are both targeted clinically with antagonists and agonists, respectively, for the treatment of elevated intraocular pressure (IOP) [157-160]. Furthermore, such increases in IOP are often associated with glaucoma, for which β_2AR antagonists are first-line drugs [161]. Apart from the eye, β_2AR and CB1R are co-expressed in numerous tissues including brain, bone, reproductive tract and parts of the cardiovascular system [162-167]. Equilibrium between aqueous humour production in the ciliary body epithelium and outflow via the trabecular meshwork and uveoscleral pathways exists to keep IOP normal in humans [168]. Not only are both β_2AR and CB1R expressed in the two tissues, both receptors help regulate this balance [164, 165, 169-171]. β_2AR typically couples to Gαs, as well as Gαi, the latter to a lesser degree [172]. CB1R typically couples to Gαi, leading to mitogen-activated protein kinase (MAPK) activation, and to some extent to Gαs and Gαq/11 as well [173-175]. Hudson et al. demonstrated using

bioluminescence resonance energy transfer (BRET) that β_2AR and CB1R interact physically when co-expressed in HEK 293 cells [161]. They also observed a number of functional interactions between the two receptors. For one, simply co-expressing β_2AR in HEK 293 cells, increased cell surface expression of CB1R and decreased CB1R constitutive activity in terms of MAPK activation. Moreover, both receptors were co-internalized following treatment with either a β_2AR or CB1R agonist. Lastly, they revealed functional interactions at the level of MAPK and CREB for both receptors in response to their respective ligands alone or in combination. They further confirmed similar interactions in primary human trabecular meshwork (HTM) cells endogenously co-expressing both receptors.

Labour contractions gave birth to the β_2AR and OTR interaction

β_2AR and OTR are two common pharmacological targets for the prevention of preterm labour contractions due to their critical roles in uterine mechanical regulation. Both receptors are co-expressed in human myometrial smooth muscle cells, but have seemingly opposing signalling in these cells. Simplistically, the Gαs-coupled β_2AR stimulates uterine relaxation via increased intracellular cAMP levels, and downstream intracellular calcium sequestration [176-178]. The Gαq-coupled OTR, on the other hand, mediates uterine contraction via increased intracellular calcium concentrations and consequently myosin light chain phosphorylation by an activated calcium/calmodulin-dependent myosin light chain kinase (MLCK) [179, 180]. OTR is also thought to mediate calcium-independent contraction via Gαi coupling that does not involve

phosphorylation and activation of MLCK, but rather activation of the extracellular signal-regulated MAPK (ERK), which phosphorylates cytoskeletal proteins [181-184]. Both OTR and β_2AR signalling lead to ERK phosphorylation and activation despite their apparently opposing functions in the uterus, which suggests the potential for more complicated interactions between the two receptors depending on the signalling output measured. A β_2AR agonist, ritodrine, inhibited oxytocin-induced calcium-free contractions pointing to an interaction at the level of ERK [183]. Indeed, Wrzal et al. demonstrated a functional interaction at the level of ERK, whereby the β_2AR agonist, isoproterenol, and antagonists, propranolol or timolol, individually inhibit OTR-mediated ERK signalling in human myometrial cells, whereas a β_2AR inverse agonist potentiated OTR-mediated ERK signalling [185]. Likewise, OTR antagonists, atosiban and [1-D(CH2)5,Tyr(ME)2,Thr4,Tyr-NH2(9)] ornithine vasotocin (OTA), each prevented β_2AR-mediated ERK signalling [185]. Under normal signalling conditions, antagonist action at its receptor alone, should result in no signalling nor change in signalling without agonist activity. However, they showed an antagonist at one receptor modulated agonist signalling at the other and vice versa. Moreover, not all β_2AR antagonists had the same effect on OTR-mediated ERK signalling. Based on the antagonistic effects and protein-protein interaction studies, they conclude the interactions between β_2AR and OTR to be allosteric in nature instead of downstream signalling crosstalk, in the context of a heterodimer [185]. Wrzal et al. showed further, that the functional interactions are in part due to the switching of β_2AR

coupling to a non-classical Gαi-linked PKC pathway when co-expressed with OTR in HEK 293 cells or human myometrial cells [186].

Rationale for this study

In collaboration with Dr. Jonathan Javitch, I started by replicating what Han et al. showed with the D2R homodimer, and noted that the D2R agonist, quinpirole, dose-dependently increased calcium signalling in cells co-expressing the untagged D2R with the D2R-fused Gqi5 protein (D2R-Gqi5), but not in cells expressing D2R-Gqi5 (data not shown) or D2R alone (see Figure 7). I tried to apply the same strategy to known heterodimers CB1R/D2R and adenosine A2A receptor (A2AR)/D2R, but was unable to detect transactivated calcium signalling of D2R-Gqi5 by either CB1R or A2AR agonists, WIN55,212-2 or adenosine, respectively [187-193]. After learning that D2R, CB1R, and A2AR form a hetero-oligomer, I decided to test the effect of CB1R and A2AR on D2R/D2R-Gqi5 homodimer signalling, however never achieved a consistent, recurring result [194]. I then simplified the experiment to include only the D2R/D2R-Gqi5 homodimer in the presence of stimulated or un-stimulated A2AR. I noted the presence of un-stimulated A2AR consistently lowered the efficacy of quinpirole-induced D2R homodimeric signalling, whereas adenosine induced no further change. In order to determine whether this was an A2AR-specific phenomenon or simply an artifact of any generic receptor competing at the level of D2R expression in a heterologous system, I tested another GPCR, OTR, in its stead. Serendipity revealed a surprising, dampening effect of oxytocin on D2R homodimer

signalling in cells co-expressing D2R and OTR (see Figure 8). These intriguing results were worth examining further and so I pursued studies of allosteric interactions in the context of a putative D2R and OTR heterodimer. Previous *in vitro* and *in vivo* studies have alluded to crosstalk between the D2R and OTR that suggest they may form dimers, including their colocalization in lactotroph cells and a shared downstream target, PRL secretion. Based on the text above, there are putative physiological interactions of dopamine and oxytocin, however no direct molecular or mechanistic link has been made in any of the aforementioned settings. Therefore I begin here by analyzing potential interactions of D2R and OTR at the molecular level, both physical and functional.

Beyond signalling, I would also like to examine the consequences of ligand occupancy on the conformational dynamics and asymmetries within GPCR/G protein/effector (RGE) complexes. I was not ready to give up on the CB1R completely, and wanted to test it in the context of my second aim with the β_2AR. The two receptors are co-expressed endogenously and functionally interact *in vitro* in HTM cells as well as HEK 293 cells, in which they have reportedly physically interacted [161]. β_2AR and OTR also make an interesting pair to study conformational dynamics since they have already been shown to interact functionally, both allosterically and physically, in the form of a heterodimer [185, 186]. I want to determine how conformational changes in β_2AR/CB1R and β_2AR/OTR heteromeric complexes manifest in accordance with these interactions following a similar ligand regimen as described in earlier studies from the lab [186]. In other words, we want to see if what holds true for downstream signalling,

holds true at the level of the RGE complex. For instance, β_2AR typically signals through Gαs and OTR through Gαq. However pertussis toxin (PTX) was capable of inhibiting agonist stimulated β_2AR-mediated ERK1/2 phosphorylation only when co-expressed with OTR, likely a consequence of their dimerization, suggesting the heteromer favours coupling to Gαi [186]. Taking advantage of the BRET technique, it would be interesting to see if this would be reflected in terms of conformation, in that BRET between the receptor and Gαi would be more favourable relative to Gαs when β_2AR is co-expressed with OTR versus alone.

Hypothesis

I hypothesize that heterodimeric GPCRs will have distinct signalling outputs based on their organization with respect to the G protein, which would be influenced by the presence of different ligands. More specifically, ligands for either the D2R or OTR will affect signalling through the other depending on the class of ligand and pathway assayed, which may reflect and explain their roles physiologically. I also predict that ligands at one or both receptors will induce conformational changes within the β_2AR/CB1R and β_2AR/OTR complexes that mirror and influence the signalling outcomes reported previously.

If allosteric modulation does occur, we predict that the conformational dynamics measured by BRET, aequorin, and other signalling assays will be sensitive to 1) the ligand(s) involved (i.e. an agonist at one protomer alone or in combination with an agonist, antagonist or inverse agonist at the other protomer

and vice versa), and 2) the conformational vantage point taken, in terms of the BRET pair as discussed below.

Objective

Based on the notion of allosteric communication between homo- and heterodimers, I proposed to study the implications at the level of complexes containing receptors, G protein and effector in response to different combinations of ligands at each receptor equivalent. Firstly, I wanted to study consequences of signalling upon ligand occupation specifically at D2R and/or OTR, using a combination of effector assays. Secondly, I wanted to know how the physical asymmetries within a heterodimeric RGE complex manifest in parallel to the signalling asymmetries for the β_2AR/CB1R and β_2AR/OTR heterodimers.

To address the first objective, I tested D2R and OTR expressed alone or together in HEK 293 cells in the presence of different combinations of ligands at either receptor using a collaboration of signalling effector assays. Combinations of ligands included one agonist or antagonist, dual agonist, agonist at one receptor and antagonist at the other and vice versa. I assessed D2R involvement in OTR-mediated calcium signalling, OTR involvement in D2R-mediated inhibition of cAMP signalling, and D2R and OTR effects on the partner ERK signalling. I also looked at the effects stimulation of one receptor has on internalization patterns of the other and vice versa using immunofluorescence and confocal microscopy. In addition to the functional interactions, I wanted to assess possible physical

associations between the two receptors by performing competition BRET studies and immunoprecipitation assays.

The second objective used BRET to study positional and conformational changes within RGE complexes. Different components of the RGE complex in pairs were tagged with *Renilla* luciferase (RLuc) or green fluorescent protein (GFP), to test different known ligands in the heterodimeric context of two receptors, either β_2AR and CB1R or β_2AR and OTR. By repositioning the RLuc between different Gα and Gβγ classes, either protomer, or the effectors, for example, AC, I could potentially map the dynamics of signalling complexes containing multiple receptors, G proteins and effectors following ligand occupation on one or both receptors from different conformational vantage points (Figure 5). Preliminary data suggested that if I were to continue down this path however, I would have to commit to a Doctor of Philosophy (PhD) – or a "Pledge of Heavy Dedication," which I am not prepared to do at this point in my life. That being said, I focused my time on a Master of Science (MSc) – or a "My Summer Closure," more realistically on the first objective.

MATERIALS & METHODS

Reagents and Antibodies

Reagents were obtained from the following sources: high glucose Dulbecco's Modified Eagle Medium 1X (DMEM), fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) were from Wisent (St-Bruno, QC); G418 sodium sulfate, blasticidin, and hygromycin were also from Wisent (St-Bruno, QC), and zeocin from InvivoGen (San Diego, CA); Lipofectamine 2000 was from Invitrogen (Carlsbad, CA) and polyethylenimine (PEI) from Polysciences (Warrington, PA); oxytocin, quinpirole, and isoproterenol were all from Sigma-Aldrich (St. Louis, MO); OTA was obtained from Bachem (Torrence, CA); raclopride was from Tocris Bioscience (Bristol, UK); coelenterazine h and coelenterazine 400a were obtained from Biotium (Burlington, ON); PermaFluor Aqueous Mounting Medium was from Thermo Fisher Scientific (Fremont, CA). Monoclonal anti-HA from raw ascites (Covance purchased from Cedarlane Labs (Hornby, ON)) and polyclonal anti-Flag (Sigma-Aldrich, St. Louis, MO) were used at 1:5000 and 1:3000, respectively, for western blot analysis, and 1:1000 for immunofluorescence, polyclonal Anti-ERK/p44/42 (T202/Y204, Cell Signaling Technology, Denver, MA) 1:1000, monoclonal anti-GAPDH (Ambion, Streetsville, ON) 1:5000, goat-anti-mouse-Alexa488 and goat-anti-rabbit-Alexa555 (Invitrogen, Burlington, ON) both used for immunofluorescence at 1:1000, secondary antibodies horseradish peroxidase-conjugated (HRP) anti-rabbit and anti-mouse (Sigma-Aldrich, St. Louis, MO) 1:20000 and anti-Flag M2

agarose beads and 3X Flag peptide (Sigma-Aldrich, St. Louis, MO). Polyclonal anti-ERK-CT was generously contributed by Dr. Bruce Allen (Montréal Heart Institute, Montréal, QC) and used at a dilution of 1:5000.

DNA Constructs

The inducible, signal peptide Flag-tagged dopamine D2 receptor short (SFD2s/FRT/TO, referred to as Flag-D2R in the results section) expression plasmid was generously donated by Dr. Jonathan Javitch (Columbia University, New York, NY, see [19]). D2R and Gγ2-GFP10 were from Dr. Michel Bouvier's laboratory (Université de Montréal, Montréal, QC). *E*xchange *P*rotein *A*ctivated by *c*AMP (EPAC) tagged with RLuc at its carboxy-terminus and tagged with citrine GFP at its amino-terminus was obtained from Dr. Ali Salahpour (University of Toronto, Toronto, Ontario) and was validated previously [195]. Flag-Gβ1 was obtained from the UMR cDNA Resource Center (www.cdna.org). HA-β_2AR was generated in our laboratory and verified by sequencing. Darlaine Pétrin, in our lab, generated HA-OTR, HA-OTR-Venus (OTR-Venus), cMyc-OTR-RLuc (OTR-Rluc) and the ACII-RLucII constructs. All sequences were verified using bidirectional sequencing analysis. The first three were created based on a hOTR-YFP plasmid template obtained from Dr. Hans Zingg (McGill University, Montréal, QC). OTR was amplified by polymerase chain reaction (PCR) using the following oligonucleotides: for HA-OTR, forward 5'-ttatgcctgcggatccgagggcgcgctcgcagccaact -3' and reverse 5'-tttaaacgccggatctcacgccgtggatggctggga -3', for HA-OTR-Venus, forward 5'-

ttatgcctgcggatccgagggcgcgctcgcagccaact -3' and reverse 5'-cgccacctccggatcccgccgtggatggctggga -3', and for cMyc-OTR-RLuc, forward 5'-ggacctgtgcggatccgagggcgcgctcgcagccaact -3' and reverse 5'-cgccacctccggatcccgccgtggatggctggga -3'. The PCR fragments were introduced into restriction enzyme, BamHI, linearized pIRESpuro3-HA, pIRESpuro3-HA-Venus, or pIREShyg3-cMyc-RLuc plasmids, respectively, by recombination using the In-Fusion cloning system (Clontech Laboritories, Mountain View, CA). All three newly generated constructs were mutated at a single nucleotide to yield an alanine amino acid at position 218, which is the most commonly found single nucleotide polymorphism (SNP) in the population, instead of threonine. The ACII-RLucII construct was generated by subcloning rat ACII into a pRLuc-N2 vector, and the RLuc was replaced by *Renilla* luciferase II (RLucII). Rat ACII was removed from another plasmid using the NheI restriction enzyme and the 5' ends were filled using Klenow fragment. The resulting blunt-ended DNA fragment was ligated into restriction enzyme, SmaI, linearized pRLucII. Gαs-RLuc8 and Gαi2-RLuc8 constructs were gifts from Dr. Céline Galés (INSERM, Toulouse, France). RLucII and *Renilla* luciferase 8 (RLuc8) have improved enzymatic properties and consequently enhanced BRET over RLuc.

Cell Culture and Transfection

Flp-In T-REx-293 cells were stably transfected with apo-aequorin-containing vector, pcin4, designated Flp-In T-REx-293 pcin4AEQ#3 cells. This cell line was further transfected with SFD2s in pcDNA5/FRT/TO vector,

designated Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO. Flp-In T-REx-293 pcin4AEQ#3, Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO (these were both generated and donated by Dr. Jonathan Javitch) and HEK 293F cell lines were cultured in high glucose DMEM, supplemented with 5% FBS and 1% P/S at 37ºC with 5% CO_2. Flp-In T-REx-293 pcin4AEQ#3 and Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO cells were grown in the presence of G418 sodium sulfate (700 μg/mL) to maintain selection of the apo-aequorin-containing vector, pcin4, and blasticidin (15 μg/mL) to maintain selection of the pcDNA6/TR vector that allows for expression of the Tet repressor gene, which controls expression of genes of interest within the pcDNA5/FRT/TO vector. In this case the gene of interest is SFD2s. Flp-In T-REx-293 pcin4AEQ#3 cells are also grown in presence of zeocin (10 μg/mL) to maintain selection for the Flp-in target site vector, pFRT/lacZeo, which introduces the flippase recognition target (FRT) for recombination of the pcDNA5/FRT/TO vector. Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO cells were also grown with hygromycin (100 μg/mL, Wisent) to maintain selection of the pcDNA5/FRT/TO vector, which expresses the gene of interest, SFD2s, only when induced with tetracycline. Cells plated in six-well plates or in T75 flasks were transfected when they reached approximately seventy percent confluence. Transfections for BRET in HEK 293F cells were performed using PEI (1 mg/mL stock) at a DNA to PEI ratio of 1:3. Lipofectamine 2000 (Invitrogen) was used for transfections in all other experiments at a DNA to lipofectamine ratio of 1:2 (with the exception of the confocal and immunoprecipitation experiments for which the ratios were 1:1 and 1:2.5,

respectively). Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO cells were induced with tetracycline (1 μg/mL) approximately twenty-four hours before harvest for expression of the transfected D2R in pcDNA5/FRT/TO. All cells are harvested about forty-eight hours post transfection.

Aequorin Assay

Flp-in Trex pcin4 Aeq#3 or Flp-in Trex pcin4 Aeq#3 SFD2s/FRT/TO cells were transfected with receptors of interest in poly-D-lysine (PDL, 100 μg/mL)-treated six-well plates and induced with tetracycline (1 μg/mL), for stable and transient SFD2s/FRT/TO expression, twenty-four hours before harvest. Forty-eight hours after transfection, cells were washed twice with Krebs buffer (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 and 5.9 mM glucose) at room temperature (RT), then were re-suspended in 2 mL Krebs buffer supplemented with 1 mM $CaCl_2$, in the presence of 5 μM coelenterazine *h*. After four hours rotating at RT in the dark, luminescence was measured using the BioTek Synergy 2 Multi-mode Microplate Reader (Fisher Scientific, Ottawa, ON). For single drug treatments, 50 μL of a 2X concentration of the desired compound was injected into wells of a 96-well microplate (white Optiplate; PerkinElmer, Waltham, MA) containing 50 μL of cell suspension. For dual drug treatments, 25 μL of a 4X or 2X (for half logs) concentration of each desired compound or vehicle, was added manually (in case of antagonist and its vehicle) or injected per well of a 96-well microplate containing 50 μL of cell solution. For treatment with three drugs, 20

μL of a 5.5X or 2.75X (for half logs) concentration of each desired compound or vehicle was added manually or injected per well of a 96-well microplate containing 50 μL of cell solution. Luminescent signals were measured for the first fifteen seconds after injection and averaged per concentration to be plotted as dose-response curves.

EPAC Assay

HEK 293F cells were transfected in PDL-treated six-well plates with receptors of interest and the EPAC construct. Forty-eight hours post-transfection, cells were washed twice with phosphate buffered saline (PBS) 1X (137 mM NaCl, 2.7 mM KCl, 10.3 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5)-RT and re-suspended in 500 μL PBS 1X-RT per well. The cells were harvested and 70 uL distributed per well of a 96-well microplate (white Optiplate; PerkinElmer, Waltham, MA). The plate was left to incubate for two hours at RT in the dark. For inhibitor treatments, 1 μM OTA or 1 μM raclopride final was added to cells for the last fifteen minutes, and for non-inhibitor studies, vehicle was added instead. For the raclopride dose response curves, increasing concentrations of raclopride were added at this point. The EPAC assay is based on BRET and signals were obtained on a BioTek Synergy 2 Multi-mode Microplate Reader (Fisher Scientific, Ottawa, ON) using 458/20-nm (RLuc) and 528/20-nm (GFP10) band pass filters. Following the fifteen-minute vehicle or antagonist pretreatment, total fluorescence was measured first, then immediately after, 50 μM coelenterazine h (Biotium, Burlington, ON) was added per well and total luminescence measured for five

minutes. The average of these BRET ratios represent the basal BRET of the cells. The cells were then treated with 25 μM forskolin alone, or simultaneously with increasing concentrations of quinpirole with or without 100 nM oxytocin, increasing concentrations of oxytocin with or without simultaneous 25 μM forskolin, and BRET ratios were read for one hour. BRET ratios collected over the final five minutes were averaged to represent final BRET. Net BRET was calculated by subtracting basal BRET from final BRET. The data was plotted as dose-response curves, in which the Y-axis represents the percent of maximum forskolin-induced response for each experiment, which was set at one.

MAPK Assay

HEK 293F cells were transfected in PDL-treated six well plates with receptors of interest. Forty-eight hours post-transfection, cells were serum-starved for ninety minutes at 37ºC. The cells were transferred to a 37ºC water bath following one hour. For inhibitor studies, cells were pretreated with one of two antagonists or their respective vehicles diluted in DMEM for the last fifteen minutes: 1 μM OTA or H_2O, or 1 μM raclopride or DMSO. Cells were then treated with one of two agonists or their respective vehicles diluted in DMEM for five minutes: 1 μM quinpirole or H_2O, or 100 nM oxytocin or H_2O. Following treatments for all conditions, cells were washed twice with PBS 1X on ice and then lysed in lysis buffer (50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethyleneglycoltetraacetic acid

(EGTA), 1 mM Na_3VO_4, 1% Triton-X-100, 1 μM microcystin, 5 mM dithiothreitol (DTT), 10 μg/mL leupeptin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) for five minutes. Cells were scraped and collected on ice. Samples were spun down at 16,100 X *g* at 4ºC for ten minutes and the supernatant subjected to protein quantification. 50 μg per sample was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE**)** for western blot analysis.

Crude Membrane Preparation and Solubilization

HEK 293F cells were grown in T75 flasks, and transfected with SFD2s/FRT/TO and/or HA-OTR at about 70% confluence. Cells transfected with the SFD2s/FRT/TO (Flag-D2R) were induced with tetracycline 1 μg/mL approximately twenty-four hours prior to harvest. Thereafter, cells were washed twice with 4ºC PBS 1X and re-suspended in 10 mL lysis buffer (5 mM Tris-HCl pH 7.5, 2 mM EDTA, trypsin inhibitor 5 μg/mL, benzamidine 10 μg/mL and leupeptin 5 μg/mL) on ice. Samples were then homogenized using the Polytron (Ultra Turrax T18 basic, IKA) twice for ten seconds each at fifty percent maximal setting. Cellular debris were spun down with a 209 X *g* spin at 4ºC and the supernatant was collected and further centrifuged with a 30,600 X *g* spin for twenty minutes at 4ºC. Pelleted crude membrane preparations were re-suspended in 0.5-1 mL solubilization buffer (0.5% *n*-dodecyl-β-D-maltoside (DDM), 75 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 2 mM EDTA, trypsin inhibitor 10 μg/mL, benzamidine 50 μg/mL and leupeptin 5 μg/mL) and incubated overnight on a

rocker at 4ºC. The next day, insoluble fractions were separated from solubilized membranes by a thirty second spin at 5,900 X *g* at 4ºC. Samples were quantified for protein content and 40 μg of solubilized crude membranes were kept at -20ºC prior to immunoprecipitation for western blot analysis, which represents the total cell lysate and protein input prior to immunoprecipitation (Fraction A).

Immunoprecipitation

25 μL Flag M2 agarose beads were pre-washed three times with solubilization buffer. 700 μg of solubilized crude membranes as collected in above text, were incubated with the beads on a rocker, overnight at 4ºC. The next day, the supernatant was collected and 60 μL was kept at -20ºC for western blot analysis to assess for non-binding proteins (Fraction B). The beads were washed three times in solubilization buffer and remaining bound proteins eluted with 150 ng/μL 3X flag peptide in Tris-Buffered Saline 1X solution (TBS 1X, 50 nM Tris-HCl pH 7.5 and 150 mM NaCl). The supernatant was collected and 60 μL separated for western blot analysis, which represents the immunoprecipitated protein (Fraction C).

Western blotting

Protein samples were diluted in 4X loading buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 16.3% glycerol, 0.025% bromophenol blue and 5% β-mercaptoethanol) and heated at 65ºC for fifteen minutes prior to analysis by SDS-PAGE using 10-12% acrylamide gels. Following electrophoresis,

proteins were transferred onto activated PVDF membranes (BioRad). Membranes were then blocked with 5% milk dissolved in TBS 1X-0.1% Tween 20 solution (TBST 1X) for one hour at RT in order to minimize nonspecific antibody binding. Thereafter, the membranes were incubated overnight at 4ºC with primary antibody in 5% milk at the appropriate dilutions listed in the *Reagents and Antibodies* section above. The next day, membranes were washed three times with TBST 1X prior to a one-hour incubation at room temperature with the appropriate HRP-coupled secondary antibody in 5% milk at a 1:20000 dilution. After three more washes in TBST 1X, proteins were visualized with Plus ECL (PerkinElmer). To assess for ERK1/2 activation, anti-pERK and anti-ERK primary antibodies were used as previously described [196].

Competition BRET Assay

HEK 293F cells were transfected in PDL-treated six-well plates with OTR-venus, OTR-RLuc and increasing amounts of untagged HA-OTR or D2R. Forty-eight hours post-transfection, cells were washed twice with PBS 1X-RT and re-suspended in 500 μL PBS 1X-RT per well. 80 μL of cells were plated per well of a 96-well Optiplate (PerkinElmer) and analyzed for BRET on the BioTek Synergy 2 Multi-mode Microplate Reader (Fisher Scientific, Ottawa, ON) using 458/20-nm (RLuc) and 528/20-nm (GFP10) band pass filters. Total fluorescence was measured before collecting BRET values, and total luminescence measured ten minutes from the start of reading to ensure similar biosensor expression across conditions. 5 μM coelenterazine h was added per well, and BRET measurements

started three minutes after. Background BRET was measured in cells expressing the RLuc construct alone, without GFP such that resonance energy transfer between RLuc and GFP could not occur. Net BRET was calculated as the difference between BRET for each amount of cold receptor used and background BRET. The data were plotted as bar graphs whereby net BRET was plotted against increasing amounts of untagged receptor.

Confocal Immunofluorescence Microscopy on Permeabilized Cells

HEK 293 cells were plated on PDL-treated glass coverslips in six-well plates and transfected twenty-four hours after with SFD2s/FRT/TO (Flag-D2R) and/or HA-OTR. Following another twenty-four hours, cells transfected with the SFD2s/FRT/TO were induced with tetracycline 1 μg/mL. Approximately twenty-four hours later, cells were starved in serum-free media for thirty minutes at 37ºC. Cells were then treated with 1 μM quinpirole or 100 nM oxytocin final for forty minutes at 37ºC. Thereafter, cells were fixed with 4% paraformaldehyde for five minutes at RT. Following three washes with PBS 1X-RT, cells were incubated with a blocking and permeabilizing (B/P) solution containing 2% bovine serum albumin (BSA) and 0.05% Triton-X-100 in PBS 1X-RT for twenty minutes at RT in order to simultaneously minimize nonspecific antibody binding and allow antibody binding intracellularly. Cells were then incubated with primary antibodies, mouse-anti-HA and rabbit-anti-Flag, diluted 1/1000 in B/P solution for one hour at RT. Following another three washes in PBS 1X-RT, cells were incubated in secondary antibodies, anti-mouse conjugated to fluorophore

Alexa488 and anti-rabbit conjugated to fluorophore Alexa555, diluted 1/1000 in B/P solution for forty-five minutes at RT. The cells were washed four more times with PBS 1X-RT and mounted onto microscope slides with PermaFluor mounting agent. Images were collected using a Zeiss LSM-510 Meta laser scanning microscope (Zeiss, Toronto, ON).

Bioluminscence Resonance Energy Transfer

HEK 293F cells were transfected in PDL-treated six-well plates with proteins fused to one of two biosensor tags, RLuc- (donor) or GFP- (acceptor), as well as receptors of interest and Flag-Gβ1. Forty-eight hours after transfection, cells were washed twice with PBS 1X-RT and re-suspended in 500 μL PBS 1X-RT per well. The cells were harvested and 80 μL plated per well of a 96-well microplate (white Optiplate; PerkinElmer, Waltham, MA). Experiments were performed using 2 mM coelenterazine 400a per 96-well (Biotium, Burlington, ON) and signals measured on a BioTek Synergy 2 Multi-mode Microplate Reader (Fisher Scientific, Ottawa, ON) using 400/30-nm (RLuc) and 516/20-nm (GFP10) band pass filters.

The BRET ratio is determined by the amount of fluorescence relative to the luminescence. RLuc luminescence values and GFP fluorescence levels were measured for each experiment to ensure similar biosensor expression across conditions. Therefore differences in BRET across conditions cannot be attributed to variations in their expression levels. Increased BRET ratios indicate a decrease in distance between the two, tagged proteins, whereas decreased BRET indicates

an increase in the distance between them. The BRET background served as a negative control, and was determined in cells expressing the RLuc-tagged protein alone such that resonance energy transfer between RLuc and GFP could not occur.

Data Analysis

All data were plotted, and statistics calculated using GraphPad Prism.

RESULTS

Transactivation of shared G proteins within the D2R homodimer

Initially, I replicated what Dr. Jonathan Javitch published with the D2R homodimer in my own hands. The Javitch system is made up of three components. First, it uses a functional signalling assay, aequorin, in which Gαi-coupled receptors, such as D2R, do not report. However, the second component is fusion of one receptor to a mutated G protein, Gqi5, which is essentially a PTX-insensitive Gαq that allows Gαi-coupled GPCRs to activate calcium signalling. The last five amino acids of Gαq are replaced with those of Gαi1 to alter G protein coupling, and the fourth cysteine residue in the carboxy-terminus was mutated to isoleucine to render the G protein PTX-resistant, which allows silencing of background signalling from endogenous Gαi-coupled receptors following treatment with PTX [197]. Basically, D2R is forced to talk to Gqi5 and calcium signalling. If the fusion linker between receptor and G protein is long enough, D2R can stimulate calcium release. However the linker is purposely made short enough, such that steric hindrance prevents the receptor's ability to productively interact with the associated G protein. Co-expression of the third component, an unfused D2R construct, rescues signalling (Figure 6), suggesting the unfused protomer A, either transactivates the fused protomer B, which activates the G protein, similar to the $GABA_BR$, or directly interacts with the G protein. The beauty of this approach is that it allows study of what each receptor in the dimer does in isolation. I repeated this in Flp-In T-REx-293 pcin4AEQ#3

SFD2s/FRT/TO cells, which are a special strain of HEK 293 cells expressing inducible D2R and constitutive apo-aequorin. Apo-aequorin is a calcium-sensitive photoprotein integral to the aequorin assay. Essentially, following receptor activation, Gαq or Gqi5 signals through the PLC pathway and consequently, calcium released downstream binds apo-aequorin, inducing a conformational change in the protein, which subsequently oxidizes or excites its substrate coelenterazine. Coelenterazine emits a measurable blue light upon returning to ground state. Thus in the Javitch system, intracellular calcium production and ultimately luminescent output of mitochondrial aequorin is proportional to the level of transactivation between the two protomers. In collaboration with the Javitch group, using this approach and set of tools, I was able to optimize the system and replicate their results in my hands, whereby a D2R selective agonist, quinpirole, seemed to stimulate dose-dependent transactivation of D2R-Gqi5 by unfused D2R (Figure 7). When expressed alone, neither the unfused receptor nor the fusion protein was able to signal (data not shown).

Presence of OTR affects D2R homodimeric signalling

As mentioned above, the path leading to the study of OTR for functional interactions with D2R was a serendipitous one. The first instance that revealed potential for their interaction was OTR co-expression in the D2R/D2R-Gqi5 aequorin system. D2R-Gqi5 was transfected transiently in an inducible Flag-D2R stable cell line, and calcium signalling assessed in the presence or absence of OTR, which was initially picked as a negative control for experiments described

in the introduction. As expected, quinpirole induced a dose-dependent increase in calcium mobilization in cells expressing wild type (WT) D2R and D2R-Gqi5 alone, and this is unaffected by OTR agonist and antagonist, oxytocin and OTA, respectively (Figure 8a). Interestingly, oxytocin pretreatment blocked D2R homodimeric calcium signalling in cells co-expressing OTR, and this effect was blocked with OTA (Figure 8b). I repeated the experiment with OTA alone to ascertain whether this effect is simply due to receptor occupancy, in which case a similar effect as seen with oxytocin might have been seen in response to OTA, similar to the β_2AR/OTR dimer pair [185, 186]. However, OTA alone was incapable of modifying D2R homodimeric signalling with or without OTR co-expression, suggesting receptor occupancy alone is not responsible for the effect (Figure 8c). It is possible the decrease in homodimeric D2R/D2R-Gqi5 mediated calcium signalling is a mark of functional interaction with OTR, however it cannot be ruled out, that oxytocin pretreatment may induce D2R co-internalization with OTR (see below), thus explaining the decrease in signalling. It is also possible intracellular calcium stores were decreased or quenched, following oxytocin pretreatment and action on OTR homodimers or oligomers, thus producing the lowered D2R homodimeric signalling. In an effort to determine the likelihood of this, endogenous HEK 293 muscarinic receptors, M1, M3, and M5, which naturally couple to Gαq, were assessed for changes in calcium signalling in the presence of OTR. Pretreated OTR had little effect on muscarinic receptor-mediated calcium mobilization (data not shown), suggesting pretreatment with oxytocin alone does not exhaust intracellular calcium stores.

Regardless, since D2R-Gqi5 and OTR both couple to calcium signalling, it was necessary to move away from the artificial fusion system. Thus I analyzed calcium signalling in terms of potential D2R modulation on OTR-mediated calcium mobilization in a less proscribed system as well as potential functional interactions between D2R and OTR in the context of different effectors, cAMP and ERK.

Modulation of OTR-mediated calcium signalling by different classes of D2R ligands

OTR typically couples to Gαq, resulting in downstream release of calcium from intracellular stores. Cells stably expressing apo-aequorin (Flp-In T-REx-293 pcin4AEQ#3) were transiently transfected with OTR, with or without D2R, to determine the effect of D2R on OTR-mediated calcium mobilization in the presence of different ligands. Oxytocin dose-dependently increases calcium mobilization in cells expressing OTR, and also on cells co-expressing D2R, though to a lesser extent (Figure 9a). Oxytocin is unable to elicit such response in cells expressing empty vector or D2R alone. The oxytocin-mediated response in cells expressing OTR, with or without D2R, was blocked by the OTR antagonist, OTA, which confirms the response is specific to OTR (Figure 9b). OTA pretreatment alone had no effect on calcium signalling. The reduced response in cells expressing both receptors may be a result of competition at the level of protein expression, competition for dimerization partners, and/or internalization (discussed below). The oxytocin-mediated response was unaffected by a D2R-

selective agonist or antagonist, quinpirole or raclopride, respectively, in cells expressing OTR alone (Figure 10a). However, when cells expressing both D2R and OTR were exposed to simultaneous oxytocin and quinpirole treatment, the efficacy of oxytocin-mediated calcium signalling increased relative to oxytocin treatment alone, and this effect was blocked with raclopride pretreatment, suggesting the phenomenon is specific to agonist stimulation of the D2R (Figure 10b). Quinpirole was unable to induce a dose-dependent increase in cells expressing empty vector, D2R or OTR alone, or D2R and OTR together (data not shown), which is not surprising since WT D2R does not couple to Gαq. This data suggests agonist stimulation of D2R positively modulates OTR-stimulated signalling *in vitro*. This result reveals a potential synergistic interaction between oxytocin and quinpirole, which alone have less and no effect, respectively. Whether this modulation is allosteric in nature or through canonical signalling crosstalk downstream of each receptor remains to be determined. Raclopride was able to prevent the quinpirole-induced increase in oxytocin-mediated calcium signalling, however raclopride treatment in the absence of quinpirole, had no effect on oxytocin-mediated calcium mobilization (Figure 11a,b). Thus, the effect of quinpirole on OTR calcium signalling is not simply due to D2R occupancy, and requires the presence of the OTR ligand. This suggests quinpirole binding to the D2R acts as an allosteric modulator of OTR.

Modulation of D2R-mediated cAMP inhibition by different classes of D2R and OTR ligands

D2R classically couples to Gαi, which counteracts AC activation through Gαs and hence inhibits cAMP production. HEK 293 cells transiently expressing D2R and a BRET sensor for cAMP based on the exchange protein activated by cAMP (EPAC) with or without OTR were assessed for potential effects of OTR on D2R-mediated cAMP inhibition. The EPAC assay relates BRET values in terms of cAMP production such that a decrease in BRET indicates an increase in cAMP. Essentially, EPAC is a dual biosensor, whereby the protein undergoes a conformational change that forces the sensors further apart upon cAMP binding, and thus a decrease in basal BRET. With that said, cAMP production needs to be stimulated in order to study inhibition or essentially decreased cAMP production. To accomplish this, cells were co-treated with D2R agonist and forskolin, which is a direct activator of AC. Forskolin administration with PBS, represents the maximal cAMP production in my assays. Quinpirole dose-dependently decreased forskolin-mediated cAMP production, suggesting quinpirole mediates inhibition of AC in a dose-dependent manner, as expected (Figure 12a,b). This effect was abrogated by pretreatment with raclopride, suggesting the response is specific to D2R (Figure 13). The presence of unoccupied or oxytocin-treated OTR did not elicit a significant change in quinpirole-mediated cAMP inhibition compared to D2R alone (Figure 14a). Similarly, pretreatment with OTA had no effect on D2R-mediated cAMP signalling regardless if OTR was present or not (Figure 14b). OTR is not reportedly linked to the cAMP pathway, and as anticipated, increasing concentrations of oxytocin treatment alone or co-treatment with forskolin, had no

effect on cAMP signalling in cells expressing D2R with or without OTR (data not shown).

No OTR ligand tested until this point seemed to modify D2R signalling in the context of cAMP synthesis, however the D2R antagonist, raclopride, generated intriguing results detected only in the presence of OTR. Raclopride dose-dependently decreased cAMP production in HEK 293 cells expressing both D2R and OTR, and more potently than in cells expressing D2R alone (Figure 15). Contrary to the expected null effect of a neutral antagonist in the absence of agonist, the typical D2R-selective antagonist, raclopride, had an effect on its own. Raclopride seemed to exhibit inverse agonist activity at certain concentrations in cells expressing D2R alone, suggested by increased forskolin-mediated cAMP production. In cells expressing both receptors, raclopride dose-dependently decreased forskolin-mediated cAMP production, similar to, though with lower efficacy than quinpirole. Thus, raclopride conversely seemed to act as a D2R partial agonist, at least under conditions of co-expression with OTR. This data suggests raclopride is actually a biased ligand, which exhibits different properties for different signalling pathways. Furthermore, potential D2R-interacting partners, such at the OTR, influenced the nature of raclopride signalling.

Modulation of D2R and OTR-mediated ERK phosphorylation by partner ligands

Both D2R and OTR have been implicated in activating MAPK, and therefore I wanted to study potential changes in MAPK activity in response to

their ligand occupied partner, i.e. OTR and D2R, respectively. Ligand combinations were limited to agonist and antagonist or inverse agonist, since both receptors stimulate ERK phosphorylation, and thus dual agonist would likely mask their mutual effects. Firstly, I carried out agonist stimulation time courses to determine the optimal time frame for treatment with oxytocin and quinpirole, which were both identified at five minutes (data not shown). Secondly, I wanted to determine ideal concentrations of antagonist to use, such that they noticeably blocked agonist stimulation of their respective receptors. 1 μM raclopride pretreatment was capable of blocking 100 nM quinpirole-mediated ERK phosphorylation in HEK 293 cells expressing D2R, with or without OTR co-expressed (Figure 16). This suggests the response is specific to D2R, and furthermore, quinpirole was unable to elicit a response in cells expressing OTR alone. Likewise, 1 μM OTA pretreatment abrogated 100 nM oxytocin-induced ERK phosphorylation (Figure 17). Oxytocin failed to induce ERK activation in cells expressing D2R alone. Raclopride and OTA treatment alone, respectively, did not result in ERK activation. It appears as though the presence of un-stimulated D2R had little effect on oxytocin-mediated ERK-phosphorylation, when compared to cells expressing OTR alone, although the experiment would need to be repeated a number of times to confirm this. It is difficult to make conclusions about the effect that the presence of OTR had on D2R-mediated ERK signalling since the levels of D2R expression were not equal between cells expressing it alone, or together with OTR. Unlike the untagged D2R, transfection of HA-OTR was optimized such that equal expression was assured in cells

expressing OTR alone or together with D2R (data not shown). It was difficult to do the same for D2R since the construct lacks a suitable epitope tag. In preliminary experiments, D2R was transfected in the same manner as OTR. Quinpirole-mediated ERK activation was unaffected by pretreatment with OTA (Figure 18) and oxytocin-mediated ERK activation was likewise unperturbed by pretreatment with raclopride (Figure 19). Inverse agonists and different antagonists should be tested further for potential changes in ERK activation. Based on the ligands tested, D2R and OTR may not modulate each other in terms of ERK signalling, suggesting that only a subset of signalling pathways are modulated allosterically in the context of a putative heterodimer.

Physical interactions between D2R and OTR

In order to assess potential physical interactions between D2R and OTR, I used two experimental strategies, beginning with immunoprecipitation. The two receptors co-immunoprecipitated in HEK 293 cells only when they were co-expressed in the same cells, but not in membranes from cells expressing each receptor alone, nor when these membranes were mixed (Figure 20). Furthermore, D2R pulled down both immature and mature molecular weight forms of OTR suggesting the two receptors interact in the ER during receptor biosynthesis as well as at the plasma membrane, respectively. The early interaction is missed in mixtures of solubilized membranes expressing each receptor alone, and therefore substantiated by the fact that the solubilized receptors did not interact unless co-expressed. This suggests the early D2R and OTR interaction is necessary for their

association as mature receptors. Lastly, both D2R and OTR oligomers co-immunoprecipitated, suggesting the complexes may exist as larger multimers. I attempted a reverse immunoprecipitation to see if HA-OTR-Venus could pull down Flag-D2R, however had little success using two different methods – HA beads, and anti-GFP, incubated immunoglobulin G beads. It is possible the reverse is not optimal if the receptor interaction induces a conformation such that the OTR amino- and carboxy-terminal tags are not readily accessible to antibody.

To confirm the results obtained from immunoprecipitation of D2R and OTR, I next performed BRET competition experiments in HEK 293 cells. There is no good biosensor-tagged D2R such that the receptor is still functional, therefore I could not use direct fluorescence resonance energy transfer (FRET) or BRET. First, I performed BRET with increasing, equal amounts of OTR-Venus and OTR-RLuc to determine the most optimal amount of donor, OTR-RLuc, to use (250 ng, Figure 21a). Next I carried out an acceptor saturation assay using said amount of OTR-RLuc to determine the most ideal amount of acceptor, OTR-Venus, to use (500 ng, Figure 21b). Lastly, I performed a BRET competition assay based on the determined, fixed amounts of BRET pair, co-expressed with increasing amounts of untagged- or cold-D2R. With increasing cold-D2R there was a slight decrease in BRET between OTR-RLuc and OTR-Venus, suggesting D2R is capable of competing out OTR/OTR interactions, reaching significance at 1 μg of cold-D2R (Figure 21c). I repeated the experiment with smaller increments of cold-D2R up to 1.5 μg, and still saw saturation at the maximal amount of cold receptor. I also repeated the experiment with increased OTR-Venus (750 μg), in

case there was not enough acceptor to see strong competition, however, the results were similar and actually saturated BRET already at 1 μg cold-D2R. It is possible this technique needs to be optimized to detect D2R and OTR receptor interaction, especially if their interaction affinity is low. It may be possible with high amounts of cold-D2R but this would compromise expression of the biosensor-tagged receptors or saturate competition. Taken together, the data indicates the presence of D2R- and OTR-containing complexes. To my knowledge, no one has yet demonstrated a physical interaction between the two receptors.

Co-internalization of D2R and OTR

I next assessed changes in internalization patterns for D2R and OTR, which would further support a physical interaction between the two proteins. D2R does not reportedly internalize following stimulation with increasing concentrations of quinpirole, however OTR does in response to oxytocin in HEK 293 cells [198, 199]. Interestingly, So et al. demonstrated that D2R co-expression with another dopamine receptor subtype, dopamine D1 receptor (D1R), allowed for quinpirole-mediated internalization of both receptors, and vice versa with a D1R-selective agonist. I wanted to test whether a similar functional interaction occurred between D2R and OTR. This would not only substantiate their heterodimerization, but also help elucidate a mechanism for the OTR-mediated decrease in homodimeric D2R/D2R-Gqi5 calcium signalling. HEK 293 cells were transfected to transiently express Flag-D2R and/or HA-OTR to assess for agonist-mediated internalization patterns by confocal microscopy. Cells were treated with

1 μM quinpirole or 100 nM oxytocin, which have previously been shown to internalize D2R in the presence of D1R or OTR, respectively [198, 199]. The two receptors co-localized at the cell membrane in untreated cells (seen in yellow), corroborating their potential to form heterodimers (Figure 22). Following a forty-minute stimulation with quinpirole, D2R remained at the cell surface in cells expressing D2R alone, which fits with previous reports. Also as expected, oxytocin mediated OTR internalization in OTR-expressing cells. Quinpirole and oxytocin did not induce OTR and D2R internalization, respectively, non-specifically in cells expressing either receptor alone. Interestingly, endosomal structures can be seen in cells expressing both receptors, which is a hallmark of internalization. Oxytocin stimulation caused internalization of both receptors, suggesting D2R co-internalizes with OTR, post OTR-activation. On the other hand, quinpirole possibly mediated internalization of D2R, as well as OTR when co-expressed with D2R, although to a lesser extent. To support this data, cell surface immunofluorescence assays could be performed, whereby the levels of receptors on the plasma membrane are measured before and after drug treatment. The co-internalization of at least D2R with activated-OTR is strong support for a novel function, associated with their heterodimerization.

Ligand-induced conformational changes within established β_2AR/CB1R and β_2AR/OTR heterodimer pairs

As the second aspect of my project, I wanted to test conformational changes within RGE complexes following ligand stimulation. By repositioning

the RLuc within the Gα, or tagging instead the effector or either protomer, I aimed to construct a collective picture of the dynamics of signalling complexes containing multiple receptors, G protein and effector upon ligand activation from different vantage points (refer to Figure 5). Using BRET, where the Gα subunit is tagged with RLuc and the Gγ subunit with GFP, I wanted to test different known β_2AR heterodimers in the context of this BRET pair to begin.

β_2AR and CB1R are co-expressed in HTM cells, among other cell types, and heterodimerize *in cellulo* [161]. I transiently expressed β_2AR, with or without CB1R, in HEK 293 cells in order to control their expression and to see the effects of ligand stimulation of β_2AR, with and without CB1R co-expression. As discussed above, β_2AR can couple to both Gαs and Gαi, whereas CB1R couples primarily to Gαi. Thus, cells were co-transfected with the Gγ2-GFP10 and either Gαs-RLuc8 or Gαi2-RLuc8 constructs in order to measure BRET for both possible G protein partners. BRET is the measure of energy, non-radiatively transferred from an excited luminescent donor, e.g. RLuc, to a fluorescent acceptor, e.g. GFP. RLuc8 is an improved variant of RLuc for the purpose of BRET because it has brighter and more stable luminescence, due to higher enzymatic activity. Preliminary results with the β_2AR and CB1R heterodimer suggests the presence of CB1R alters signalling through β_2AR following isoproterenol treatment, and does so differentially with Gαs versus Gαi2 (Figure 23a). Co-expression of CB1R did not seem to alter the Gαs BRET pair dynamics after isoproterenol treatment, suggesting that the presence of unstimulated CB1R does not necessarily disrupt agonist-stimulated β_2AR coupling to Gαs. However

unstimulated CB1R seemed to increase the BRET between Gαi2-Gγ2, relative to β_2AR expressed alone, though statistically insignificant. Therefore, it is possible CB1R promotes putative β_2AR/CB1R coupling and/or further β_2AR coupling to Gαi2 as well. On the other hand, the CB1R agonist, WIN55,212-2, also had little effect on Gαs-Gγ2 BRET in cells co-expressing both receptors. Interestingly, stimulated CB1R in the presence of unstimulated β_2AR had a comparable Gαi2-Gγ2 BRET result as stimulated β_2AR alone. Based on this data, it is possible co-stimulation of the co-expressed receptors may increase Gαi2-Gγ2 BRET, higher than stimulated β_2AR alone or co-expressed with unstimulated CB1R. WIN55,212-2 did result in a CB1R-specific change in Gαi2-Gγ2 BRET, and perhaps a minor one in Gαs-Gγ2 BRET, seen by a decrease and increase in BRET, respectively, in cells co-expressing CB1R versus cells expressing β_2AR alone (Figure 23b). The decrease in BRET likely reflects dissociation of Gα and Gβγ subunits upon CB1R activation, or arguably a positional reorientation of biosensors further apart from each other. The increase in BRET, though small, is intriguing since CB1R does not reportedly interact with Gαs, and may depend on the presence of β_2AR, though this cannot be concluded based on these experiments.

Wrzal et al. recently demonstrated that β_2AR and OTR heterodimerize in human myometrial cells, and exhibit an altered pattern of coupling to Gαi, different from either parent receptor, which couple to Gαs/Gαi and Gαq, respectively [186]. I transiently expressed β_2AR, with or without OTR, in HEK

293 cells, to assess the effect of OTR on BRET following ligand stimulation. Gαs-Rluc8 or Gαi2-Rluc8 was also co-transfected in the same cells with Gγ2-GFP10 to analyze BRET changes in the context of both G proteins, since β_2AR couples to each. Furthermore, Wrzal et al. reported allosteric interactions between the two receptors centered on the Gαi [186]. Following isoproterenol treatment, BRET appears to decrease for both assay partners Gαs- and Gαi2-Rluc with Gγ2-GFP10 when OTR is present versus β_2AR alone, though statistically insignificant (Figure 24a). The OTR agonist, oxytocin, seemingly induces increases for both BRET pairs when OTR is co-expressed with β_2AR compared to unoccupied OTR with stimulated β_2AR or stimulated β_2AR alone. However, it does seem to have increased BRET values in cells expressing β_2AR alone as well (Figure 24b). This may be an artifact of oxytocin activating endogenous vasopressin receptors (AVPR1) in HEK 293 cells. It appears as though the two ligands induce different G protein conformations when β_2AR and OTR are co-expressed such that the β_2AR agonist promotes a separation in Gα and Gγ subunits, whereas the OTR agonist stimulates a closer association, particularly for Gαi2. Further studies need to be performed to ascertain the involvement of β_2AR in oxytocin activation of OTR and the level of AVPR1 influence in this system.

I next assessed BRET changes using another RGE complex subunit pair, effector and G protein, adenylyl cyclase II (ACII)-RLucII and Gγ2-GFP10. RLucII has increased stability and enzymatic activity over RLuc in a manner similar to RLuc8. As expected, isoproterenol stimulated a significant BRET specifically in cells expressing β_2AR versus cells expressing OTR, since β_2AR

couples to Gαs, which is upstream of ACII recruitment to the cell membrane (Figure 25a). BRET following isoproterenol treatment was even significantly higher in cells expressing both receptors, suggesting a positive effect of OTR on effector recruitment. Oxytocin was unable to stimulate BRET values as high as the β_2AR agonist (Figure 25b). This is not entirely unexpected, since OTR couples to Gαq, which does not lead to ACII activation. Interestingly, even significantly less BRET was seen in cells expressing both receptors. It is possible the OTR agonist has a negative effect on endogenous, stimulated β_2AR complex conformational changes. I suspect the similar BRET value in β_2AR alone-expressing cells to that of cells expressing OTR alone may be due to endogenous AVPR1 activation. That being said, the effect seen when both β_2AR and OTR are expressed may be a result of the endogenous AVPR1 interacting with OTR. Further studies should be done to see the effect of oxytocin when the AVPR1 is blocked with antagonist, to see if the BRET changes are eradicated or not. Simultaneous, dual agonist treatment with isoproterenol and oxytocin produced similar and not significantly different results from isoproterenol alone (Figure 25c). More studies using different BRET pairs for both β_2AR/CB1R and β_2AR/OTR heterodimers are needed to understand better the movement of subunits within the complex.

DISCUSSION

D2R and OTR potentially interact at many levels *in vivo*, which warranted their further study in terms of molecular interactions, both functional and physical. To this end, I have demonstrated functional implications for the putative heterodimer in the calcium and cAMP signalling pathways. *In vitro,* agonist stimulation of D2R seems to positively modulate OTR-mediated calcium signalling, and unoccupied OTR seems to alter D2R ligand activity in cAMP signalling. However, there appears to be little modulation by either receptor on the other in the context of ERK activation based on these experiments. These functional interactions may be a consequence of physical interactions between D2R and OTR. I have demonstrated the two receptors physically associate *in vitro*, as well as internalize together following OTR stimulation. Apart from distinct signalling effects, I have also shown receptors within a heterodimer can influence the conformational dynamics of the entire RGE complex. This is the case for both β_2AR/CB1R and β_2AR/OTR, following ligand stimulation of one receptor or the other. Together, these data highlight how much more complicated GPCRs really are than originally believed.

Our understanding of GPCRs has advanced significantly from thinking of them as monomeric, single ligand responsive, on/off signalling entities to multimeric, multi-modulated, multi-pathway activating, allosteric machines. This is extraordinary when considering the organization it must take to coordinate the vast number of GPCRs within a given cell or tissue, not to mention in a whole

organism. A number of these receptors are expressed in multiple tissues, performing many different tissue-specific functions. For example, very simply the β_2AR induces contraction of cardiac myocytes in the heart, and conversely, dilation of the smooth muscle cells in blood vessels and the uterus [176, 178, 200]. It is becoming more apparent that GPCRs can also have different roles dependent on interaction with different types of receptors. For instance, the D2R and GHSR1a "individually" (or perhaps with a yet unknown silent partner) promote feeding, however, together in the form of a heteromer, they inhibit food intake [152]. To that same effect, D2R exhibits another completely different role in PRL secretion. Based on previous studies in concert with my findings, it is possible the function of D2R in PRL secretion evolved from its association with OTR, and vice versa. Apart from the D2R/OTR heterodimer, there are likely so many other GPCR heterodimers yet to be discovered!

Based on the knowledge gained from emerging heterodimers, organization of the GPCRs may in part be achieved through multiple physical receptor interactions as part of a large GPCR network. These interactions may be regulated by a combination of factors, including tissue localization, timing of receptor transcription and translation, as well as potentially orchestrated by Gβγ subunits that help mediate receptor complex formation in the ER [12, 33]. GPCR functions may be tailored based on the receptor types making up a given signalling complex. Thus, it is no longer accurate to refer to functions of a receptor, but better yet, functions of a homo- or heterodimer. Standard drug design approaches rely on the notion that drugs binding the two receptors in a given dimer likely function

independently of one another. However, based on my findings, together with other groups, it is becoming more obvious that this is not the case. We have been deluded previously by looking *in vivo* only at signalling, which may hide a dimer that essentially reports as a monomer when studied in a simple signalling assay. An allosteric dimer may deliver what appears to be a monomeric signal, since one receptor is silent, even though it is allosterically modulating that signal. With that said, we need to take advantage of this phenomenon in discovering new, previously unknown heterodimers and oligomers. Moreover, the heterodimers that are known need to be reanalyzed for allosteric properties. This may explain some of the side effects of so many drugs. For example, a ligand made to target a receptor's orthosteric site, may not only be acting on its receptor as the primary signalling receptor, but simultaneously acting on its receptor as an allosteric modulator to multiple other receptors. As one can imagine, this has huge implications in drug design. Receptor-selective drugs are no longer adequate, and instead, modern drugs need to target a specific homo- or heterodimer selectively.

In the case of the D2R/OTR heterodimer, of which there may exist a D2R signalling complex, modulated by a silent OTR, or an OTR signalling complex modulated by a silent D2R, OTR and D2R-targetting drugs may induce unanticipated side effects, respectively. For example, animal studies allude to increased uterine resistance or tone following dopamine administration, which may develop from stimulated, silent D2R positively modulating OTR in the uterus [201]. This is not entirely out of the realm of possibility since D2R has actually been recorded as being expressed in the human endometrium [202]. Moreover, the

D2R antagonist, metoclopramide, exerts an opposite or relaxing effect on the uterus [203]. On the other hand, downregulated dopamine signalling as well as oxytocin treatment have been linked to seizures, which may be in part due to activated, silent OTR negatively modulating D2R in the brain [204-206]. Interestingly, both oxytocin and plasma concentrations of PRL increase dramatically during prolonged epileptic seizure [207]. Apart from the clear indication for oxytocin stimulation of PRL release, it is possible D2R signalling is simultaneously downregulated in this system. Other side effects of oxytocin include tachycardia and hypotension in pregnant women, which may stem from the β_2AR/OTR interaction [208, 209]. OTR may molecularly positively enhance β_2AR-mediated heart contraction in this instance, though this is physiologically undesirable. Apart from treatment in preterm labour, oxytocin and another OTR agonist, carbetocin, are also administered for prevention of post pregnancy hemorrhaging, which may act via negative modulation of β_2AR-mediated vasodilation [210]. Similarly, tachycardia and hypotension afflict cannabis users as well, and can result from endogenous CB1R ligand, 2-arachidonoyl glycerol (2-AG), action [211, 212]. This phenomenon may result from CB1R stimulation in the context of β_2AR.

Aside from preventing certain unwanted side effects, dimers should also be examined for their therapeutic potential. β_2AR antagonists and CB1R agonists are currently used clinically for the treatment of glaucoma. Based on what Han et al. demonstrated, that optimal D2R homodimeric signalling was obtained with an inverse agonist in combination with an agonist, it would be interesting if a similar

phenomenon were true for heterodimers [19]. For example, if co-administration of an antagonist at β_2AR and an agonist at CB1R are actually acting in the context of the β_2AR/CB1R heterodimer, which is responsible for the effective treatment of glaucoma, and if their co-treatment is more effective than either agonist alone. Similarly, whether co-treatment of a β_2AR agonist and OTR antagonist administered for preterm labour, act in concert at the β_2AR/OTR heterodimer more effectively than either drug alone. Based on this notion, bivalent ligands hold a lot of promise as drug candidates. The best combination of ligands, be it agonist at receptor 1 /antagonist at receptor 2 and vice versa, agonist at receptor 1/agonist at receptor 2 etc., need to be determined and thereafter linked together such that they selectively target the dimer of interest and in a conformation that permits their simultaneous binding to the respective receptors within the dimer. Little is known about the physical and molecular interactions between D2R and OTR, let alone the effects of different ligands on these interactions, which is what I have attempted to introduce as part of my thesis.

To date, the physiological functions of D2R and OTR have been described extensively in the scientific literature in which, the two receptors overlap markedly in regulation of PRL secretion. Taking it all together, as mentioned above, the two GPCRs potentially regulate together, olfactory- and stress-stimulated PRL secretion, as well as food intake and social interaction. However, less is known about their interaction at the molecular level. My thesis comprises a series of experiments contributing some novel findings, including heterodimerization of D2R and OTR, as well as some of their functional

interactions. In an attempt to improve on simple signalling assays, effector responses were analyzed in the presence of one or both receptors, using different combinations of ligands at both receptors.

OTR typically couples to Gαq and thus calcium signalling, however D2R does not couple to Gαq, or Gαq-independent calcium signalling. I have shown that the D2R agonist, quinpirole, cannot stimulate calcium signalling in cells expressing WT D2R alone. Furthermore, quinpirole was unable to elicit a response in cells expressing OTR alone as well. Therefore it was interesting to see that simultaneous oxytocin and quinpirole mediate synergistic calcium mobilization higher than oxytocin stimulation alone. Furthermore, this effect was seen only in cells co-expressing the two receptors, and blocked with the D2R-selective antagonist, raclopride, suggesting the effect is specific to the D2R. This phenomenon cannot simply be attributed to D2R occupancy, since raclopride alone, had no effect on OTR-mediated calcium signalling. This is an intriguing result considering the two GPCRs have supposed opposing roles in PRL regulation. *In vitro* studies demonstrated oxytocin administration to lactotrophs resulted in PRL secretion in tandem with increased intracellular calcium, suggesting calcium signalling plays a central role in PRL secretion [91]. In addition, the regulation of PRL has a huge temporal influence, likely channeling into its rhythmic pattern of release. Simply, oxytocin stimulates PRL secretion, which in turn stimulates dopamine release in a time-delayed fashion. Subsequent activation of D2R on PRL-secreting cells eventually inhibits further PRL release through the inhibition of AC and consequent down-regulation of cAMP and

PKA/CREB responsive elements. However, oxytocin-mediated PRL secretion parallels a decrease in dopamine, suggesting the oxytocin feedback can overcome the dopamine negative feedback on PRL release when necessary. Taking this all together, it is possible continuous dopamine release originally stimulated by PRL reverts from inhibition of PRL via D2R and cAMP downregulation to stimulation of PRL secretion in a time-delayed manner, via simultaneous stimulation with oxytocin, of D2R/OTR complexes coupled to Gαq and calcium signalling, which may overcome the inhibitory function via D2R alone and begin the proposed cycle over again.

D2R seemingly modulates OTR signalling in the context of calcium signalling, therefore I tested next whether OTR could likewise modulate D2R-effector signalling. D2R classically couples to Gαi, which acts to inhibit AC activation and subsequent cAMP production. I have shown that the D2R agonist, quinpirole, inhibits cAMP production in a dose-dependent fashion, which is prevented by co-treatment with the D2R antagonist, raclopride. Interestingly, raclopride treatment alone, also inhibited cAMP production dose-dependently, though less dramatically than quinpirole. If raclopride is truly a neutral antagonist, it should have no effect in the absence of an agonist at its respective receptor. The concept of intrinsic efficacy relies on the notion that one agonist or partial agonist can activate maximally, or sub-maximally, respectively, all ligand binding-induced cellular responses, and that antagonists can only block this agonist activity with no intrinsic activity of its own [23]. However, raclopride did have an effect on its own, and appeared to act more like an inverse agonist for this

pathway in cells expressing D2R alone, alluding to its potential biased ligand activity. To my knowledge, raclopride has not previously been documented to exert inverse agonism, thus, this should be confirmed by other independent signalling assays. Moreover, raclopride acted like yet another type of ligand, a partial agonist, in cells co-expressing D2R and OTR. Interestingly, this activity is only evident when OTR is co-expressed with D2R, suggesting receptor interacting partners can influence ligand activity. In this case, OTR did not directly modulate D2R signalling, however it may have modulated D2R ligand properties and ligand-mediated signalling. Interestingly, many previous reports have also demonstrated biased ligand activity for the D2R [22, 24, 213, 214]. There are clear implications for biased ligands in therapy, to generate drugs acting on the physiologically beneficial pathways and/or sparing pathways that mediate the negative side effects.

Apart from studying effects of one receptor on specific effector signalling of the other, I also studied a downstream effector common to both receptors, ERK. Gαq-coupled OTR signalling leads to intracellular calcium signalling, however it also triggers an epidermal growth factor receptor (EGFR)-dependent MAPK cascade that culminates on the phosphorylation and activation of ERK [215, 216]. I have shown that oxytocin mediates ERK activation in cells expressing OTR, with or without D2R. This is a key mechanism in the anxiolytic activity of oxytocin. Inhibition of the MAPK responsible for phosphorylating ERK, MEK1/2, completely abrogates the anxiolytic effect of oxytocin within the rat hypothalamic PVN [217]. Furthermore, oxytocin likely exerts some of its anxiolytic effects

indirectly via stimulation of PRL secretion. Studies in rats show downregulation or upregulation of PRL activity in the brain parallels an increase or decrease in anxiety-related behaviour, as well as brain levels of the major stress hormone mediator, adrenocorticotropic hormone (ACTH), suggesting PRL dampens the responsiveness of the HPA axis or stress response [131-133]. The short isoform of PRL also mediates ERK phosphorylation in the rat hypothalamic PVN and SON, including sub-populations of cells expressing the ACTH precursor, corticotropin-releasing hormone (CRH), and oxytocin [35, 218]. Contrary to decreasing CRH activity, inhibition of MEK1/2 actually increases CRH expression *in vitro*, suggesting phosphorylated ERK enhances HPA axis activity [218]. Blume et al. suggest a model whereby PRL has an acute stimulatory effect on CRH expression and a chronic inhibitory effect on HPA axis activity [124]. Unlike OTR and PRLR, which seem to oppose stress responses, the D2R has been implicated in mediating certain stresses, via decreased PRL secretion. Based on all this, one might predict D2R and OTR have a functional interaction at the level of ERK signalling in regulating stress. However, neither receptor antagonist, OTA nor raclopride, seemed to affect quinpirole- or oxytocin-mediated ERK activation, respectively, in cells co-expressing D2R and OTR. More ligands, for example, different antagonists and inverse agonists, should be tested before it is concluded D2R and OTR have no direct functional interaction in the context of MAPK activation. Moreover, it would be interesting to test D2R and OTR ligands in cells co-expressing either receptor with the PRLR since both receptors have been

implicated with PRL in stress. Perhaps in this system, D2R and OTR interact indirectly via PRL signalling.

D2R and OTR have potentially pathway specific modulating effects on one another in response to different combinations of ligands. It remains to be determined whether these functional interactions are mediated distal to the receptors, via convergence of downstream effector signalling or directly at the level of the receptors via allosteric communication. A greater scope of ligands tested in each assay would help solve this quandary, such as different antagonists and inverse agonists for each receptor in combination with an agonist for that pathway. For example, an effect of having an antagonist at the silent modulating receptor on signalling through the primary receptor would imply allosterism. Of note, the aequorin and EPAC assays done for putative D2R and OTR interactions involved co-treatment with agonist simultaneously, which would leave relatively little time for downstream signalling of one receptor to alter calcium or cAMP signalling, respectively, of the other receptor, which argues against downstream effector signalling crosstalk. For further convincing evidence, in the case of D2R modulation of OTR-mediated calcium mobilization, signalling could be assessed in the presence of an inhibitor against each D2R-Gαi downstream player. Likewise, inhibitors against downstream OTR-Gαq effectors can be used to assess D2R-mediated cAMP signalling in the presence of OTR. If these inhibitors prevent the synergistic calcium response and altered D2R ligand activity seen, respectively, it would suggest receptor signalling crosstalk is responsible for the functional interactions each receptor has on one another. Furthermore, this could

help confirm expected or elucidate new players involved in the signalling pathway in question. Heterodimers have reportedly exhibited altered G protein coupling and downstream signalling from one or both receptors individually [186, 219]. I have also shown that D2R and OTR physically interact in the form of a heterodimer and a hetero-oligomer, which suggests allosterism. Further study is required in order to determine the minimal stoichiometry of the D2R/OTR heteromer required for the aforementioned functional interactions. For simplicity, this putative interaction will be referred to as heterodimeric.

D2R and OTR co-immunoprecipitate *in vitro,* suggesting they are at least in a multimeric protein complex together, though not necessarily directly interacting. However, D2R is able to compete with OTR homodimerization as measured using BRET, suggesting the two receptors may in fact associate directly. This is substantiated by immunofluorescence and confocal microscopy data showing the two receptors co-localize at the cell membrane as well as in endosomes. Not only do D2R and OTR interact at the cell surface, but they also internalize together supporting their physical association in a complex. The dimer favours internalization similar to OTR alone, and exhibits altered internalization patterns from D2R alone. D2R is normally retained at the plasma membrane following quinpirole treatment, but is internalized following oxytocin treatment with OTR, and potentially following quinpirole treatment as well, only when co-expressed. To help clarify and quantify internalization, immunofluorescence surface assays could be performed with fluorescently conjugated antibodies labeling each receptor. If internalization were indeed occurring, less fluorescence,

and thus fewer receptors, would be measured at the cell surface following drug administration. If quinpirole were not inducing internalization, an interesting follow up experiment would be to test co-stimulation of both D2R and OTR agonist to see if pre-treatment with quinpirole actually prevents oxytocin-mediated OTR internalization as is the case for the β_1AR/β_2AR heterodimer for example, whereby β_1AR inhibits agonist stimulated internalization of β_2AR [196]. Some D2Rs and OTRs may exist throughout their life cycles together in a heterodimer. Immunoprecipitation data reveals they associate already during receptor biosynthesis, and confocal microscopy shows them at the cell surface where they have heterodimeric-specific functional implications on signalling, as well as shows them intracellularly, indicating they internalize together. It remains to be determined whether these receptors continue signalling in endosomes, and/or are degraded or recycled back to the plasma membrane thereafter. The fact that mixed membranes expressing either D2R or OTR, do not co-immunoprecipitate the mature receptors, suggests the proteins must interact during biosynthesis and/or trafficking to the cell surface, if they are to interact at all. These early interactions are lost in mixed membranes and correlate with lack of mature receptor interactions in the immunoprecipitate.

Oxytocin mutually endocytosed both receptors in cells co-expressing D2R and OTR. This may explain the reduction in quinpirole-mediated calcium signalling of the D2R/D2R-Gqi5 homodimer in the presence of stimulated OTR versus its absence. OTR may complex with the D2R homodimer, in line with the immunoprecipitation results revealing oligomeric interactions, and induce

internalization of the entire complex following oxytocin pretreatment. Therefore, the decreased calcium signalling may correspond with a decreased pool of available D2R homodimers to signal at the cell surface. Unfortunately, it is also possible the drop in signalling can be attributed to competition for the transcriptional machinery and expression between D2R-Gqi5 and OTR. This may also be the case for oxytocin-stimulated OTR-mediated calcium signalling, which reaches a higher efficacy in cells expressing OTR alone than cells co-expressing D2R and OTR. Arguably, it is also possible, at least in addition to expression competition, OTR in the case of D2R/D2R-Gqi5 and D2R in the case of OTR are competing with the respective homodimers to form D2R/OTR heterodimers, which are less efficient in calcium signalling. The decrease in efficacy may parallel a change in the ratio of dimers such that there are fewer homodimers and more heterodimers. This raises the question of why heterodimers exist at all then. As mentioned above, the efficiency and organization of a diverse signalling system potentiated by heterodimers and their interactions still holds. Heterodimers may sacrifice signalling efficiency for additional functional signalling capabilities.

According to the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NU-IUPHAR), there are three standards, of which at least two need to be met in order for two receptors to be recognized and accepted by the scientific community for putative heterodimers [220]. The criteria include evidence for a physical interaction in native tissue or primary cells, a specific functional property to the heterodimer, and disruption in knockout or knockdown animals for either receptor [220]. The D2R/OTR

heterodimer succeeds in at least the first two conditions. I have demonstrated that D2R and OTR heterodimerize *in vitro* by two different methods in primary HEK 293 cells, as well as their co-localization in common subcellular compartments by confocal microscopy. I have also shown two specific pharmacological properties of the D2R/OTR heterodimer, including positive modulation of OTR-mediated calcium signalling, as well as a specific ligand-altered activity at the heterodimer, such that raclopride exhibits different signalling properties in the cAMP pathway. As further evidence, it would be interesting to test whether D2R and OTR physically interact endogenously in the pituitary gland. This could be done with labeled ligands or antibodies against either receptor, by co-immunoprecipitation, confocal microscopy and/or BRET and FRET, in pituitary tissue.

Based on the physical and functional interactions between D2R and OTR observed, it would be interesting to assess how RGE conformations are affected following similar ligand regimens. I have begun exploring this concept in two already reported heterodimers very superficially. I have shown unstimulated and stimulated partner receptors have an effect on G protein subunits in terms of BRET, suggesting some sort of conformational alteration. For instance, the presence of unstimulated CB1R, had a positive effect on BRET between Gαi2-Gγ2 following β_2AR activation versus in the absence of CB1R. However, this effect was not seen with CB1R agonist treatment instead, which is a little unexpected considering CB1R couples to Gαi. Stimulation of CB1R actually reverted the increase mediated by unstimulated CB1R to similar BRET levels following isoproterenol treatment in cells expressing β_2AR alone. It is possible

the effect depends on β_2AR stimulation, or that WIN55,212-2 confers a conformation in CB1R that keeps the biosensors further apart than they would be with unoccupied CB1R. Since unoccupied CB1R potentially induces an increase following isoproterenol stimulation, stimulated CB1R may synergistically or additively increase Gαi2-Gγ2 BRET with stimulated β_2AR. It is necessary to repeat these experiments testing the CB1R agonist on cells expressing CB1R with or without β_2AR, to see the effect of β_2AR on CB1R signalling at the level of the receptor-G protein. This may shed light on whether β_2AR plays a role in CB1R agonist-mediated increase in Gαs-Gγ2 BRET. This result is intriguing because WIN55,212-2 triggers a small increase in BRET when CB1R is expressed, which does not normally couple to Gαs, compared to β_2AR alone. Thereafter, it would be interesting to test co-stimulation of both β_2AR and CB1R agonists in cells expressing one or both receptors. The same could be said for the β_2AR/OTR heterodimer. I observed a decrease in isoproterenol-mediated Gαs- and Gαi2-Gγ2 BRET when OTR was present versus when it was absent. Intriguingly, an increase in oxytocin-mediated BRET for both biosensor pairs is observed in cells expressing β_2AR alone or with OTR. That being said, another experiment assessing BRET following oxytocin treatment in cells expressing OTR alone or with β_2AR is imperative to illuminate the role of β_2AR on oxytocin-mediated BRET, if any. Furthermore, assessing the effects of oxytocin following treatment with an AVPR1-selective antagonist, to isolate the effects of OTR would be useful. I would also like to assess using BRET, conformational changes between Gαq and Gγ2 to assess the effects of β_2AR on OTR coupling to Gαq. BRET

between the Gαs-related effector, ACII, and Gγ2 revealed a positive effect of unstimulated OTR, which suggests it positively modulates or increases isoproterenol-mediated β_2AR signalling, including receptor activation, G protein conformational changes and ACII and Gβ1γ2 interactions. However co-stimulation with oxytocin had no further apparent effect. Together, the BRET data, for the different biosensor pairs following ligand stimulation, suggest allosteric modulation can occur at the level of the RGE complex. It would be interesting to carry out BRET experiments with tagged effectors of the OTR-Gαq pathway to see whether β_2AR has any allosteric effects as well. Many other different BRET pairs need to be assessed to generate a portrait of the conformational changes that occur within the RGE complex. Simultaneously along the way, allosteric effects might be recognized as with the Gαs-Gγ2, Gαi2-Gγ2, and ACII-Gγ2 pairs. BRET between the Gα and Gγ2 subunits represents one conformational vantage point for each complex containing Gαs, Gαi or Gαq, and ACII with Gγ is another conformational perspective. Other BRET pairs may include G protein (Gα or Gγ) to receptor 1 or receptor 2, including differently tagged locations within the G protein sequence to distinguish dissociation versus reorientation while remaining associated [221]. Had I more time, in addition to single and dual agonist treatment, I would test the effects of antagonists or inverse agonists at one receptor on different BRET pairs following stimulation through the other receptor. The same could be repeated for the D2R/OTR heterodimer. Furthermore, it would be interesting to assess changes in D2R and GRK or β-arrestin interactions in the presence of both receptors. GRK is implicated in OTR internalization and could

help explain D2R internalization with OTR, if the BRET interaction increases versus in cells expressing D2R alone.

My findings support the notion that in addition to the multiple sites within a receptor, a GPCR can be modulated via sites on a completely different receptor. These influences include altering both signalling patterns and potentially RGE complex conformations. Several experiments performed in this study, outline altered signalling for the D2R receptor in the presence of OTR occupied by different ligands, and vice versa. Based on the results above, it is possible that the effects D2R and OTR have on each other's signalling profiles may be a function of allosteric communication within a heterodimer.

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SUMMARY

In vitro co-expression of D2R and OTR lead to unique functional interactions compared to when either receptor was expressed alone, following ligand occupation of one or both receptors. Quinpirole-stimulated D2R positively modulated OTR signalling in the calcium pathway, and unoccupied OTR modulated D2R antagonist activity in the cAMP pathway. D2R and OTR modulation of one another may be pathway-selective, since there was no apparent modulation of either receptor on the other with regards to ERK signalling based on my experiments. The functional interactions may be a consequence of allosteric communication between a novel D2R/OTR heterodimer. D2R and OTR heterodimerize *in vitro,* and internalize together following OTR stimulation. This is in line with their co-localization at the cell surface as well as in intracellular compartments. Thus, D2R/OTR exhibits heterodimer-specific functional interactions in terms of signalling and internalization. Two previously characterized heterodimers, β_2AR/CB1R and β_2AR/OTR, exhibit altered RGE complex conformations following ligand stimulation of one receptor or the other. Thus, allosteric modulation can occur upstream of effector signalling and may be in part responsible for the modulated signalling output. These heterodimers are three examples of how GPCR dimers are considerably more complicated than originally perceived, and validate the importance of studying new and existing heterodimers for their allosteric properties if new drugs are ever to improve on current therapies.

FIGURES

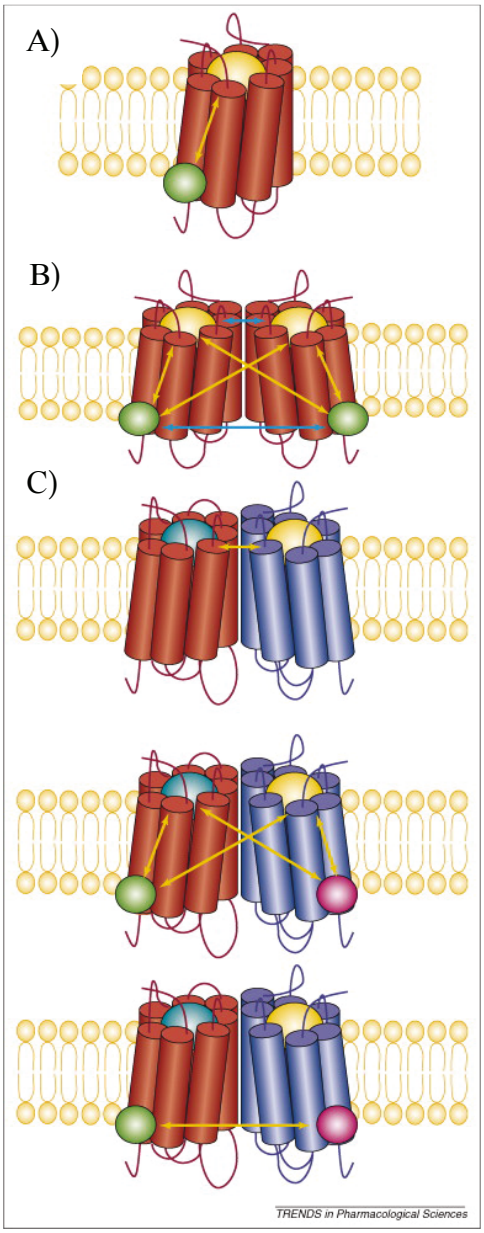

Figure 1: *Cooperativity and allosterism in homo- and heterodimers.* (A) In a receptor monomer, ligands at a different site from the orthosteric site interact allosterically with endogenous ligand, yellow arrow. (B) Within a homodimer, the same ligands binding equivalent sites on two separate receptors exhibit cooperativity with each other, blue arrows, and allosterism with ligands binding to different sites within the same or partner receptor, yellow arrows. (C) Within a heterodimer, all ligands act allosterically with each different ligand binding the same or partner receptor. Figure taken from [2].

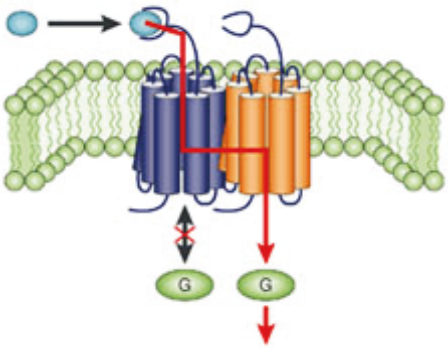

Figure 2: *GABA-mediated transactivation of the $GABA_BR$.* GABA binds the orthosteric site only on $GABA_BR$-1 and transactivates $GABA_BR$-2. $GABA_BR$-2 then activates the G protein. Asymmetry is seen in not only ligand binding and receptor activation, but also receptor organization about the G protein. Figure taken from [17].

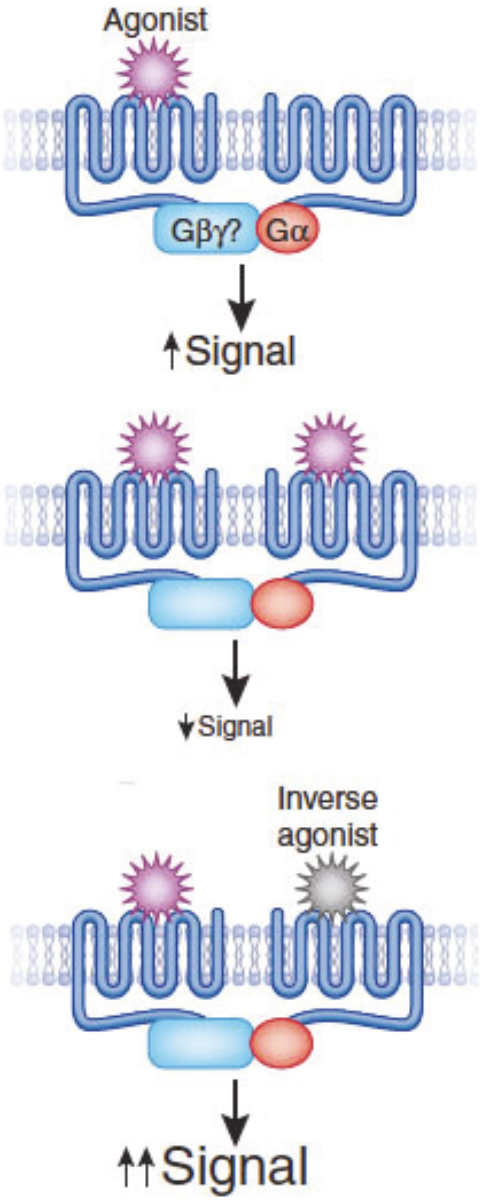

Figure 3: *Ligand and protomer occupation-dependent signalling within the D2R homodimer.* An agonist at protomer A with a mutant protomer B incapable of binding ligand results in a signal (top), that is comparatively decreased with agonist at both protomers (middle), and increased with an inverse agonist at protomer B (bottom). Ligand occupation of protomer B allosterically modulates signalling through protomer A, and does so differently depending on the ligand [19]. Figure taken from [222].

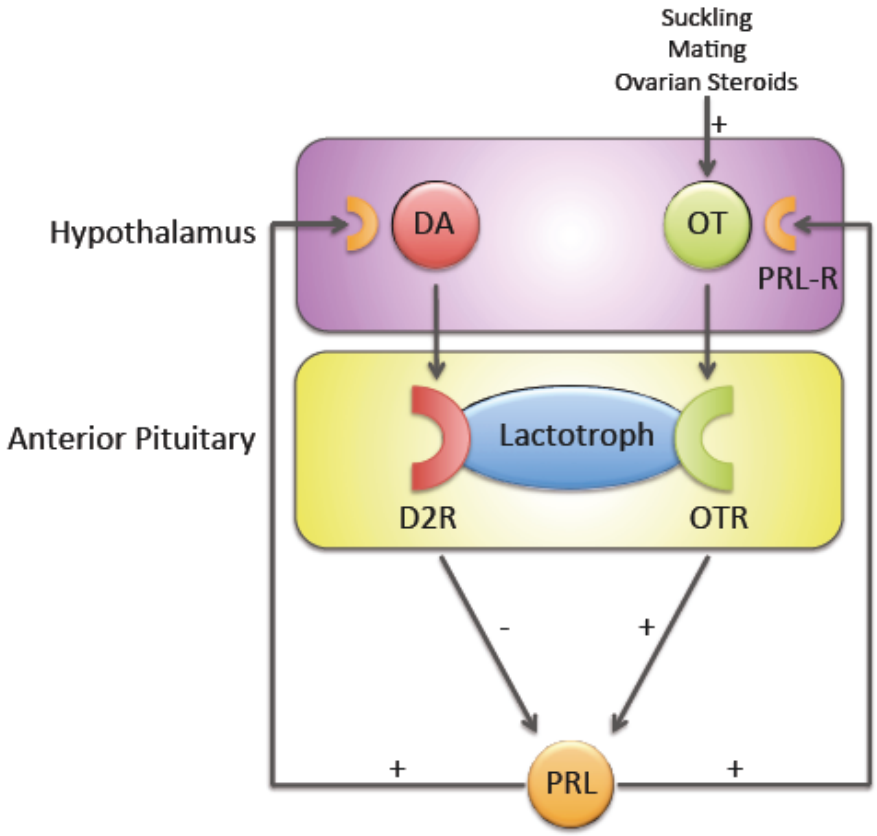

Figure 4: *Opposing dopamine and oxytocin regulation of PRL secretion.* Oxytocin (OT) released from the hypothalamus stimulates prolactin (PRL) secretion from the anterior pituitary in response to suckling, mating and ovarian steroids. PRL in turn positively feeds back and potentiates oxytocin release in a positive feedback loop. PRL also promotes dopamine (DA) release from the hypothalamus, which in turn inhibits PRL secretion in a negative feedback loop. Figure based on image taken from [38].

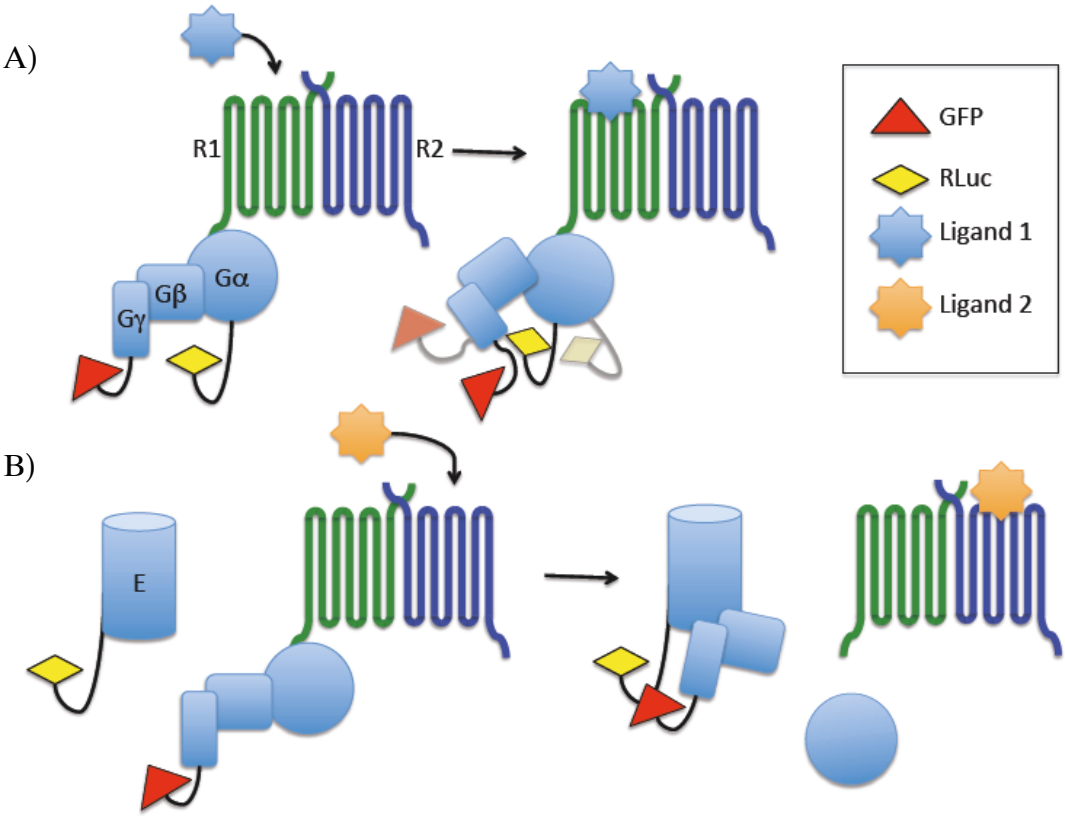

Figure 5: *Understanding conformational dynamics by measuring BRET from different conformational vantage points.* Measuring BRET for distinctly tagged, paired subunits of the RGE complex provides multiple different conformational vantage points in order to map the overall RGE complex conformational changes following ligand stimulation. A) For example, ligand occupancy of one (or both, not shown) receptors may induce a conformational change within the G protein such that GFP (red triangle) tagged to Gγ and RLuc (yellow diamond) tagged to Gα shift into closer proximity resulting in an increase in BRET. In contrast, the two biosensors may move further apart (shown as faded) resulting in a decrease in BRET. These tags can also be moved to different residues within the same protein and BRET measured, which would help determine, in the case of decreased BRET, whether a given change represents dissociation of tagged subunits or a positional change in an ongoing interaction. The goal is to eventually build a repertoire of the conformational changes inferred by different combinations of ligands at the two receptors. B) Potential interactions between effector and G protein following ligand stimulation can also be determined using this method.

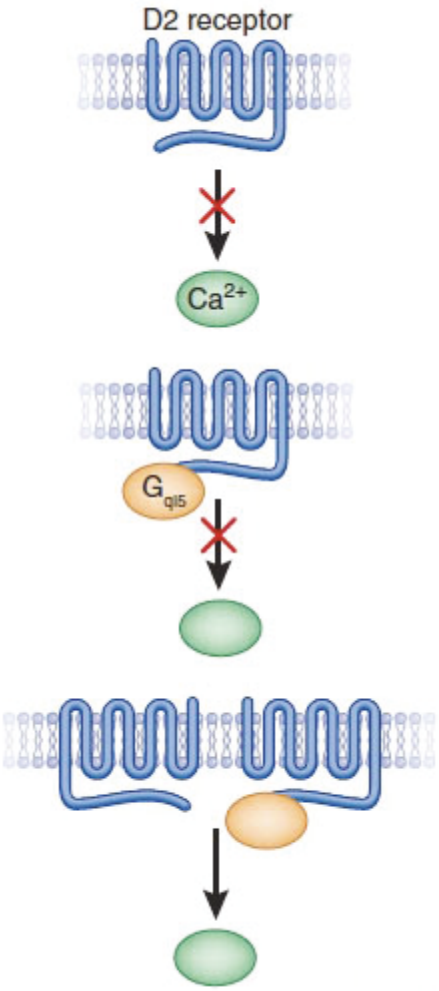

Figure 6: *The Javitch experimental paradigm forces the Gαi-coupled D2R to talk to Gαq and calcium signalling.* Neither the WT D2R (top) nor the Gqi5-fused D2R alone (middle) can couple to calcium (Ca^{2+}) signalling. However, co-expression of the two constructs rescues signalling (bottom) [19]. Figure taken from [222].

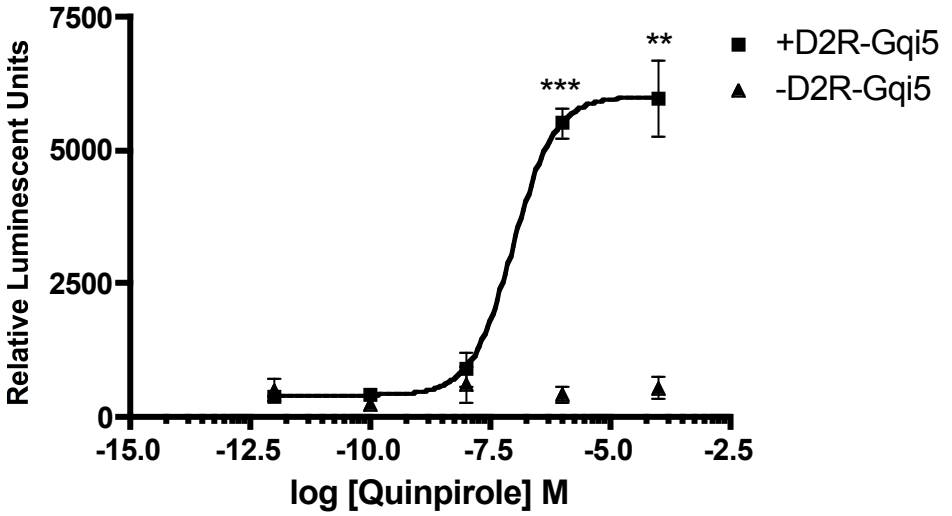

Figure 7: *D2R agonist-mediated transactivation within the D2R homodimer.* Agonist stimulation of Flp-In T-REx-293 pcin4AEQ#3 SFD2s/FRT/TO cells stably expressing WT D2R alone (-D2R-Gqi5), or co-transfected with the D2R-Gqi5 fusion protein (+D2R-Gqi5). WT D2R alone was incapable of coupling to calcium signalling in response to quinpirole. However, co-expression of WT D2R and D2R-Gqi5 rescued signalling. The data represent the mean ± SEM for three independent experiments in duplicate for each concentration point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus lowest concentration point.

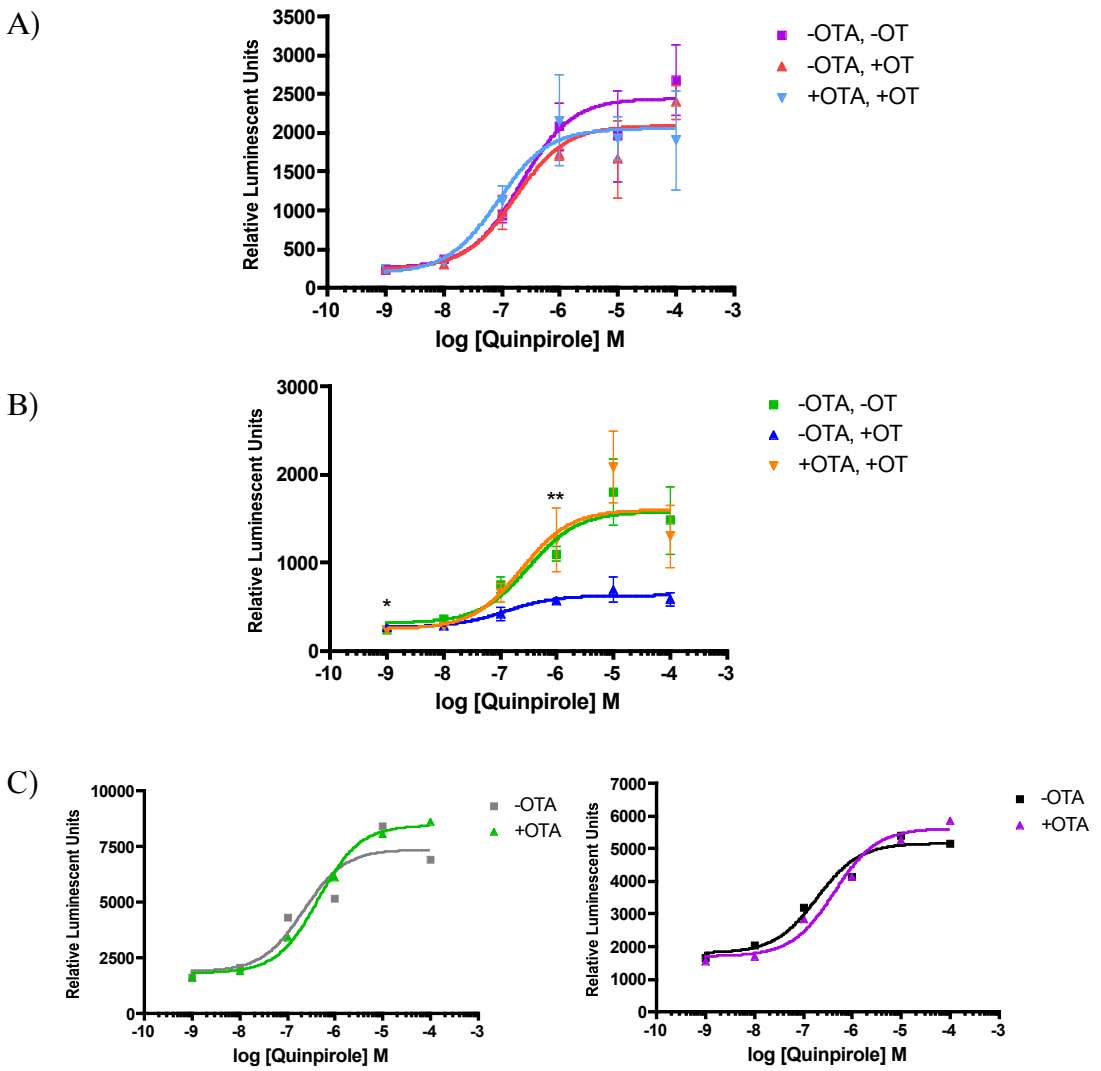

Figure 8: *Oxytocin pretreatment decreases D2R homodimer calcium signalling.* Flp-In T-REx-293 pcin4AEQ#3 cells were transiently transfected with WT D2R and D2R-Gqi5, with or without OTR. Cells were pretreated fifteen minutes with 1 μM OTA and/or three minutes with 100 nM oxytocin and subsequently treated with increasing concentrations of quinpirole. A) D2R homodimer signalling was not significantly different in cells expressing WT D2R and D2R-Gqi5, with (+OT, -OTA) or without oxytocin (-OT, -OTA) and OTA (+OT, +OTA). B) Oxytocin decreased D2R homodimer signalling efficacy in cells expressing both D2R constructs and OTR. This effect was blocked with OTA pretreatment. C) OTA pretreatment did not significantly change calcium signalling in cells, with (right) or without OTR (left). The data in A and B represent the mean ± SEM for three independent experiments in duplicate for each concentration point (except 10 μM quinpirole, for which one experiment was omitted as an outlier). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between -OTA, -OT and -OTA, +OT, for each respective concentration point. The data in C represent one experiment conducted in duplicate.

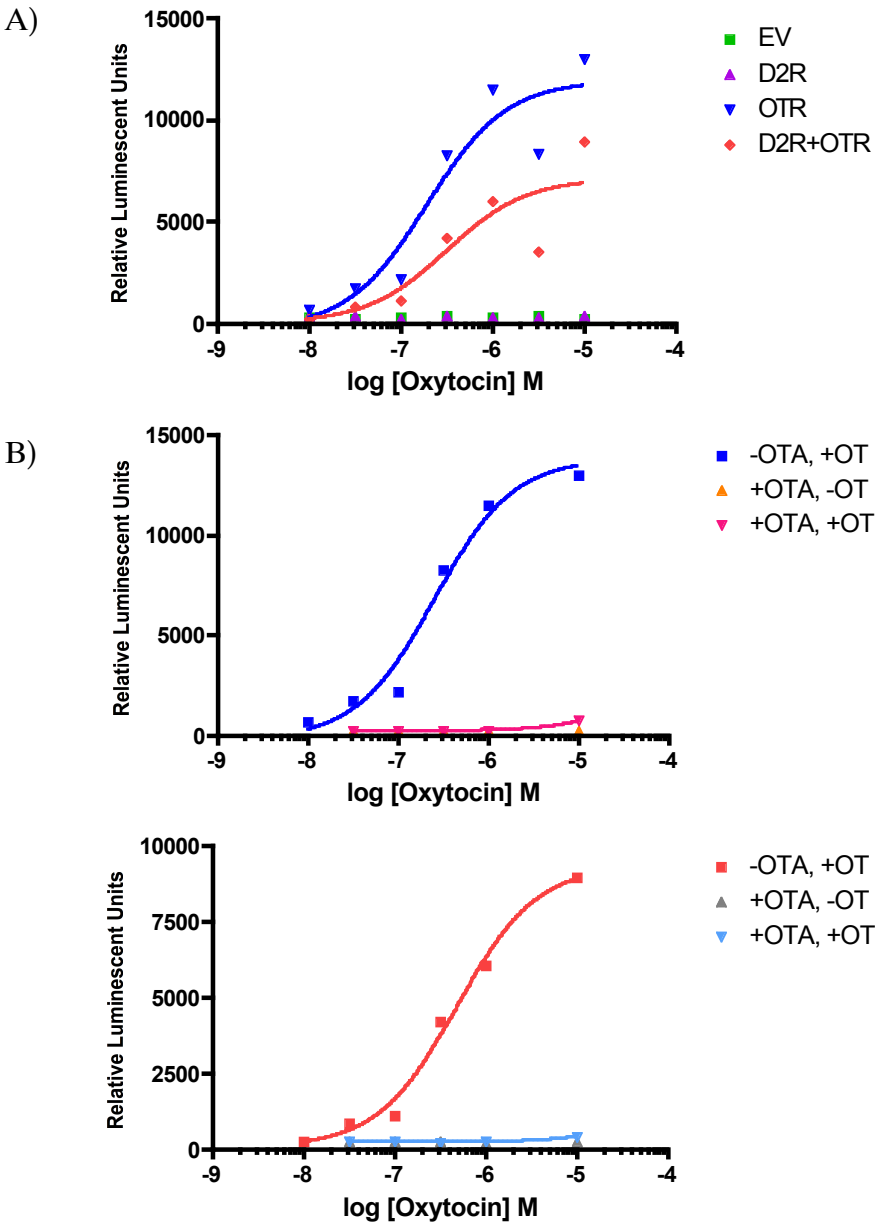

Figure 9: *Oxytocin dose-dependently increases calcium signalling only in cells expressing OTR.* Flp-In T-REx-293 pcin4AEQ#3 cells were transiently transfected with empty vector (EV), WT D2R alone, OTR alone, or WT D2R together with OTR. Cells were treated with increasing concentrations of oxytocin. A) Oxytocin mediated dose-dependent calcium mobilization only in cells expressing OTR alone or together with D2R to a lesser extent. B) Oxytocin-mediated calcium signalling (-OTA, +OT) was blocked by a fifteen minute pretreatment with 1μM OTA (+OTA, +OT) and OTA pretreatment alone (+OTA, -OT) had no effect in cells expressing OTR alone (top) or co-expressing D2R (bottom). Data are representative of three independent experiments conducted in duplicate, then averaged.

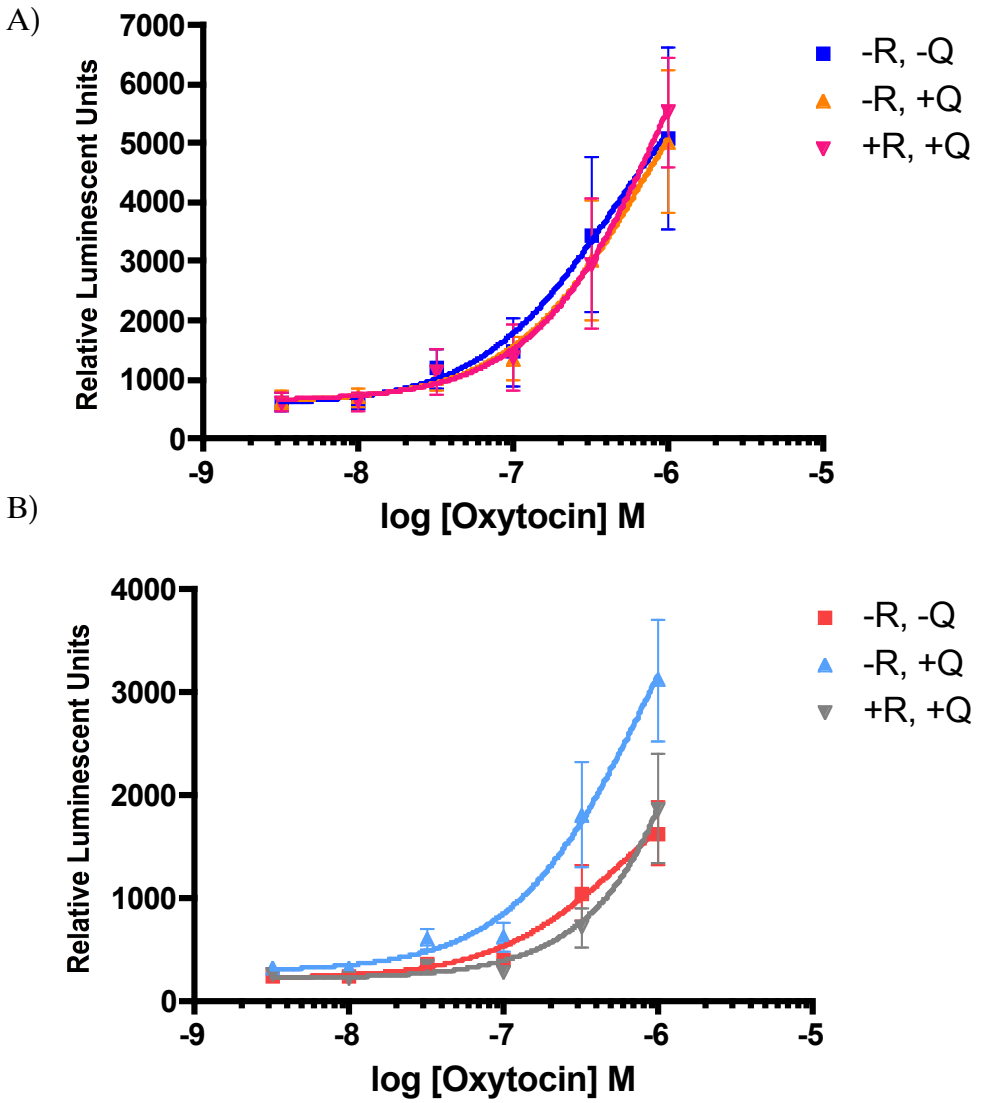

Figure 10: *Quinpirole positively modulates oxytocin-mediated calcium signalling in cells co-expressing D2R and OTR.* HEK 293 cells transfected with OTR alone (A) or with D2R (B), were pretreated with vehicle (-R) or 1 μM raclopride (+R) for fifteen minutes prior to simultaneous treatment with vehicle (-Q) or 100 nM quinpirole (+Q) and increasing concentrations of oxytocin. A) Oxytocin induced a dose-dependent increase in calcium mobilization in cells expressing OTR alone, which was unaffected by D2R agonist and antagonist, quinpirole and raclopride, respectively. The difference with or without quinpirole was insignificant for all data points measured. B) Quinpirole increased the efficacy of oxytocin-mediated calcium signalling in cells co-expressing both D2R and OTR, which was abrogated by raclopride pretreatment, though insignificant for all data points. The data represent the mean ± SEM for four independent experiments conducted in duplicate for each concentration point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between -R, -Q and -R, +Q, for each respective concentration point.

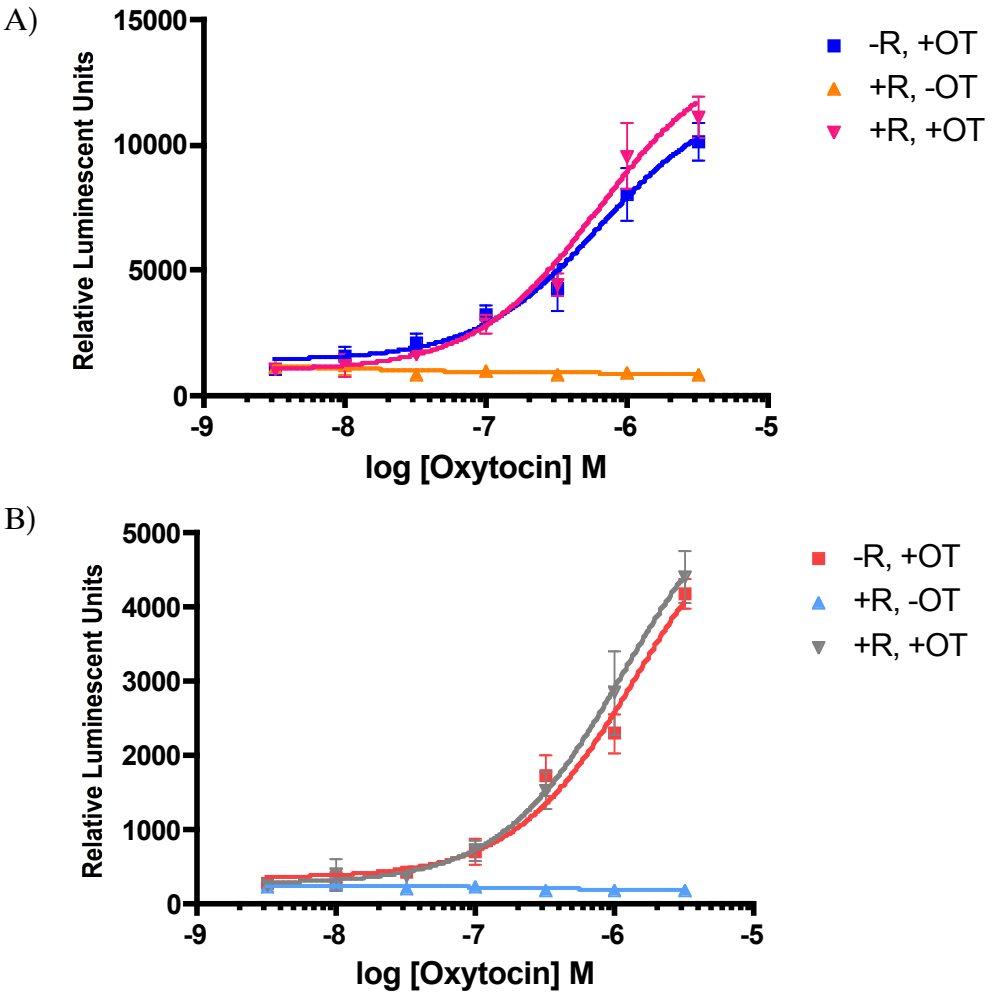

Figure 11: *Raclopride-occupied D2R has no effect on oxytocin-mediated calcium signalling in cells co-expressing D2R and OTR.* HEK 293 cells transfected with OTR alone (A) or with D2R (B), were pretreated with vehicle (-R) or 1 μM raclopride (+R) for fifteen minutes prior to simultaneous treatment with vehicle (-OT) or increasing concentrations of oxytocin (+OT). A) Oxytocin induced a dose-dependent increase in calcium mobilization in cells expressing OTR alone, which was unaffected by the D2R antagonist, raclopride, and was insignificant for all data points measured. B) Raclopride also had no effect in cells co-expressing D2R and OTR, and was insignificant for all data points measured. The data represent the mean ± SEM for four independent experiments in which n=2-4 for each concentration point conducted in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between -R, +OT and +R, +OT, for each respective concentration point.

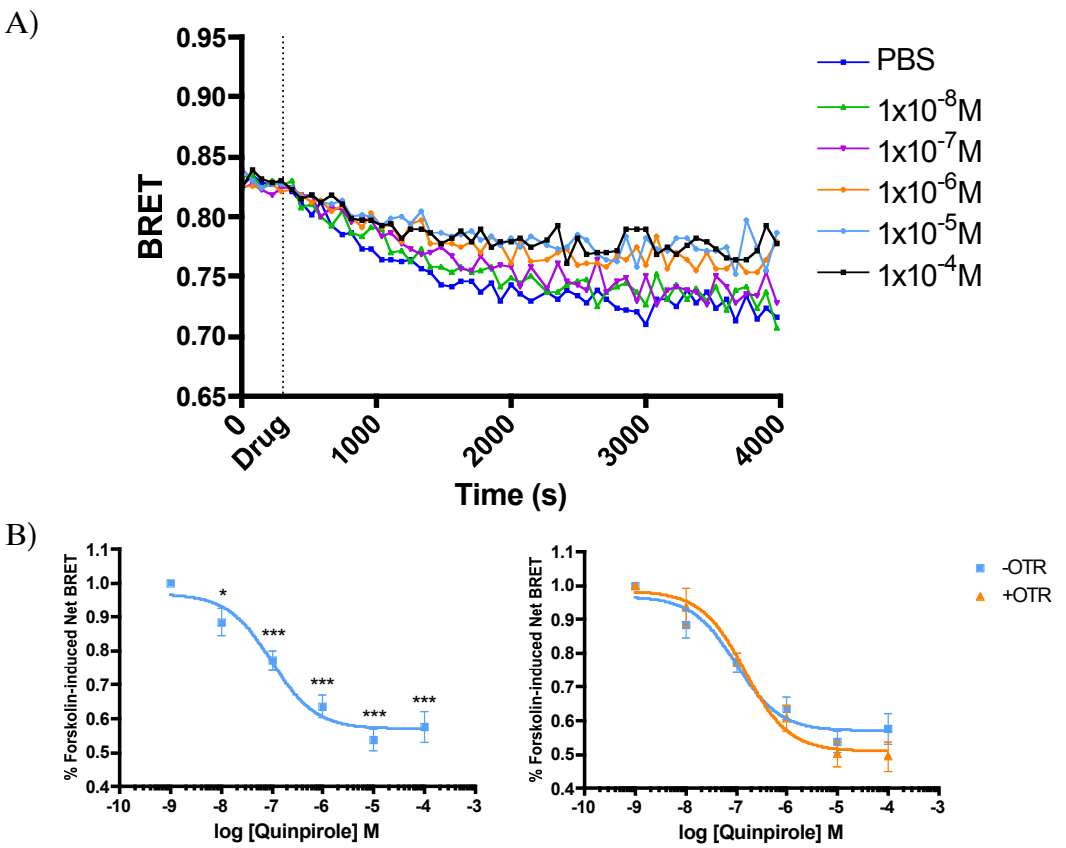

Figure 12: *Quinpirole inhibits forskolin-mediated cAMP production via D2R in a dose-dependent manner.* 25 μM forskolin alone (PBS) induced a decrease in BRET corresponding to an increase in cAMP production that was dose-dependently inhibited by the D2R agonist, quinpirole. A) Time course of drug treatment in cells expressing D2R: substrate was added at time 0, and drug cocktail at time 300 seconds. BRET values averaged over the first five minutes represent basal BRET and the last five minutes represent treatment-stimulated BRET. The difference between these two averages is presented as net BRET. B) The net BRET data was transformed to percentage of maximal cAMP production induced by forskolin, which was set at 1, and plotted as a dose-response curve (left). OTR co-expression had no significant effect on D2R-mediated cAMP inhibition (right). The data in A is one experiment representative of four independent experiments. The data in B represent the mean ± SEM for four independent experiments conducted in duplicate for each concentration point. *p < 0.05, **p < 0.01, ***p < 0.001 between each concentration point and the maximum forskolin effect.

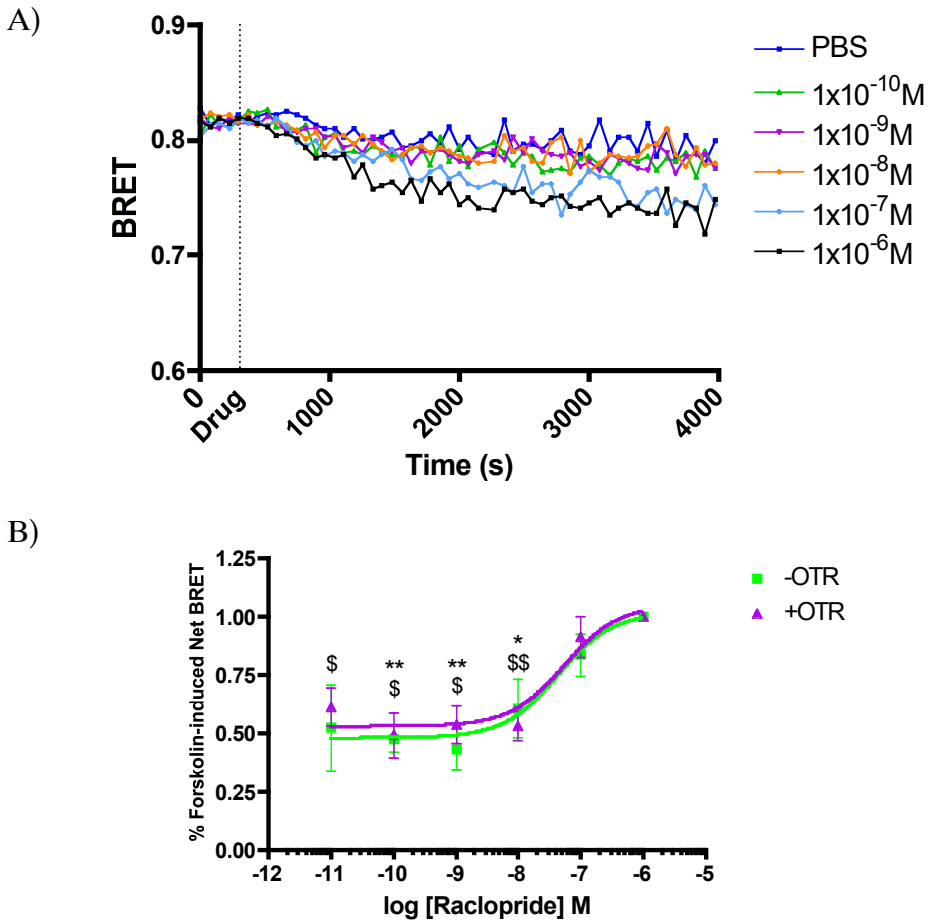

Figure 13: *Raclopride inhibits the quinpirole-mediated inhibition of cAMP production in a dose-dependent manner.* Cells were pretreated fifteen minutes with increasing concentrations of raclopride and then treated with 1 μM quinpirole plus 25 μM forskolin alone (PBS). Raclopride dose-dependently reversed quinpirole-mediated inhibition of forskolin-induced cAMP production. A) Time course of drug treatment in cells expressing D2R: raclopride was added fifteen minutes prior to substrate at time 0, and drug cocktail at time 300 seconds. BRET values averaged over the first five minutes represent basal BRET and the last five minutes represent treatment stimulated BRET. The difference between these two averages is presented as net BRET. B) The net BRET data was transformed to percentage of maximal cAMP production induced by forskolin and the highest concentration of raclopride, which was set at 1, and plotted as a dose-response curve for cells expressing both D2R and OTR. OTR co-expression had no significant effect on raclopride-mediated inhibition of the D2R effect. The data in A is representative of two independent experiments conducted in duplicate. The data in B represent the mean ± SEM for two independent experiments conducted in duplicate for each concentration point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between each concentration point and the maximum forskolin effect (-OTR). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ between each concentration point and the maximum forskolin effect (+OTR).

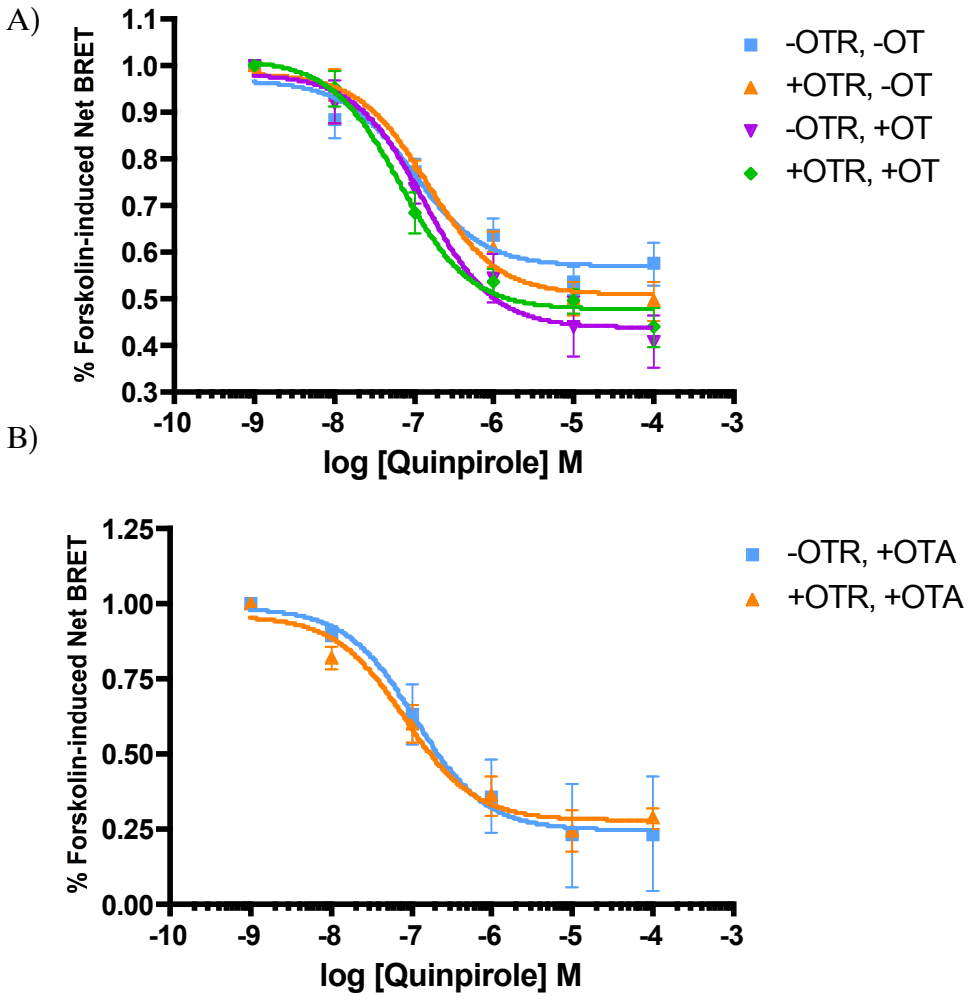

Figure 14: *Oxytocin and OTA have no effect on D2R-mediated cAMP inhibition.* HEK 293 cells transiently expressed D2R with or without OTR. A) Quinpirole dose-dependently decreased forskolin-mediated cAMP production (-OTR, -OT). The presence of OTR, treated (+OTR, +OT) or untreated (+OTR, -OT) had no significant effect on quinpirole-mediated cAMP inhibition. B) Fifteen minute pretreatment with 1 μM OTA induced no change in D2R-mediated cAMP inhibition, with (+OTR, +OTA) or without (-OTR, +OTA) OTR present. The net BRET data was transformed to percentage of maximal cAMP production induced by forskolin, which was set at 1, and plotted as a dose-response curve. The data represent the mean ± SEM for four (A) and two (B) independent experiments conducted in duplicate for each concentration point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between +OTR, -OT, -OTR, +OT, or +OTR, +OT versus –OTR, -OT (A) or –OTR, +OTA versus +OTR, +OTA (B).

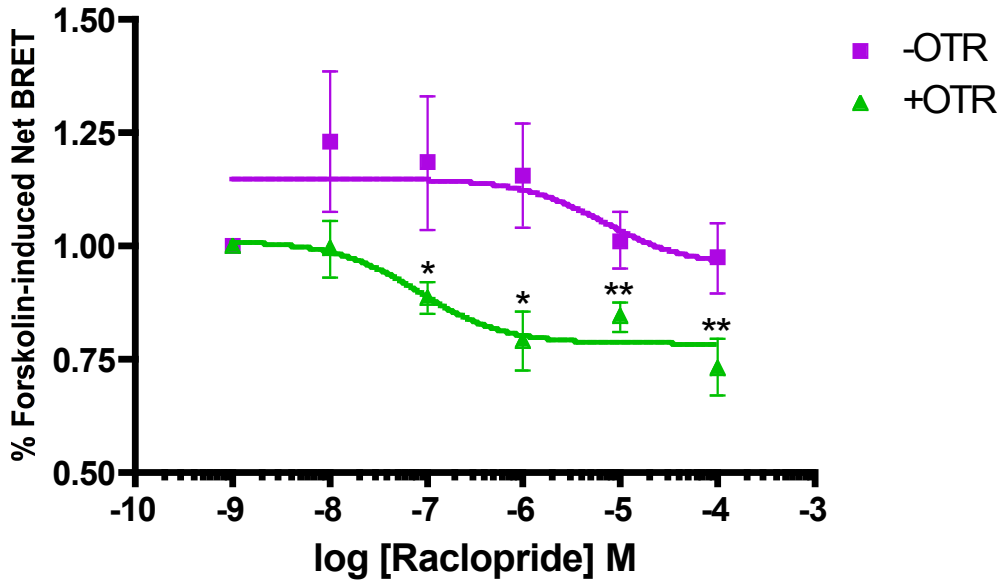

Figure 15: *OTR potentiates the effects of raclopride on D2R modulation of the cAMP pathway.* HEK 293 cells transiently expressed D2R with (+OTR) or without (-OTR). Cells were treated with 25 μM forskolin following a fifteen-minute pretreatment with increasing concentrations of raclopride. Raclopride dose-dependently inhibited forskolin-mediated cAMP production in cells expressing both D2R and OTR together. This effect was less pronounced in cells expressing D2R alone. The net BRET data was transformed to percentage of maximal cAMP production induced by forskolin, which was set at 1, and plotted as a dose-response curve. The data represent the mean ± SEM for three independent experiments conducted in duplicate for each concentration point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between each concentration point versus max forskolin effect.

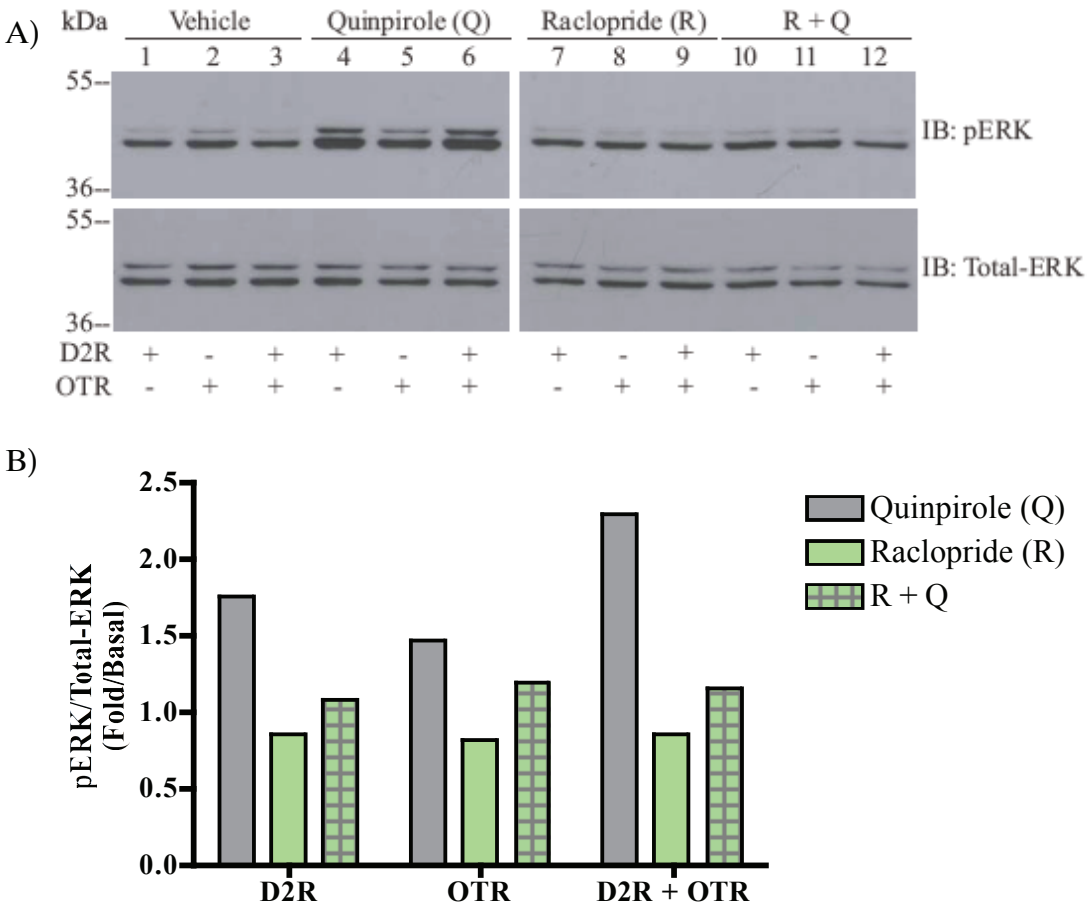

Figure 16: *Raclopride blocks quinpirole-mediated ERK phosphorylation.* HEK 293 cells transfected with D2R and/or HA-OTR were pretreated with DMSO or 1 μM raclopride for fifteen minutes and then stimulated with H_2O or 100 nM quinpirole for five minutes. A) Western blot analysis: cells were treated with DMSO and H_2O in lanes 1-3 (Vehicle), with DMSO and quinpirole in lanes 4-6 (Quinpirole (Q)), with raclopride and H_2O in lanes 7-9 (Raclopride (R)), or with raclopride and quinpirole in lanes 10-12 (R + Q). Cell lysates were immunoblotted (IB) for phosphorylated ERK (pERK, top) or Total-ERK (bottom). B) Quantification of western blots by calculating the fold of drug-treated response as a fraction of Total-ERK minus vehicle-treated response as a fraction of Total-ERK. The data represent a single experiment.

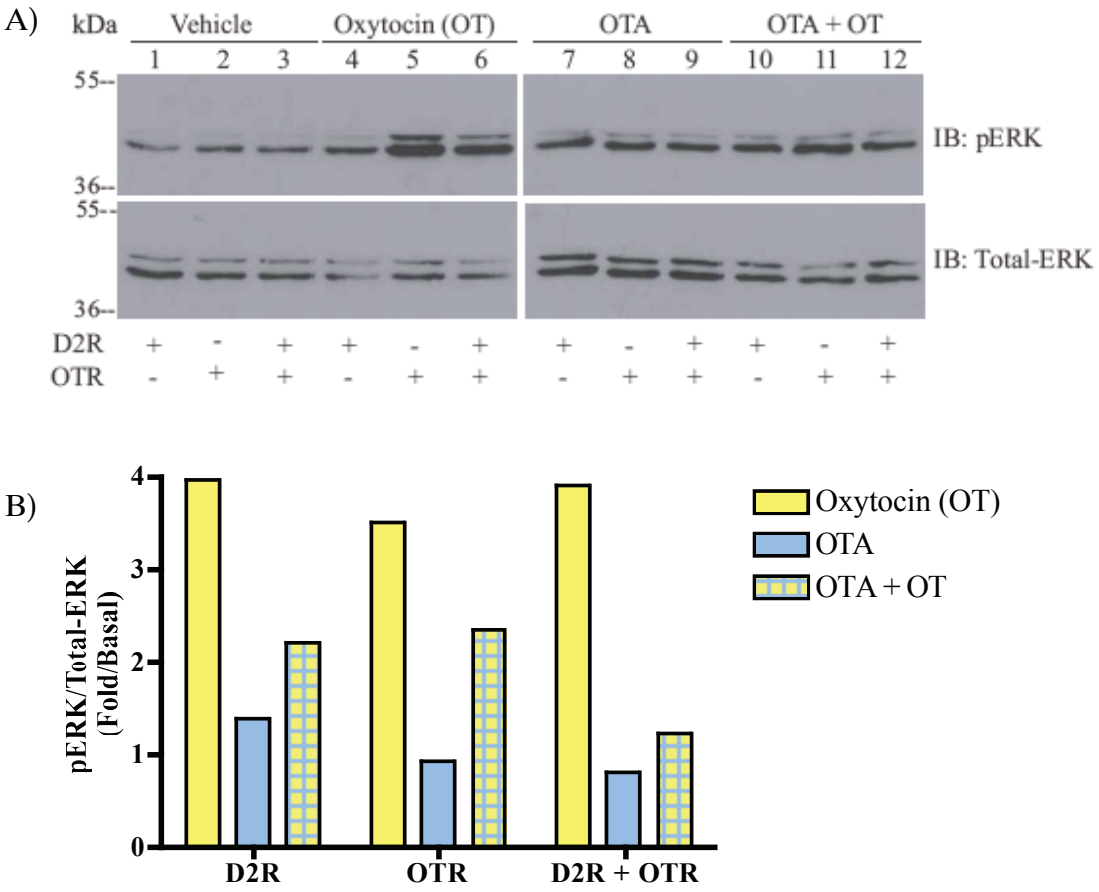

Figure 17: *OTA blocks oxytocin-mediated ERK phosphorylation.* HEK 293 cells transfected with D2R and/or HA-OTR were pretreated with H_2O or 1 μM OTA for fifteen minutes followed by five minutes stimulation with H_2O or 100 nM oxytocin. A) Western blot analysis: cells were treated with H_2O twice in lanes 1-3 (Vehicle), with H_2O and oxytocin in lanes 4-6 (Oxytocin (OT)), with OTA and H_2O in lanes 7-9 (OTA), or with OTA and oxytocin in lanes 10-12 (OTA + OT). Cell lysates were immunoblotted (IB) for phosphorylated ERK (pERK, top) or Total-ERK (bottom). B) Quantification of western blots by calculating the fold of drug-treated response as a fraction of Total-ERK minus vehicle-treated response as a fraction of Total-ERK (quantification of oxytocin-mediated ERK phosphorylation in cells expressing D2R alone does not seem to reflect accurately the western blot analysis, likely due to unequal loading). The data represent a single experiment.

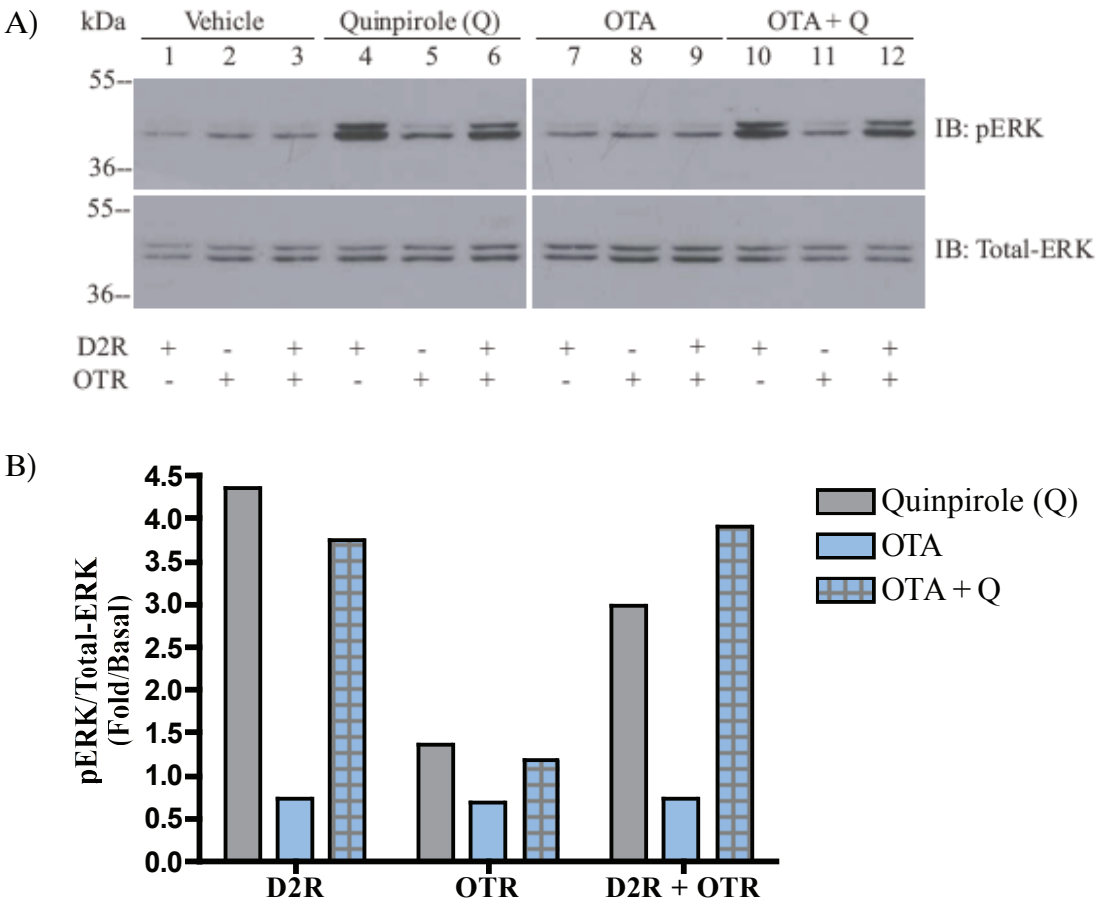

Figure 18: *OTA has no effect on quinpirole-mediated ERK phosphorylation.* HEK 293 cells transfected with D2R and/or HA-OTR were pretreated with H_2O or 1 μM OTA for fifteen minutes and then stimulated with H_2O or 100 nM quinpirole for five minutes. A) Western blot analysis: cells were treated with H_2O twice in lanes 1-3 (Vehicle), with H_2O and quinpirole in lanes 4-6 (Quinpirole (Q)), with OTA and H_2O in lanes 7-9 (OTA), or with OTA and quinpirole in lanes 10-12 (OTA + Q). Cell lysates were immunoblotted (IB) for phosphorylated ERK (pERK, top) or Total-ERK (bottom). B) Quantification of western blots by calculating the fold of drug-treated response as a fraction of Total-ERK minus vehicle-treated response as a fraction of Total-ERK. The data represent a single experiment.

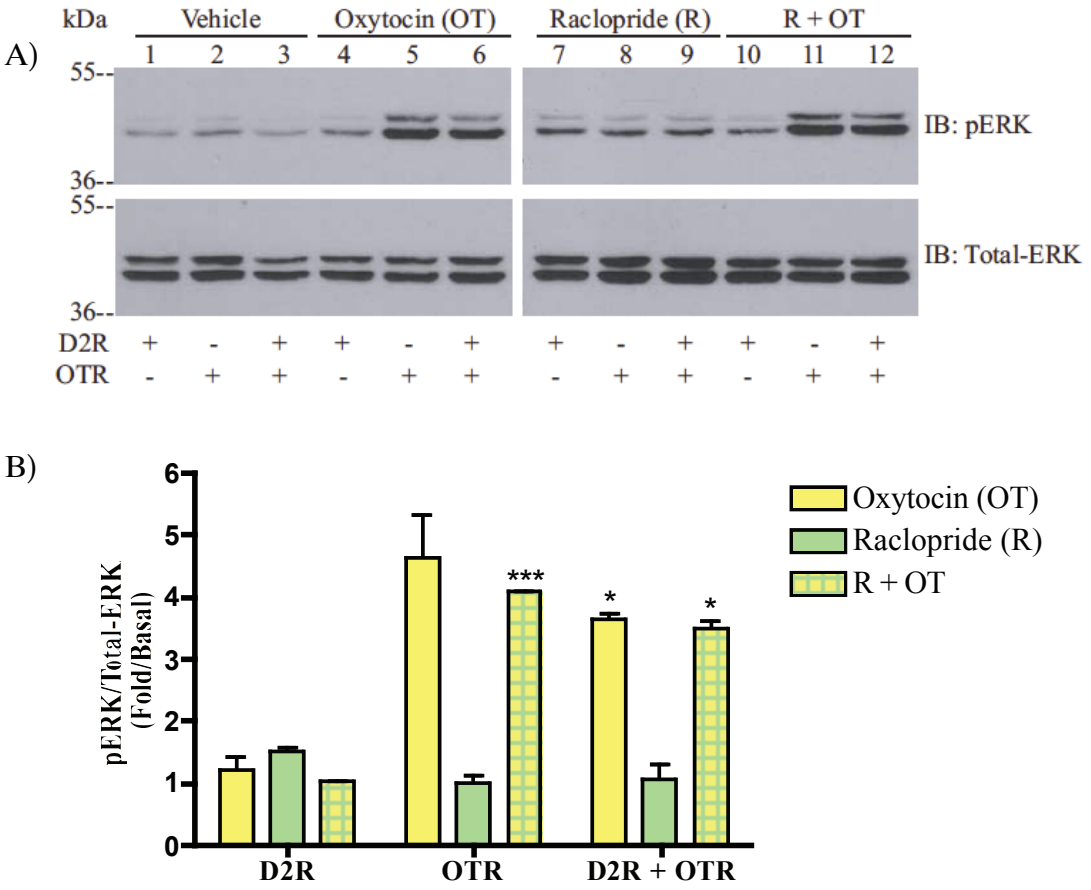

Figure 19: *Raclopride has no effect on oxytocin-mediated ERK phosphorylation.* HEK 293 cells transfected with D2R and/or HA-OTR were pretreated with DMSO or 1 μM raclopride for fifteen minutes and then stimulated with H_2O or 100 nM oxytocin for five minutes. A) Western blot analysis: cells were treated with H_2O twice in lanes 1-3 (Vehicle), with DMSO and oxytocin in lanes 4-6 (Oxytocin (OT)) with raclopride and H_2O in lanes 7-9 (Raclopride (R)), or with raclopride and oxytocin in lanes 10-12 (R + OT). Cell lysates were immunoblotted (IB) for phosphorylated ERK (pERK, top) or Total-ERK (bottom). B) Quantification of western blots by calculating the fold of drug-treated response as a fraction of Total-ERK minus vehicle-treated response as a fraction of Total-ERK. The data represent the mean ± SEM for two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus basal set at 1.

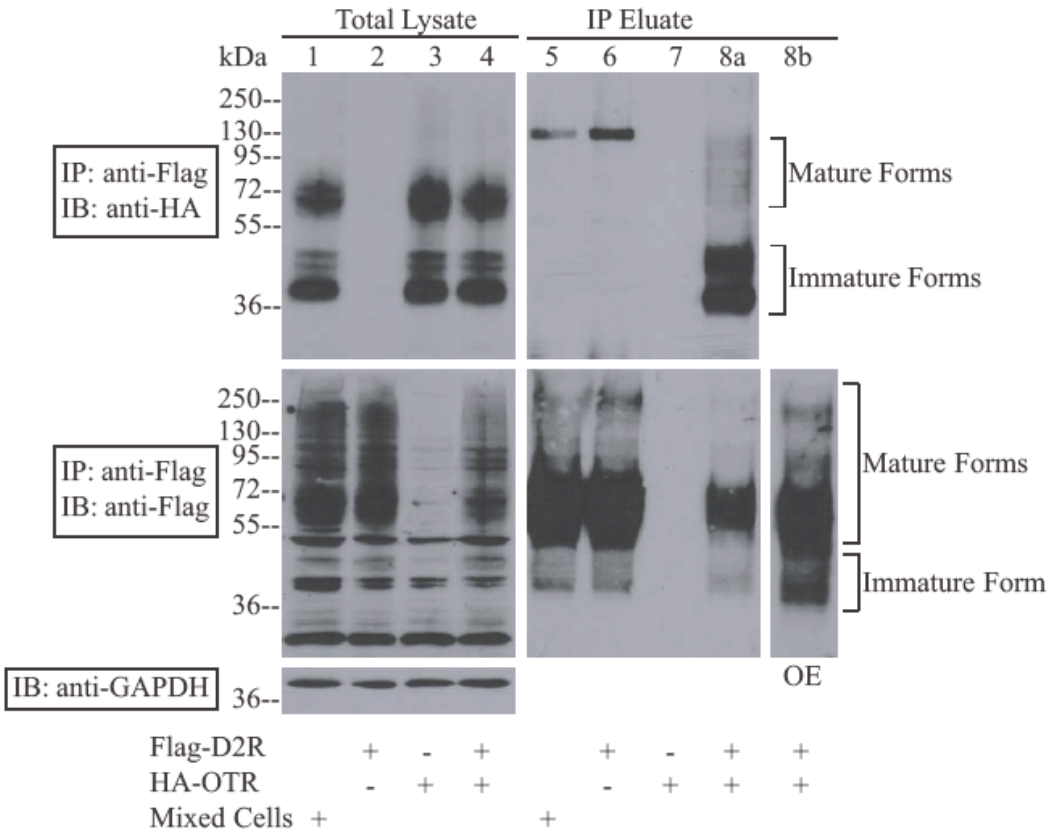

Figure 20: *D2R and OTR co-immunoprecipitate in HEK 293 cells following ligand stimulation.* Western blot analysis of total lysates and immunoprecipitates of HEK 293 cells transiently transfected with Flag-D2R (induced) and/or HA-OTR. Cells were transfected with either Flag-D2R (lanes 2 and 6) or HA-OTR (lanes 3 and 7) alone, or both receptors together (lanes 4 and 8ab). Lane 8b is an overexposure (OE) of lane 8a. As a control, two cell populations transfected with either Flag-D2R or HA-OTR were mixed together (lanes 1 and 5). Immunoprecipitation (IP) was performed using anti-Flag M2 agarose beads and immunoblotted (IB) with either anti-HA (top, lanes 5-8a) or anti-Flag (bottom, lanes 5-8ab). Total lysates are shown in lanes 1-4, and were immunoblotted (IB) with either anti-HA (top) or anti-Flag (bottom). Anti-GAPDH was used for an equal loading control. Brackets indicate the different molecular weight forms of HA-OTR and Flag-D2R. Image is representative of three independent experiments.

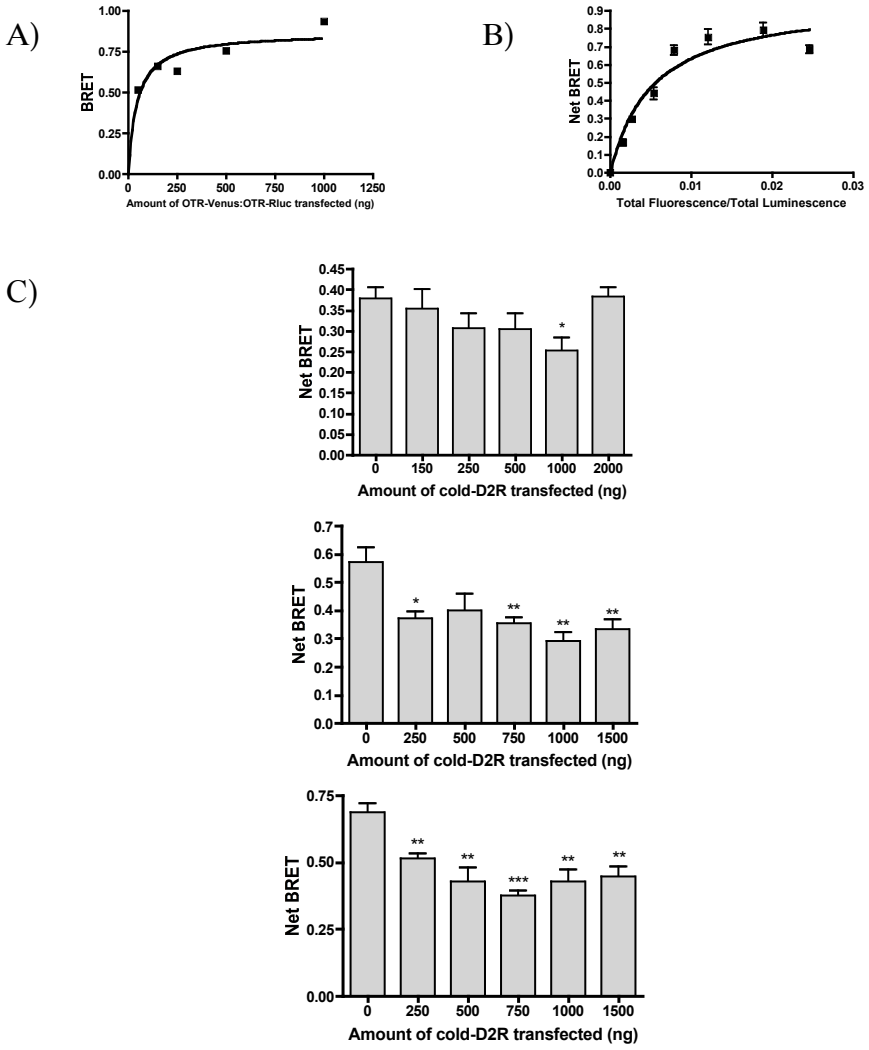

Figure 21: *D2R competes OTR homodimerization in HEK 293 cells.* A) Cells were transfected with increasing but equal ratios of OTR-venus:OTR-RLuc to determine the optimal amount of donor to use for BRET competition. B) Acceptor saturation to determine the optimal amount of acceptor to use for competition (250 ng OTR-RLuc and 0, 150, 250, 500, 750, 1000, 1500 or 2000 ng of OTR-Venus were transfected). C) BRET competition. Cells were transfected with constant amounts of OTR-Venus and OTR-RLuc, as well as increasing amounts of untagged or cold-D2R. With increasing amounts of co-transfected cold-D2R, there is a decrease in BRET between co-expressed OTR-Venus (500 ng) and OTR-RLuc (250 ng, top). Amounts of BRET pair were chosen based on saturation assays. Different amounts of cold-D2R were co-transfected (middle). Increased, fixed amount of OTR-Venus (750 ng) was transfected. The data in A represent a single experiment. The data in B represent the mean ± SEM for a single experiment conducted in five technical replicates. The data in C represent the mean ± SEM for three independent experiments (top, middle, bottom) conducted in five technical replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus no cold-OTR transfected.

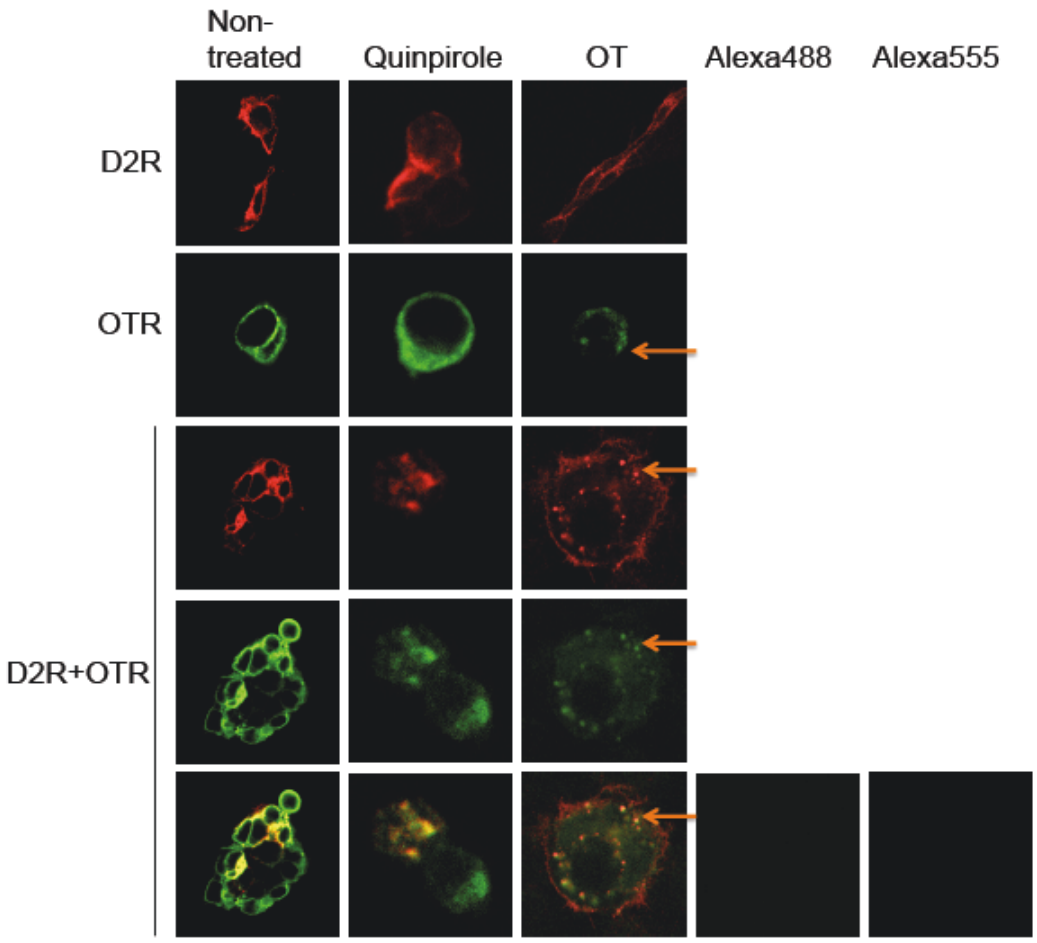

Figure 22: *D2R and OTR co-internalize in HEK 293 cells.* Cells were transfected with rabbit-Flag-D2R and/or mouse-HA-OTR. Flag-D2R was visualized by anti-rabbit conjugated-Alexa555 in red, and HA-OTR by anti-mouse conjugated-Alexa488 in green. Yellow represents co-localization as seen in merged images. Cells were left untreated, or stimulated with 1 μM quinpirole or 100 nM oxytocin (OT) for forty minutes. Orange arrows indicate endosomes. Untreated cells co-expressing both receptor constructs subject to secondary antibody alone served as a negative control for nonspecific binding. Images are representative of multiple fields within a single experiment.

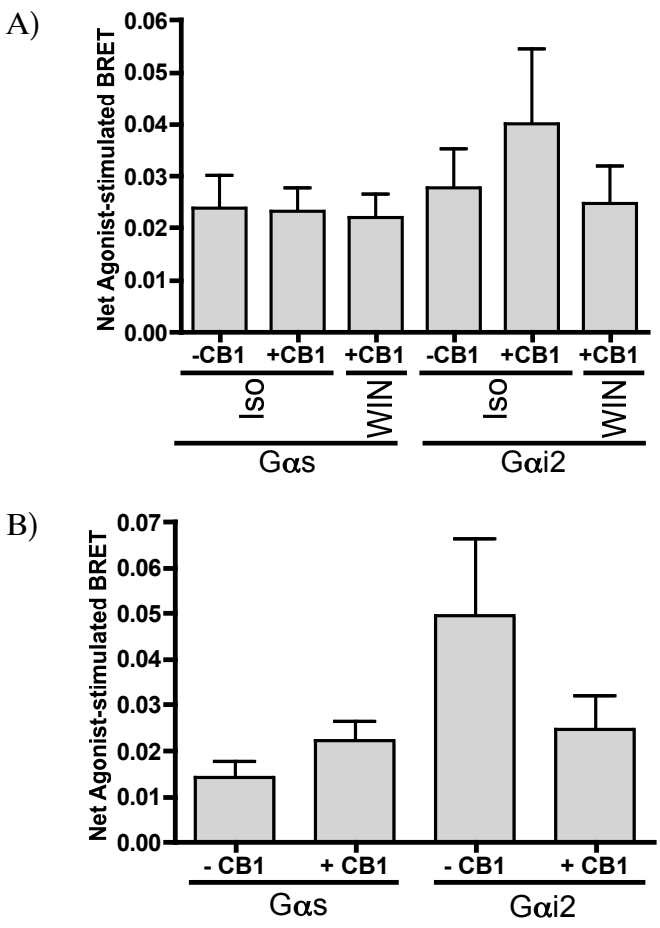

Figure 23: *Agonist-mediated BRET between G protein subunits in cells expressing β_2AR with or without CB1R.* HEK 293 were transiently transfected with β_2AR, Flag Gβ1, Gγ2-GFP10 and Gαs- or Gαi2-RLuc8, either with or without CB1R, and were treated with 10 μM isoproterenol or 10 μM WIN55,212-2. (A) BRET increased between Gαi2-Rluc8 (Gαi2) and Gγ2-GFP10 comparatively to Gαs-Rluc8 (Gαs) and Gγ2-GFP10, only when CB1R was co-expressed (+CB1R) versus expression of β_2AR alone (-CB1R), following isoproterenol treatment (Iso). WIN55,212-2 (WIN) did not seem to have an effect on BRET between Gαs-Rluc8 and Gγ2-GFP10 or between Gαi2-Rluc8 and Gγ2-GFP10 in cells expressing both receptors when compared to isoproterenol treatment of cells expressing β_2AR alone. However, in cells co-expressing both receptors, WIN55,212-2 stimulation induced markedly lower BRET than isoproterenol. (B) WIN55-212,2 treatment of cells expressing β_2AR, with or without CB1R. A small increase in BRET occurred between Gαs-Rluc8 and Gγ2-GFP10 when CB1R was expressed. A decrease in BRET between Gαi2-Rluc8 and Gγ2-GFP10 was seen when CB1R was co-expressed versus when β_2AR was expressed alone. The data represent the mean ± SEM for five to eleven (A) and four to seven (B) independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

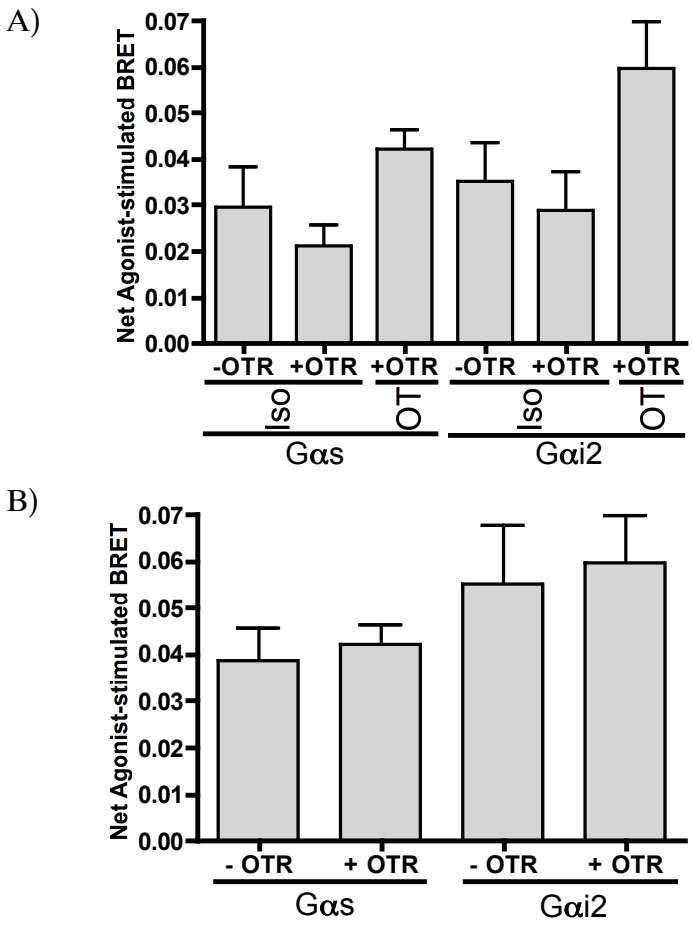

Figure 24: *Agonist-mediated BRET between G protein subunits in cells expressing β_2AR with or without OTR.* HEK 293 were transiently transfected with β_2AR, Flag Gβ1, Gγ2-GFP10 and Gαs- or Gαi2-RLuc8, either with or without OTR, and were treated with 10 μM isoproterenol or 100 nM oxytocin. A) BRET between Gαs- and Gαi2-RLuc8 with Gγ2-GFP10 (Gαs, Gαi2) seemed to exhibit a decreased trend following isoproterenol (Iso) stimulation of β_2AR in the presence of OTR (+OTR), compared to absence (-OTR). Oxytocin (OT) treatment seemed to increase BRET for both BRET pairs when OTR was co-expressed with β_2AR, however oxytocin also increased BRET to similar levels in cells expressing β_2AR alone as seen in figure B. B) Oxytocin treatment of cells expressing β_2AR, with or without OTR. Oxytocin stimulation did not seem to alter BRET, with or without OTR. The data represent the mean ± SEM for seven to eight independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

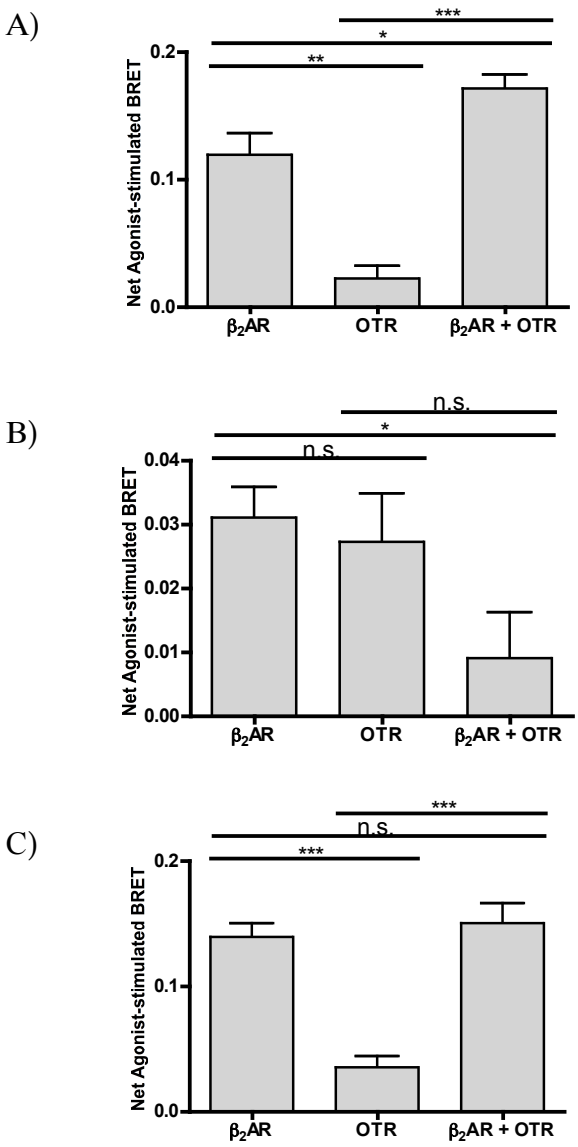

Figure 25: *Agonist-mediated BRET between effector and G protein in cells expressing β_2AR with or without OTR.* HEK 293 were transiently transfected with β_2AR, Flag Gβ1, Gγ2-GFP10 and ACII-RLucII, either with or without OTR, and were treated with 10 μM isoproterenol or 100 nM oxytocin. A) Isoproterenol treatment increased BRET between ACII-RLucII and Gγ2-GFP10 only when β_2AR was present, and did so to an even greater extent when OTR was co-expressed. B) Oxytocin stimulation had less effect in all receptor expression conditions, however significantly decreased BRET when both receptors were co-expressed. C) Simultaneous isoproterenol and oxytocin treatment increased BRET between ACII-RLucII and Gγ2-GFP10 only when β_2AR was present, which was not significantly different when OTR was co-expressed. The data represent the mean ± SEM for four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n.s. = non-significant).

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