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An investigation into ligand selectivity between dopamine receptor subtypes, biased agonism, and protean agonism

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Abstract

The G protein-coupled dopamine receptors D_2R and D_3R have long been considered major targets for antipsychotics in the treatment of disorders like schizophrenia. Typical, or first-generation antipsychotics tend to antagonise both dopamine and serotonin receptors in an effort to alleviate symptoms; the more modern atypical, or second-generation, antipsychotics (SGAs) display greater preference for antagonising serotonin receptors, and are termed 'atypical' due to their association with lower reported occurrences of extrapyramidal side effects. As these side effects are characterised by a loss of fine motor control and are thought to occur due to the mass blockade of D_2Rs, these ligands' higher relative selectivity for serotonin receptors is one potential explanation for their atypicality.

Recent findings suggest that one SGA, quetiapine, displayed biased agonism at the D_3R, favouring recruitment of and signalling via β-arrestin 2-mediated pathways over the receptor's associated G protein, resulting in modulation of calcium channels localised to the site of action potential initiation in prefrontal cortex pyramidal neurons. Further, said paper also demonstrated that treatment with quetiapine abolishes D_3R function through postendocytic receptor degradation by G protein-coupled receptor-associated sorting protein 1 (GASP1). The authors propose this as a hitherto overlooked aspect of atypicality which, if accurate, could be important for future antipsychotic drug design and the potential for developing ligands which can reduce cell surface receptor populations as opposed to just antagonising them.

The aim of this research was to optimise assay methods to support the investigation of agonist action at both D_2R and D_3R with the overall purpose of verifying these claims of biased agonism.

In order to achieve this, multiple assays were attempted and optimised for the purpose of achieving consistency and repeatability of experiments measuring the activation of the G protein and the recruitment of β-arrestin 2. Attempts were made at reducing variability between repeats using a βγ-release assay, and later a TRUPATH assay, before settling on a mG_o assay, which utilises a

modified $G\alpha_o$ G protein subunit (or 'miniG protein'), alongside a β-arrestin 2 assay, the optimisation of which were more successful.

Using these optimised assays, behaviours of various commonly prescribed SGAs were observed alongside a control dopamine receptor agonist, with particular focus on quetiapine's action.

From this, it was possible to conclude that there is no evidence of quetiapine facilitating β-arrestin 2-mediated signalling at either the D_2R or the D_3R, potentially contradicting findings by Schamiloglu *et al* (2023) which suggested that quetiapine stimulates ERK phosphorylation in a β-arrestin 2-dependent manner at the D_3R. Further, the recruitment of β-arrestin 2 to the D_3R did not appear to correlate to observed receptor internalisation, even in the case of the control agonist quinpirole, though further research would be necessary to validate any absence of receptor internalisation and to understand the previous observations of the ERK1/2 signal stimulated by quetiapine through the D_3R.

Acknowledgements

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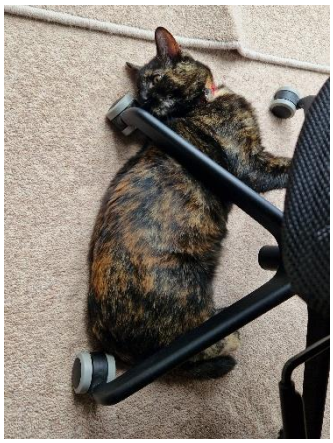
A  **B** 

Figure 1: Indispensable thesis writing assistants. Pictured: **(A)** Gil, **(B)** Mo.

1.0 Introduction

In 2006, following an evaluation of more than 21,000 drug products, J. P. Overington[1] released a review estimating that all classes of approved therapeutic drugs with a known mode of action work through just 324 distinct targets – 266 of which are human-genome-derived proteins. 60% of these targets can be found at the cell surface, despite such proteins making up only ~22% of all proteins encoded by the human genome.

As such, the importance of these interactions between drug and target receptor and their role in forming the basis of drug discovery and optimisation for decades cannot be overstated.

1.1 GPCRs

There are generally considered to be two families of post-synaptic receptors: ionotropic and metabotropic.

Ionotropic receptors are defined by their direct linkage to ion channels, possessing two functional domains: their extracellular neurotransmitter-binding site, and their ion channel-forming transmembrane domain. Upon ligand-binding, conformational changes take place, resulting in the opening of the ion channel, and as such, these are often referred to as ligand-gated ion channels.

Metabotropic receptors like GPCRs function instead through ligands binding to their extracellular binding site inducing a conformational change leading to the activation of intracellular effector proteins. These intracellular proteins may then go on to open membrane-spanning ion channels amongst the induction of other signalling cascades, though these ion channels are specifically not associated with or part of the receptor itself.

1.1.1 The structure and function of G proteins

GPCRs, or G-protein coupled receptors, are transmembrane cell surface receptors, responsible for the detection of molecules outside the cells and the subsequent activation of the relevant cellular responses, occurring through second messenger signal transduction pathways such as cyclic AMP and phosphatidylinositol.

As their name suggests, GPCRs are coupled with a heterotrimeric G protein on the intracellular side of the membrane, made up of three subunits: α, β, and γ. Upon ligand binding, a conformational change takes place in the GPCR, enabling the activation of the associated G protein through the release of GDP, bound to the G protein in its inactive state, for GTP. The α subunit, with the bound GTP, can then dissociate from the βγ subunits and go on to perform various functions (e.g. furthering the intracellular signalling cascade or simply targeting functional proteins directly); the specific resulting effect depends on the type of α subunit, of which there are currently eighteen described as encoded by the human genome[(2)].

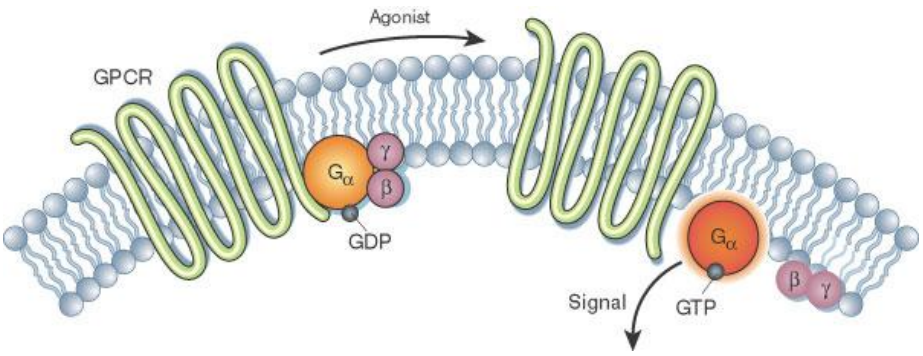


Figure 2: Activation of the Gα subunit of a G protein. Upon agonist binding, a conformational change takes place in the GPCR, enabling the activation of the associated G protein through the exchange of GDP for GTP. The α subunit may then dissociate from the βγ subunits and go on to perform its relevant functions within the cell. ([(3)]Li, J. *et al*, 2002)

Family	Members	Expression	Function
α_s (stimulatory)	s	Ubiquitous	Enhances adenylate cyclase activation, increasing accumulation of cAMP
	olf	Olfactory neurons	
α_i (inhibitory)	i1	Widely distributed	Promotes inhibition of adenylate cyclase, thereby attenuating cAMP accumulation
	i2	Widely distributed	
	i3	Ubiquitous	
	oA	Neurons	
	oB	Neuroendocrine	
	t1	Retinal rods, taste cells	
	t2	Retinal cones	
	g	Taste and brash cells	
	z	Neurons, platelets	
α_q	Q	Ubiquitous	Stimulates PLC to phosphorylate PIP^2 into IP^3 and DAG, which bind to Ca^{2+} channels on the ER, thus inducing an increase in $[Ca^{2+}]$
	11	Ubiquitous	
	14	Kidney, lung, liver	
	15	Hematopoietic cells	
	16	Hematopoietic cells	
α_{12}	12	Ubiquitous	RhoA activation
	13	Ubiquitous	

Table 1: G protein α-subunit subtypes, their functions, and their distributions. (Information derived and collated from: [2]Syrovatkina *et al* (2016), [4]Nestler *et al* (1999))

1.1.2 Clinical significance

GPCRs are the largest known family of receptors, making up around 4% of the total protein-coding human genome with over 800 different genes predicted to code for them[9], and are thought to play key roles in various physiological processes including sensory systems (vision, taste, smell, etc.), mental regulation (mood, emotional, behavioural, etc.), immunological function, the cardiovascular system, cancer progression and metastasis of some tumour types, and the endocrine system. With such wide-reaching responsibilities, it is of no surprise that GPCRs are incredibly common drug targets to treat pathologies associated with these systems; approximately 34% of all FDA approved drugs target 108 members of the GPCR family, and it is estimated

that 50% of all drugs currently on the market worldwide target some form of GPCR[10].

Further, the field of research surrounding GPCRs is still comparatively young – the first determination of the crystal structure of the GPCR rhodopsin occurred in 2000[11], and the first structure of a GPCR and G protein trimer complex was determined in 2011[12] – and is, as such, a rapidly evolving area of study.

1.1.3 Receptor regulation

During intracellular signalling cascades, receptor activation is no more important than receptor deactivation; the signal must be attenuated at some point to avoid overstimulation, and this is generally considered to occur through three 'stages': desensitisation (acute), internalisation or sequestration (intermediate timescales), and downregulation (prolonged).

Desensitisation is the quickest, and therefore first, to occur, initiating within seconds of agonist exposure and the subsequent phosphorylation of the receptor. Second-messengers activated by the stimulated GPCR further activate protein kinases such as protein kinase A (PKA) or C (PKC), and G protein receptor kinases (GRKs).

These kinases act to phosphorylate serine and threonine residues within either the C-terminal tail or intracellular loops of the GPCR, thereby impairing the receptor's ability to bind to its G protein. To do this, some GRKs require certain interactions to occur first; for example, GRKs 2 and 3 require interaction with released βγ subunits in order to function, whereas GRK5 and 6 do not. Desensitisation on its own is not associated with any measurable or significant change to either plasma membrane or intracellular receptor population and may be reversed swiftly following the agonist's removal.

Internalisation may follow desensitisation, taking place over the course of several minutes following exposure to the agonist, and involves the movement of the GPCR into a vesicle, where it may then be trafficked as needed.

Dynamin, a large GTPase, is necessary for the cleavage of these clathrin-coated vesicles from the cell surface membrane and, as such, many GPCRs undergo internalisation in a dynamin-dependent manner. Further, the phosphorylation of the GPCR by GRKs and the binding of β-arrestin facilitate endocytosis. As in the case of desensitisation, sequestered GPCRs may be recycled and returned to the plasma membrane upon the removal of the bound ligand, and the dissociation of the β-arrestin 2.

Downregulation is generally described as the removal and degradation of GPCRs from the cell surface membrane, and occurs following their prolonged activation, taking place over the course of hours or even days. Upon receptor sequestration, the GPCR may either be recycled and returned to the cell surface membrane or degraded within lysosomes, which, in the case of many GPCRs, is thought to be promoted by the ubiquitination of the receptor. It is also thought that receptor mRNA levels decrease as part of the process of downregulation, though the exact mechanisms by which this takes place are currently unknown[5,6].

This general understanding of β-arrestin 2 recruitment facilitating receptor internalisation has led to increased interest in 'biased agonism', described as the response displayed when a binding ligand does so with favourability towards a specific conformation of the receptor; for example, a conformation which leans more towards recruiting β-arrestin 2 to a GPCR than activating its associated G protein[7]. This could allow the design of a ligand which does not initiate the signalling cascade induced by G protein activation but still drives receptor internalisation as a conventional agonist would, something that could prove promising in the optimisation of treatments for various psychotic disorders.

Facilitating receptor internalisation is not β-arrestin 2's only role, though; it can also act as a scaffolding protein, enabling a second wave of intracellular GPCR signalling, often linked to the MAP kinase pathway.

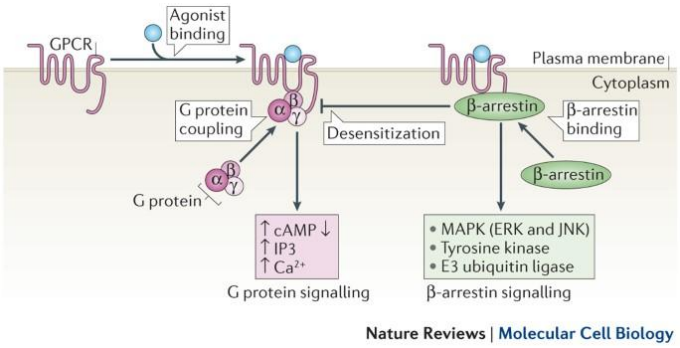


Figure 3: Simplified GPCR signalling, demonstrating potential G protein- and β arrestin-mediated signalling pathways. ([8]Ghosh, E. *et al* (2015))

1.1.4 The problem of constitutive activity

All GPCRs are activated by agonists; however, spontaneous auto-activation of receptors has also been observed. This is commonly referred to as basal or constitutive activity, where an empty receptor happens to undergo a conformational change into its active state, independent from any kind of outside stimulus such as agonist binding.

The first description of such behaviour came from Cerione *et al*., who demonstrated the β_2 adrenergic receptor (β_2AR) activating in the absence of any agonistic factors, in 1984[13]. Subsequently, constitutive activity has been reported in a number of different receptors in a number of different tissues, and the loss of said activity has been shown to result in disease in several cases, suggesting that the spontaneous auto-activation of GPCRs can play a crucial role in the regular function of the body – particularly the brain.

Evidence of the physiological effects of GPCR constitutive activity – and the consequences of its absence – include studies on the melanocortin-4 receptor, where a mutation resulting in a loss of constitutive activity is thought to cause obesity[14]. Similarly, mutations in the growth hormone secretagogue receptor resulting in a loss of constitutive activity have been linked to familial short stature[15], and dysfunction of the constitutive activity of the serotonin receptor

6 resulted in the depletion of neural stem cells and increased depression-like behaviours[16].

This is reportedly true in the case of dopamine receptors specifically[17], and so it stands to reason that receptors prone to constitutive activity in native environments would also display such behaviours *in vitro*. This can pose significant issues when it comes to performing assays in cell systems expressing dopamine receptors – if a significant portion of the receptors are already activated, can you detect an agonist's response above the basal activity?

1.2 Dopamine receptors

1.2.1 What are dopamine receptors?

Dopamine receptors are a subfamily of GPCRs, identified predominantly in the central nervous system and thought to be activated by the neurotransmitter dopamine, which is their primary endogenous ligand. Dopaminergic neurotransmission in the brain can be split into four major pathways: mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways; along with two minor pathways: hypothalamospinal tract, and incertohypothalamic pathways[18,19].

Pathway	Associated processes	Associated disorders
Mesocortico-limbic system	Reward/aversion-related cognition, Incentive salience, Pleasure, Positive reinforcement, Executive functions	ADHD, Addiction, Schizophrenia
Nigrostriatal	Motor function, Reward-related cognition, Associative learning	Addiction, Chorea, Huntington's disease, Schizophrenia, ADHD, Tourette's syndrome, Parkinson's disease
Tuberoinfundibular	Regulation of prolactin secretion	Hyperprolactinaemia
Hypothalamospinal tract	Motor function	Restless legs syndrome
Incertohypothalamic	Visceral and sensorimotor activities	Tremors

Table 2: Six major and minor dopaminergic pathways, their processes, and associated disorders. (Information derived and collated from [18]Ikemoto (2010) and [19]Hudepohl & Nasrallah (2012)).

The dopamine receptor family is made up by five receptors, labelled as the dopamine D_1 receptor (D_1R) through to the dopamine D_5 receptor (D_5R), and may then be further separated into two subfamilies: D_1R and D_5R are members of the 'D_1-like family', and D_2R, D_3R, and D_4R make up the 'D_2-like family'.

The D_1-like family are primarily coupled to G proteins with the $G\alpha_s$ (otherwise known as 'stimulatory') subtype. These α subunits are so named for their activation of adenylyl cyclase, which then leads to an increase in the intracellular concentration of cAMP, a second messenger with a variety of downstream roles in signal transduction[20]. There is some suggestion that D_1R agonism can regulate intracellular factors independent of cAMP; however, this is, at present, poorly understood and under-researched[21].

On the other hand, D_2-like receptors tend to be coupled to $G\alpha_i$ proteins, or 'inhibitory' G protein subunits. These subunits directly inhibit the formation of cAMP through the inhibition of adenylyl cyclase[22].

1.2.2 D_2-like family

In terms of structure, D_2R and D_3R bear a striking similarity to one another, with large portions of highly conserved regions, and significant sequence homology, particularly in the residues forming the orthosteric binding site (approximately 50% total amino acid sequence similarity, and 78% in transmembrane regions including the binding site)[23-25], making it exceedingly difficult to design ligands to select for one receptor over the other.

Whilst D_2R can be found throughout the entire body, D_3R displays far more restricted distribution; the regions with the greatest expression of this receptor can be found within the brain, specifically in the islands of Calleja in the nucleus accumbens within the ventral striatum. Dopamine function in these regions has been associated with roles in attention management and the reward response to stimulus[23].

1.2.3 Physiological role and clinical significance

As shown in table 2, dopamine receptors are implicated in a wide variety of neurological processes including, but not limited to, motivation, cognition, memory, learning, motor control, modulation of neuroendocrine signalling, and emesis. Their expression is not limited to the brain, however.

Dopamine receptors are also found elsewhere in the human body. For example, the cardio-pulmonary, renal, and pancreatic systems[26].

Within the cardio-pulmonary system, dopamine receptors have been described in the epicardium, myocardium, and endocardium of the heart. Dopamine has been suggested to increase myocardial contractility and cardiac output alongside heart rate, though it is important to note that it is currently under debate if the role of dopamine receptors in the heart is at all distinguishable from that of β-adrenoceptors[26].

In kidney nephrons, dopamine is thought to affect diuresis and natriuresis, playing a role in hydroelectrolytic regulation, and maintaining acid-base balance as well as blood pressure[27].

β cells, along with other endocrine and exocrine pancreatic cells, are also known to express D_2R and co-secrete dopamine alongside insulin[26].

Dopamine has been purported to be a negative regulator of glucose-stimulated insulin secretion, a connection which is supported by common side effects of traditional antipsychotics which target and block D_2R activity in the brain and are commonly reported to induce weight gain and glycaemic dysregulation[26].

Due to their varying roles and distribution, the dysfunction of dopamine receptors and the dysregulation of dopaminergic neurotransmission are implicated in numerous diseases such as social phobia, Tourette's syndrome, ADHD, Parkinson's disease, and drug or alcohol dependence and addiction[18,19].

1.2.4 The role of dopaminergic neurotransmission in psychiatric medicine

As explored extensively above, the dysfunction and dysregulation of dopaminergic signalling is associated with a variety of conditions, syndromes, and diseases throughout the body. However, one specific subset of conditions was of particular interest throughout this project, namely mental disorders linked to psychosis.

Schizophrenia, for example, is one such condition characterised by significant changes to thoughts, mood, behaviour, and even perception. These symptoms can be categorised into three groups – positive, negative, and cognitive.

Positive symptoms include delusions, hallucinations, and disorganised thoughts, speech, and behaviour[28]. Reportedly, the most common of these hallucinations involve alterations to an individual's hearing, i.e. hearing voices. These symptoms can be, understandably, particularly distressing for those suffering from the condition[29].

Negative symptoms are described as those affecting one's mood: blunted affect, wherein a person expresses very little emotions; alogia, or stunted

speech; anhedonia, wherein the individual is unable to feel pleasure; asociality, or a lack of motivation to engage in social situations or activities; and avolition, or a general apathy and lack of motivation[30].

Cognitive symptoms, though not considered to be part of the 'core symptoms' the way positive and negative are, are still a defining characteristic of schizophrenia. These tend to appear long before the onset of positive or negative symptoms and generally involve dysfunctions in neuro- or social cognition. For example, neurocognitive issues may affect speech, memory, problem solving or reasoning, sensory processing, and motor control. Symptoms affecting social cognition often result in difficulty interpreting and responding to social situations[31,32]. Where cognitive symptoms rarely respond to treatment with antipsychotics, positive and negative symptoms have been far more promising.

The dopamine hypothesis:

While the specific causes of schizophrenia are still currently unconfirmed, there are several theories on potential explanations for symptoms, the most common of which being the dopamine hypothesis which suggests dopamine systems in the mesocortocolimbic pathways, and their dysfunction, contribute to both positive and negative symptoms of schizophrenia: the mesolimbic responsible for positive symptoms[33], and the mesocortical for the negative[34] – see figure 4 below. This theory is supported by the fact that almost all current antipsychotics behave as dopamine receptor antagonists, barring xanomeline, a muscarinic agonist which exhibits functional dopamine antagonism despite lacking affinity for dopamine receptors[35].

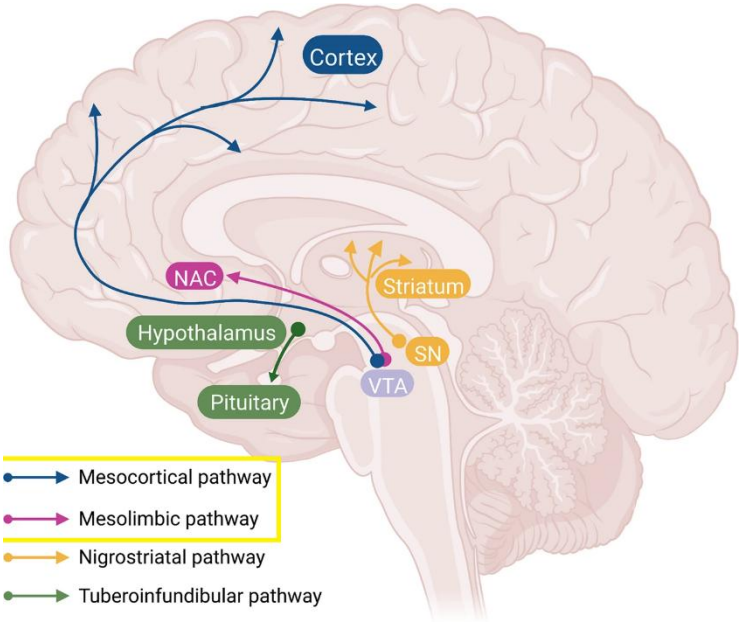


Figure 4: Dopaminergic pathways in the brain. (NAC: nucleus accumbens; SN: substantia nigra; VTA: ventral tegmental area). The mesocortical **(blue)** dopaminergic pathway (spanning from the ventral tegmental area (VTA) to the cortex), and the mesolimbic **(pink)** dopaminergic pathway (spanning from the VTA to the nucleus accumbens (NAC)), and their dysregulation are implicated in both the positive and negative symptoms associated with schizophrenia. ([36]Xu & Yang (2022), with minor modification)

These antipsychotics are separated into two groups, typical and atypical, based on their mechanism of action. Typical, or first generation, antipsychotics antagonise dopamine receptors (D_2R especially) alongside serotonin ($5\text{-}HT_{2A}$) receptors (also implicated in schizophrenia)[37], whereas atypical, or second generation, antipsychotics tend to display higher selectivity towards serotonin receptors[38]. The more recently introduced third generation antipsychotics, display partial agonism rather than antagonism of dopamine receptors[39].

While all antipsychotics are associated with extrapyramidal side effects, there is some evidence that second and third generation drugs have a lower risk of causing side effects and that, when they do, those symptoms display reduced severity[40]. These side effects – such as dystonia (involuntary muscle contraction), dyskinesia (difficulty with fine motor control), akathisia (inability to remain still), and pseudoparkinsonism (symptoms similar to those displayed

in Parkinson's disease) – can seriously impact a patient's everyday life, often reducing their willingness to continue with their treatment.

1.3 Biased agonism

1.3.1 What is biased agonism?

GPCR signalling is incredibly complex: one GPCR may be able to promiscuously couple to multiple G proteins, and there is evidence that GPCRs can engage a distinct wave of signalling through the recruitment of arrestin, which then acts as a scaffold for other signalling proteins.

Further, different ligands have been reported to display the ability to stabilise distinct conformations of the same GPCR, which might then differentially engage different subsets of the receptor's signalling cascade, such that one ligand might favour signalling through one pathway and another might favour signalling through another pathway; in the case of GPCRs, the two available options are the β-arrestin-mediated signalling events or the G protein(Gα/Gβγ)-mediated pathways. This ability of ligands to 'select' downstream cascades, as explored above in section 1.1.3, is generally referred to as either biased agonism or functional selectivity.

For example, serotonin at the 5-HT_{2A} receptor favours the activation of phospholipase C (PLC) – leading to the accumulation of inositol triphosphate (IP_3) – over phospholipase A2 (PLA2) – which is involved in the arachidonic acid signalling cascade. Lysergic acid diethylamide (LSD), on the other hand, demonstrates bias in the opposite direction to serotonin, activating PLA2 but displaying no significant activation of PLC[41].

Protean agonism:

Named after the shape-shifting Greek god Proteus, a protean agonist is generally defined as a ligand whose observed behaviour changes depending on the overall constitutive activity of a given receptor system. In a generally

quiescent system, the ligand is able to behave as a partial agonist, inducing partial activation of the system. In a receptor system displaying greater levels of basal activity, the ligand will behave as an antagonist and act to reduce overall receptor activity levels[42].

1.3.2 Potential clinical implications of biased agonism at the D_2-like dopamine receptors

As explored briefly above, the discovery that certain receptors, including the D_2R, can signal through both G protein- and β-arrestin 2-mediated pathways has provided potential avenues for the research into and design of optimised pharmacotherapies for the treatment of psychosis-related disorders[43,44]. According to findings from [45]Donthamsetti *et al* (2019), arrestin recruitment to the D_2R plays a role in mediating locomotion, without impacting incentive motivation, suggesting that these two pathways play distinct, and occasionally discrete, roles, providing an opportunity to potentially exploit these differences.

In that vein, previous reports suggest that the recruitment of β-arrestin 2 facilitates endocytosis of GPCRs, which may then be targeted for degradation in the lysosome via interaction with 'GASP1', or GPCR-associated sorting protein 1[46]. Receptor endocytosis and, thereby, the removal of receptors from the cell surface membrane means fewer available receptors for endogenous G protein agonists to interact with. So, theoretically, a β-arrestin 2 biased agonist would not just block ligand binding, and thus G protein activation, but could also lead to fewer functional receptors available in the first place[47,48].

Further supporting this theory, there has been some evidence that a reduction in functional D_2R affects responses to cocaine and is further supported by the claim that mice treated with a β-arrestin 2 biased second-generation antipsychotic (SGA) seem to develop tolerance to the locomotor and extrapyramidal side effects of the drug. Moreover, this tolerance was abolished in mice lacking GASP1, indicating a direct relation between tolerance and receptor removal and degradation[49].

If the above is the case, a β-arrestin 2 biased agonist could prove promising for the treatment of both psychotic disorders and psychostimulant-use disorders, though functional selectivity and bias at the D_3R is far less well explored than D_2R.

1.3.3 Quetiapine

In 2023, a paper[49] was released purporting the antipsychotic quetiapine as a potential biased agonist at D_3R, supposedly having an antagonistic effect on G protein activation and an agonistic effect on β-arrestin 2 recruitment. This bias could be important for several reasons. Firstly, one of the most common experiences reported by patients taking antipsychotics are extrapyramidal side effects or 'medication-induced movement disorders', causing Parkinson's-like symptoms and inhibiting fine motor control[50]; as locomotion is reportedly mediated though the recruitment of arrestin to the D_2R[45], the ability to maintain G protein antagonism for therapeutic effect without hampering β-arrestin 2-mediated signalling pathways could prove promising in the optimisation of treatments and reduction of side effects, increasing patient compliance and willingness to continue said treatments.

Secondly, if, as discussed, the recruitment of β-arrestin 2 to the D_3R facilitates receptor endocytosis, then a dopamine receptor agonist would drive internalisation, decreasing surface receptor levels over time whilst increasing G protein signalling, and an antagonist which blocks both G protein activation and β-arrestin 2 recruitment would prevent agonist-mediated endocytosis, thereby maintaining receptor levels over time. Chronic treatment with an arrestin-biased SGA, however, could offer an alternative route, resulting in a decreased number of functional D_3Rs present at the cell surface membrane, as the traditional agonist would, while offering similar G protein inhibition to that displayed by the traditional antagonist.

The data presented by Schamiloglu *et al*[49] utilised cAMP inhibition to demonstrate G protein activation, alongside imaging and the phosphorylation

of extracellular signal-regulated kinase (ERK) to measure β-arrestin 2 recruitment.

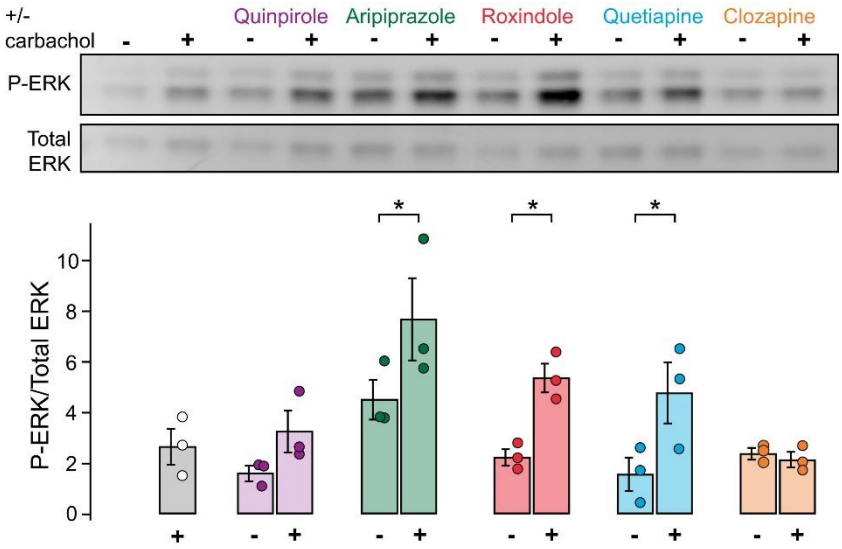


Figure 5: Measurement of total ERK and phosphorylated ERK (P-ERK) following drug treatment, extrapolated to represent β-arrestin 2 recruitment. Data as taken from [49]Schamiloglu *et al*. (2023): (Top) Representative immunoblot of P-ERK and total ERK after 5-minute drug treatment, with or without carbachol to activate PKC, in a D_3R-expressing stable HEK-293 cell line. (Bottom) Quantification of P-ERK/total ERK. Data displayed as mean ± SEM with a significance value of $p<.05$ compared to drug alone – one-way ANOVA (analysis of variance) with Holm-Šídák multiple comparisons test.

This use of ERK phosphorylation, visualised by immunoblot, while not inappropriate thanks to some research indicating β-arrestin 2 recruitment does facilitate ERK phosphorylation[51], is limited as it works on the assumption that all ERK phosphorylation can be considered a consequence of β-arrestin 2 recruitment. Similarly, it also relies on the assumption that all β-arrestin 2 recruitment led to ERK phosphorylation.

The potential of quetiapine's reported biased agonism, and the methods used to display this behaviour, led to the proposal of this research project.

1.4 Aims

1.4.1 Initial aims

Initially, the aim of this research was to investigate the behaviour of various ligands – common antipsychotics, specifically – at both the D_2R and D_3R, analysing the similarities and variations in their responses upon both G protein activation and the recruitment of β-arrestin 2. However, upon attempting the first assay, meant to measure the dissociation of the βγ-subunits from the GPCR and associated α-subunit, it was quickly determined that the initial stage of the project would need to be spent developing and optimising a functional assay to measure G protein activation at D_3R in particular.

1.4.2 Evolved aims

With the above issues in mind, new aims were established as such:

1. Develop functional BRET assays which could produce reliable and consistent data to measure both G protein activation and β-arrestin 2 recruitment to the two dopamine receptors, D_2R and D_3R.
2. Investigate properties and behaviour of the interaction between various commonly prescribed antipsychotics and D_3R in comparison to their impact at the more thoroughly researched D_2R.
3. Assess the validity of claims of biased agonism displayed by quetiapine at the D_3R, attempting to verify and reproduce this data and to further explore and better understand the implications of such behaviour.

2.0 Materials and Methods

2.1 Cell lines

Human embryonic kidney (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% v/v Foetal Bovine Serum (FBS) (Sigma Aldrich) at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. Cells were passaged via washing with 3 mL Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma Aldrich), followed by 0.05% Trypsin-EDTA (Thermo Fisher Scientific, Loughborough, UK) to dislodge cells. 4 ml DMEM was then added, and cells aspirated, before centrifugation at 1000 rpm for 3 minutes. The supernatant was discarded, and cells resuspended in 12 mL DMEM, before counting using a Countess II Automated Cell Counter (Thermo Fisher Scientific).

HEK293 cells were chosen for this study for several reasons. First, their high proliferation rate allowed for swift turnaround and high data throughput over the course of this study, which was ideal for the proposed project timeframe. Similarly, they allowed for the use of standard class II microbiological hoods and tissue culture apparatus, where (e.g.) neuronal cells may have called for the use of specialised equipment, becoming more time-consuming. Further, [49]Schamiloglu *et al* (2023) made use of HEK cells, thus the use of these cells was deemed appropriate for maintaining comparability between studies and ensuring work could be completed within a reasonable timeframe.

2.2 Transient transfections for BRET assays

HEK293 cells were seeded into 10cm plates with 10 mL of DMEM at 2.2 x 10^6 cells per dish. DNA was transfected in polyethyleneimine (PEI) in a 1:6 PEI ratio (summarised in tables 2 - 8), in sterile NaCl, mixed well, and incubated for 10 minutes at room temperature. The DNA-PEI mix was then slowly added, in a dropwise manner, to the cells in fresh media supplemented with 5% penicillin-streptomycin.

βγ-release assay:

DNA				PEI
Receptor of interest ($D_{2/3}R$-WT)	0.25 µg	0.125 µg	0.0625 µg	17.25 µg
pcDNA-3.1	0.00 µg	0.125 µg	0.1875 µg	
$G\alpha_{oA}$	2.00 µg			
Gβ1-Venus(156-259)	0.25 µg			
Gγ2-Venus(1-155)	0.25 µg			
masGRK3ct-RLuc8	0.125 µg			

Table 3: Quantities of DNA used in transfection for βγ-release assays. During the assay optimisation process, modifications were made to the mass of DNAs used in transfections mixes. Where necessary, the 'missing' DNA was supplemented with 'empty vector' (pcDNA3.1) to maintain the DNA:PEI ratio.

TRUPATH assay:

DNA		PEI
Receptor of interest ($D_{2/3}R$-WT)	1 µg	24 µg
$G\alpha_{oA}$/B-RLuc8	1 µg	
Gβ3	1 µg	
Gγ8-GFP2	1 µg	

Table 4: Quantities of DNA used in transfection for TRUPATH assays.

mG_o assay:

DNA						PEI
Receptor of interest ($D_{2/3}R$-RLuc8)	1.00 µg	0.50 µg	0.25 µg	0.125 µg	0.10 µg	24 µg
pcDNA-3.1	0.00 µg	0.50 µg	0.75 µg	0.825 µg	0.90 µg	
NES-mG_o-mVenus	4.00 µg					

Table 5: Quantities of DNA used in transfection for mG_o assays. During the optimisation process, quantities of receptor DNA were decreased and supplemented with pcDNA3.1 to maintain DNA:PEI ratio.

DNA				PEI
Receptor of interest ($D_{2/3}$R-RLuc8)	1.0 µg	1.5 µg	2.0 µg	36 µg
pcDNA-3.1	1.0 µg	0.5 µg	0.0 µg	
NES-mG$_o$-mVenus	4.00 µg			

Table 6: Quantities of DNA used in transfection for mG$_o$ assay for assessment of assay stability above 1 µg receptor DNA.

DNA			PEI
Receptor of interest ($D_{2/3}$R-RLuc8)	1.0 µg		30 µg
pcDNA-3.1	2.0 µg	0.0 µg	
NES-mG$_o$-mVenus	2.0 µg	4.0 µg	

Table 7: Quantities of DNA used in transfection for mG$_o$ assay for assessment of assay stability with decreasing mG$_o$ DNA.

β-arrestin 2 recruitment assay:

DNA		PEI
Receptor of interest ($D_{2/3}$R-RLuc8)	1.0 µg	42 µg
β-arrestin 2-mVenus	4.0 µg	
GRK2 or pcDNA3.1	2.0 µg	

Table 8: Quantities of DNA used in transfection for β-arrestin 2 recruitment assay. Initially, modifications were made to determine the necessity of GRK2 over-expression; in these cases where 2 µg GRK2 was not added, mixes were supplemented with 2 µg pcDNA-3.1. It was later determined that GRK2 was necessary for optimal assay performance.

CAAX and FYVE assays:

DNA		PEI
Receptor of interest ($D_{2/3}$R-RLuc8)	1.0 µg	42 µg
CAAX-neonGreen or FYVE-mVenus	4.0 µg	
GRK2	2.0 µg	

Table 9: Quantities of DNA used in transfection for CAAX and FYVE internalisation assays. The in-house CAAX-mVenus construct was non-functional and therefore unavailable for use in this assay, thus CAAX-nGreen was utilised in its place.

2.3 Cell plating for BRET assays

White sterile 96-well CulturPlate plates were coated with Poly-*D*-Lysine (PDL) (50 µL/well of 50 µg/mL in Phosphate Buffered Saline (PBS)) and incubated for 30 minutes before aspiration. 2.2 x 10^4 cells were then plated per well onto the PDL-coated 96-well plates and incubated overnight at 37 °C in a 5% CO_2 humidified atmosphere.

2.4 Drug preparations

All drugs were made up to a stock concentration of 10^{-2} M in DMSO (dimethyl sulfoxide, Sigma Aldrich) – with the exception of dopamine, which was made up in either DPBS or Hank's Balanced Salt Solution (HBSS) – then diluted in either DPBS or HBSS, as needed, to make up a range of concentrations (~10^{-4} – 10^{-10} M).

Ligand	Source
Aripiprazole	Kind gift of B. Capuano, Monash Institute of Pharmacy (Parkville, Australia)
Blonanserin	Sigma Aldrich (Dorset, UK)
Cariprazine	Kind gift of B. Capuano, Monash Institute of Pharmacy (Parkville, Australia)
Clozapine	Kind gift of B. Capuano, Monash Institute of Pharmacy (Parkville, Australia)
Dopamine	Sigma Aldrich (Dorset, UK)
Haloperidol	Sigma Aldrich (Dorset, UK)
Naloxone	Hello Bio (Bristol, UK)
Olanzapine	ToCris Bioscience (Bristol, UK)
Quetiapine hemifumarate	ToCris Bioscience (Bristol, UK)
Quinpirole	ToCris Bioscience (Bristol, UK)

Table 10: Ligands used in this study.

2.5 BRET assays

Bioluminescence resonance energy transfer, or BRET, can be used to approximate proximity between two or more labelled molecules. While individual methods and BRET assay subtypes will vary, all follow the same general principles.

BRET involves the transferral of energy from a donor (usually a luciferase molecule) to an acceptor fluorescent molecule. When these two molecules are separate, only luminescence from the donor will be measured; when in close proximity, energy transfer can occur, allowing for the measurement of luminescence from both the donor and acceptor molecules, on two distinct wavelengths. The ratio of luminescence is then calculated, and the resulting

'BRET ratio' can be used as a direct measurement of proximity between the labelled molecules[52].

2.5.1 Standard wash protocol

All assays used this standard wash protocol prior to coelenterazine and ligand loading, unless stated otherwise:

24 hours after replating, media was aspirated from the wells, and cells were washed once with ~80 µL per well of DPBS, then incubated with 80 µL per well for 30 minutes at 37 °C.

2.5.2 BRET1 – βγ-release

10 µL per well of h-coelenterazine (Nanolight, 50 µM) was added, before 10 µL per well of appropriate ligands were added, to a range of final concentrations (10^{-5} to 10^{-11} M), alongside DPBS as a control. The cells were then moved to and incubated in a PHERAstar FSX (BMG Labtech) and read using dual emission detection at 475 nm (RLuc8 donor) and 535 nm (BRET signal) for 10 minutes at a rate of one cycle per minute with a BRET1 filter. They were then returned to the 37 °C incubator for a further 20 minutes before being read for the last time at the 30-minute timepoint for 1 cycle.

2.5.3 BRET2 – TRUPATH

Wash protocol was followed utilising either DPBS or HBSS as needed. 10 µL per well of coelenterazine 400a (50 µM) was added, before 10 µL per well of the relevant ligand(s) was added. The plates were then read with the same protocol as 2.5.2, though instead using a BRET2 filter and dual emission detection at 395 nm (RLuc8 donor) and 510 nm (GFP2).

2.5.4 BRET1 – mG_o

10 µL of h-coelenterazine was added before plates were read for 3 minutes at a rate of 1 cycle per minute with a BRET1 filter to establish a baseline reading. 10 µL of ligands were then loaded, and plates were returned to the 37 °C incubator for 30 minutes, before being read for a final time for a further 3 cycles.

2.5.5 BRET1 – β-arrestin 2 recruitment

An identical assay protocol was followed as in 2.5.4.

2.5.6 BRET1 – CAAX and FYVE internalisation assays

Following h-coelenterazine loading (10 µL/well), the plates were read for five 1-minute cycles to establish a baseline reading, then were removed for ligand loading, before being returned to the reader for a further twenty 1-minute cycles.

2.6 Analysis

Data was exported through MARS (BMG Labtech) and statistical analysis carried out using PRISM 10 (GraphPad Software Inc., San Diego, CA, USA). Where possible, the results were normalised to baseline initially, then to vehicle, and curves were fitted using the following established equation for concentration-response signalling:

$$Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{(\log EC_{50} - \log[A])})}$$

Where [A] is the molar concentration of agonist, top and bottom, respectively, represent the maximum and minimum asymptotes, and EC_{50} equates to the molar concentration of agonist that produces a 50% maximal response (i.e. halfway between the maximum and minimum asymptotes).

For all data presented, error bars were calculated as SEM (standard error of the mean).

Where possible, experiments were performed in triplicate for each ligand used. Following this, results were collated from at least three individual repeats, which had been averaged. However, due to time constraints and high data variability, it was not always possible to supplement individual repeats where data had to be discarded. The specific number of repeats for each set of data is presented as 'n=*x*'.

3.0 Results and discussion

3.1 Assay optimisation

3.1.1 BRET1 βγ-release assay

The first aim of this research project was to establish robust functional assays for both D_2R and D_3R in order to accurately measure their activation.

There is extensive literature, dating back decades, to suggest that both D_2R and D_3R can couple to $G\alpha_o$ inhibitory G proteins, and while D_2R is able to couple promiscuously to all $G\alpha_{i/o/z}$ variations, D_3R is far more discerning in its coupling, appearing selective for $G\alpha_o$ in particular[53]. As such, the first assay attempted was the βγ-release assay using a $G\alpha_{oA}$ subunit; an assay which has the advantage of an unmodified G protein alpha subunit – providing a stronger link to endogenous conditions[54].

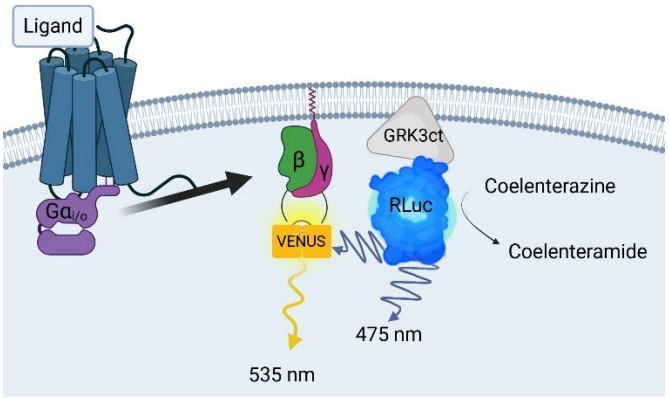


Figure 6: Representation of βγ-release assay. Upon agonist binding to the dopamine receptor, the receptor can bind and activate the heterotrimeric G protein, leading to the release of the mVenus-labelled βγ-subunit, and its subsequent increase in proximity to the RLuc8-labelled masGRK3ct (C-terminal fragment GPCR kinase 3), resulting in an increase in BRET.

Initial data:

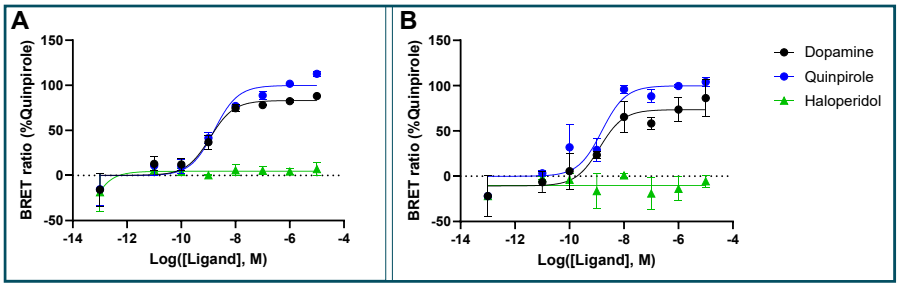


Figure 7: The ability of the agonists dopamine and quinpirole and the inverse agonist haloperidol to modulate G protein activation at the D_2R (A) and D_3R (B). HEK293 cells expressing the hD_2R or hD_3R along with $G\alpha_{oA}$ and the components of the BRET βγ-release assay as detailed in the methods exposed to the three compounds, and βγ-release was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole. Data presented is the average of two independent experiments performed in triplicate ±SEM (n=2, Tables 11,12)

As demonstrated by data in figure 7, this assay did indeed provide sensible data at times.

Analysis of potencies:

Repeat	Dopamine	Quinpirole	Haloperidol
1	9.296	8.704	ND
2	8.670	8.851	ND
Avg.	**8.983**	**8.778**	**ND**

Table 11: pEC_{50} values of D_2R for both repeats presented in Fig. 7 (A). In the case of haloperidol, no response could be identified so no curve could be fitted; ND = parameter could not be determined.

Repeat	Dopamine	Quinpirole	Haloperidol
1	8.956	8.738	8.687
2	8.833	8.876	7.471
Avg.	**8.895**	**8.807**	**8.079**

Table 12: pEC_{50} values of D_3R for both repeats presented in Fig. 7 (B).

Dopamine and quinpirole are agonists at both the D_2R and D_3R, and in this assay, they display similar potency to one another at both receptors.

The absence of a decrease in signal in the presence of increasing concentrations of haloperidol, a ligand known to act as an antagonist/inverse

agonist for both D_2R and D_3R[55,56], would indicate that there is very little constitutive activity in these particular experimental repeats.

However, these data were highly variable, particularly those produced using D_3R, and in many cases the agonists displayed only a small response above basal, accompanied by a high degree of inverse agonist activity for haloperidol. A hypothesis was reached that this inconsistency could be linked to differing degrees of constitutive activity within the receptor system, perhaps due to changes in cell surface receptor expression levels between the repeats; this is something dopamine receptors are reportedly known for[17] and can make producing reliable and repeatable data difficult.

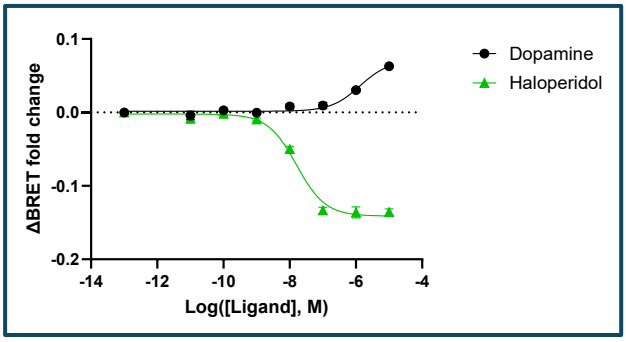


Figure 8: Representative data demonstrating lack of signal window displayed by hD_3R stimulation by dopamine and haloperidol. HEK293 cells expressing the hD_3R along with $G\alpha_{oA}$ and the components of the BRET βγ-release assay as detailed in the methods exposed to the two compounds, and βγ-release was measured as an increase in BRET ratio. Responses are normalised to baseline and presented as net change from baseline. Data presented is the average of two independent experiments performed in triplicate ±SEM (n=2).

In order to verify the magnitude and impact of constitutive activity in this assay, an investigation into the effect of increasing concentrations of the inverse agonist haloperidol on the action of dopamine in such conditions was performed.

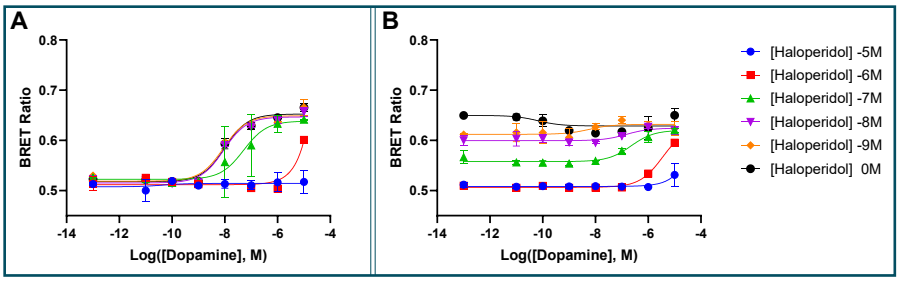


Figure 9: The effect of increasing concentrations of the inverse agonist haloperidol on the action of dopamine at the D_2R (A) and D_3R (B). HEK293 cells expressing the hD_2R or hD_3R along with $G\alpha_{oA}$ and the components of the BRET βγ-release assay, as detailed in the methods, exposed to haloperidol and dopamine, and βγ-release was measured as an increase in BRET ratio. Data presented is representative of one independent experiment performed in triplicate ±SEM (n=1, Table 13).

log([Haloperidol], M)	pEC_{50} of Dopamine Response at D_3R
-5	2.219
-6	5.454
-7	6.647
-8	6.833
-9	8.228
0	ND

Table 13: Decreasing potency of dopamine at D_3R with increasing concentrations of inverse agonist haloperidol. Data is derived from figure 9 **(B)**. (ND = parameter could not be determined).

The above data (Figure 9, **(B)**) produced by hD_3R fits the earlier hypothesis suggesting that, in cases where no signal window was observed, this is due to the high constitutive activity, as evidenced by the fact that an increase to the concentration of inverse agonist led to a lower initial baseline reading, and lower haloperidol concentrations led to the same maximal reading for D_3R; as baseline drops, the window for a dopamine response increases.

This is especially obvious when viewed in comparison to responses from D_2R, where all responses begin from the same baseline ratio and most end with the same (or similar) E_{max}, their only differences being progressive right-ward shifts as the concentration of haloperidol increases. As haloperidol and dopamine compete for the same orthosteric binding site on the receptor, haloperidol acts as a competitive antagonist and thus starts to shift the

concentration response curve to the right for both receptors with the decrease in dopamine's potency (see table 13 above).

Following the collection of the above data, it was decided that the next logical step would be to investigate whether or not reducing the mass of receptor cDNA included in the DNA:PEI mixes at transfection would result in lower constitutive activity, and thus more consistent results – i.e. 'fewer receptors, less basal activity'.

The hope was that, if there were fewer functional receptors present overall, it would be more difficult for those which constitutively activated independent of agonist stimulation to overwhelm the system and therefore obscure the agonist response. The more receptors there are, the greater the number of constitutively active receptors there will be and so will have a greater impact on the BRET readout of G protein activation from an already incredibly sensitive assay; a fact which is obvious given the data presented in figure 7, as both dopamine and quinpirole display high potency (within a 1.0-1.7 nM range for both agonists at both receptors), but have affinities much lower (for dopamine, reportedly K_is of 540 nM and 60 nM for D_2R and D_3R, respectively)[57]. This difference reflects the degree of amplification of the signalling endpoint measured.

The effect of varying receptor cDNA mass:

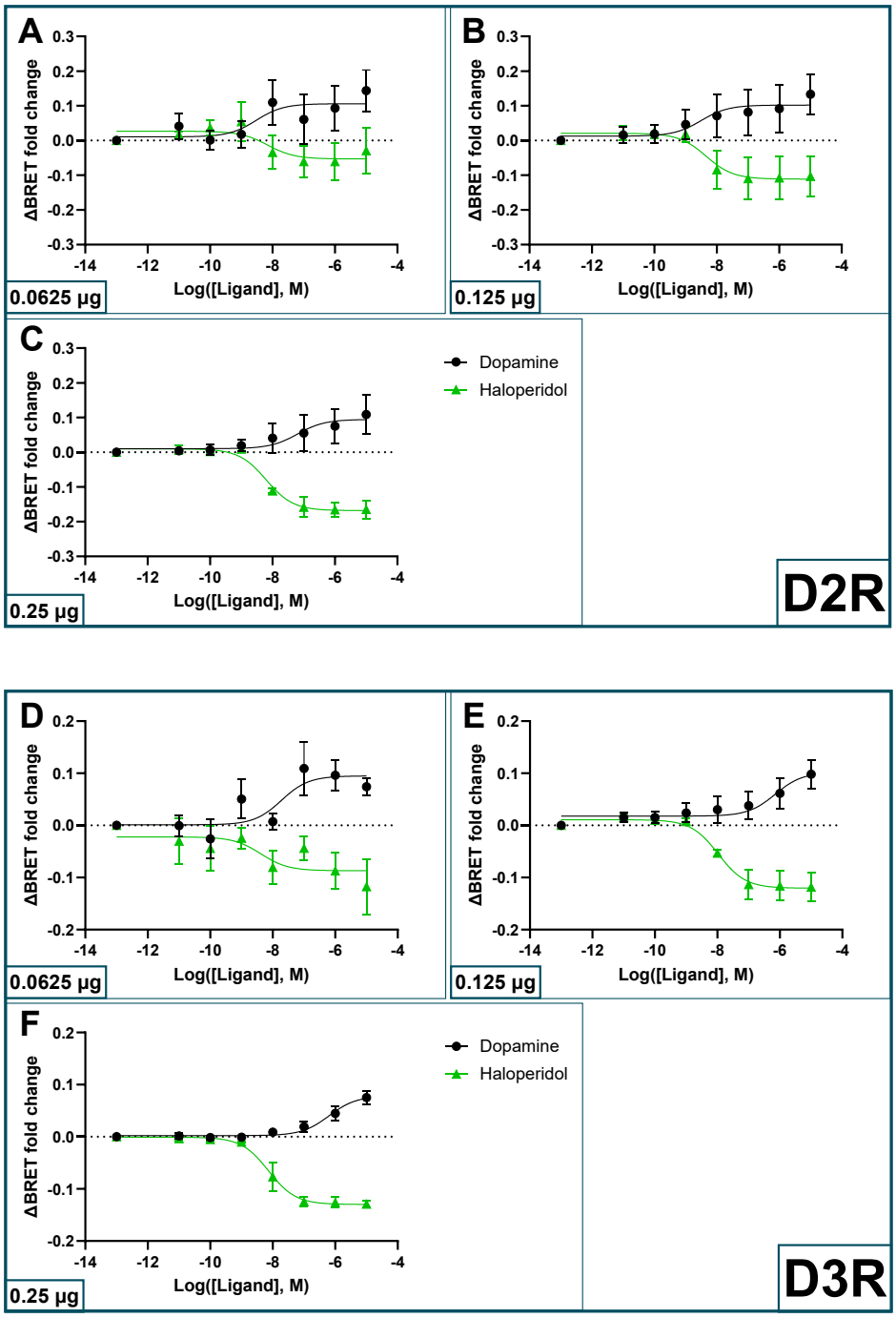


Figure 10: The effect of altering mass of D_2R (A-C) or D_3R (D-F) cDNA on the activity of the agonist dopamine, and the inverse agonist haloperidol. HEK293 cells expressing the hD_2R (**(A)**: 0.0625 µg; **(B)**: 0.125 µg; **(C)**: 0.25 µg) or hD_3R (**(D)**: 0.0625 µg; **(E)**: 0.125 µg; **(F)**: 0.25 µg) along with $G\alpha_{oA}$ and the components of the BRET βγ-release assay as detailed in the

methods, exposed to the two compounds, and βγ-release was measured as an increase in BRET ratio. Responses are normalised to the control vehicle-only response and presented as a change from baseline. Data presented is the average of four independent experiments performed in triplicate (n=4), with the exception of **(C)** and **(F)**, which are the average of two independent experiments performed in triplicate ±SEM (n=2).

In the case of D_2R, reduction of receptor cDNA mass seemed to result in higher variability. This may be due to a lower limit of receptor expression being reached such that the assay window decreased to a level from which it was difficult to obtain consistent concentration response curves.

However, even at such low levels of receptor expression, there appeared to be high levels of constitutive activity in this assay. This would suggest that even with relatively low levels of receptor, the assay is sensitive enough to pick up and amplify said constitutive activity.

The reduction in haloperidol response for D_3R (comparative to dopamine response) may indicate a reduction in constitutive activity. However, the results do appear to become even more variable with lower receptor cDNA; this is particularly obvious when observing the error bars in the dopamine responses.

While ±SEM error bars are not necessarily a direct indicator of assay validity, the observation of error bars in data resulting from different methodologies can indicate which of them tended to produce results with greater variation between experimental repeats. This knowledge could then be used to inform decisions on whether or not an alteration to the experimental method resulted in more reliable or 'usable' data than that of the original method.

Ideally, further experimentation would be performed to validate these conclusions, though it was ultimately decided that, given the limited timescale of this project, other methodologies should be explored for their potential to produce more consistent data.

3.1.2 BRET2 TRUPATH assay

The TRUPATH assay follows a similar principle to βγ-release but instead uses an RLuc8-labelled alpha subunit and a GFP2-labelled gamma subunit. As the βγ subunits are released, the BRET ratio decreases with their decreasing proximity. Though this assay does not possess the same unmodified Gα subunit as the βγ-release assay did, it does have the benefit of directly measuring the dissociation of the G protein, as opposed to the accumulation of free βγ subunits. Therefore, it may be less prone to amplification and, thereby, display lower levels of basal or constitutive activity.

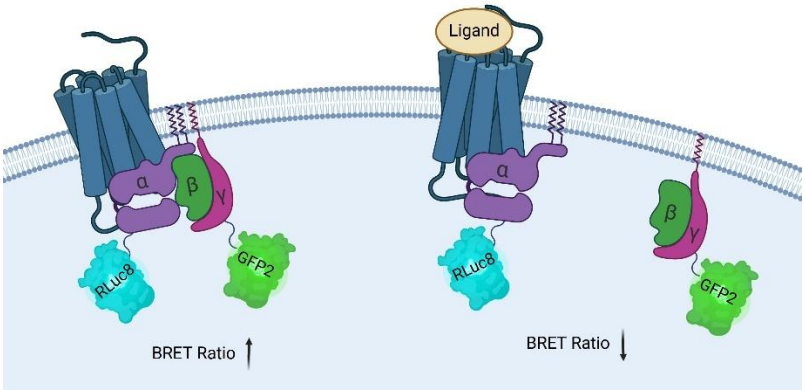


Figure 11: Representation of TRUPATH assay. Upon ligand binding and GPCR activation, the associated G protein is activated, and the GFP2-labelled βγ subunit is released, increasing its distance from the RLuc8-labelled α subunit, which can then be measured as a detected decrease in BRET ratio[58].

$G\alpha_{oA}$ vs. $G\alpha_{oB}$:

The TRUPATH methodology, as presented by [58]Olsen *et al* (2020) suggested this assay could be performed using either an RLuc8-tagged $G\alpha_{oA}$ or $G\alpha_{oB}$ subunit, so it was important to prioritise checking and deciding which would produce more reliable and less variable data.

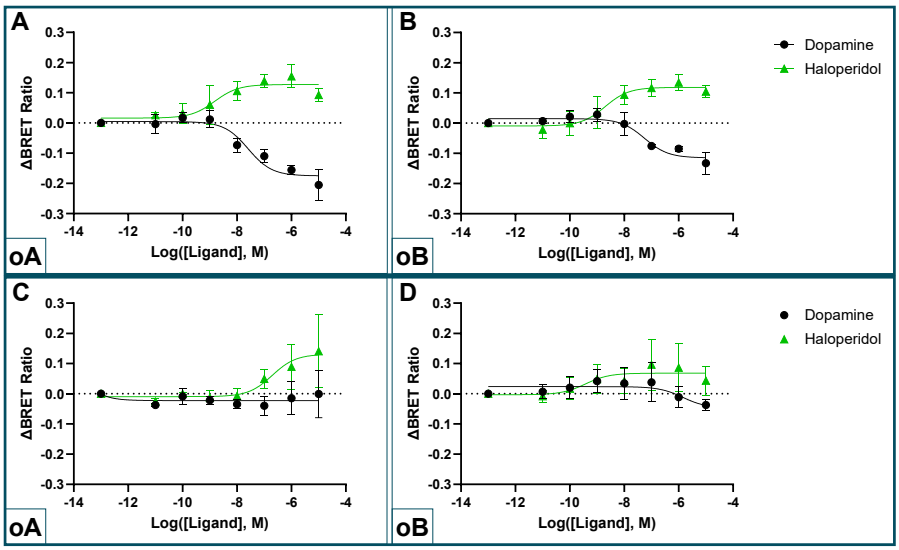


Figure 12: Comparison of the impact of using the $G\alpha_{oA}$ (A,C) subunit vs. using $G\alpha_{oB}$ (B,D) on the response of the agonist dopamine and the inverse agonist haloperidol. HEK293 cells expressing the hD_2R **(A,B)** or hD_3R **(C,D)** along with $G\alpha_{oA}$ or $G\alpha_{oB}$ and the components of the BRET TRUPATH assay, as detailed in the methods, exposed to the two compounds and activation of the receptor was measured as a decrease in BRET ratio. Responses are presented as a change from baseline. Data presented is the average of three independent experiments performed in triplicate ±SEM (n=3)

At D_2R, the use of $G\alpha_{oB}$ resulted in more variable data than $G\alpha_{oA}$, and although there was little change in the haloperidol response, there was a marginal decrease in assay window from dopamine from a span of 0.1797 (± 0.047) (BRET ratio units) from $G\alpha_{oA}$ to 0.1297 (± 0.035) from $G\alpha_{oB}$. There was little effect on dopamine's observed potency as, though it decreased from a pEC_{50} of 7.593 ± 0.877 (25.5 nM) using the $G\alpha_{oA}$ construct to 7.256 ± 0.744 (55.5 nM) with the $G\alpha_{oB}$ subunit, this change remained within the wide error margins.

This, combined with the immense difficulty faced from the borderline indecipherable data at D_3R, likely due to the lack of assay window, led to the use of $G\alpha_{oA}$ moving forward.

The influence of assay buffer on constitutive activity:

The above experiments were performed in the assay buffer DPBS. The degree of constitutive activity observed in current data is not consistent with prior data from the laboratory, in which assays were performed in HBSS, (ref: personal communication from Rob Lane). Previous evidence suggests that buffer composition, particularly sodium ions, can influence observed levels of constitutive activity in receptor systems, including D_2R and D_3R[59]. This is thought to occur due to sodium ions binding to a conserved site on the extracellular domain of class A GPCRs (of which dopamine receptors are a member), where it may then act as a negative allosteric modulator, pushing the receptor toward a less active conformation.

This knowledge, combined with the original TRUPATH methodology making use of HBSS rather than DPBS, made clear the need to investigate whether or not the problematic increase in constitutive activity could be connected to the change in standard assay buffer.

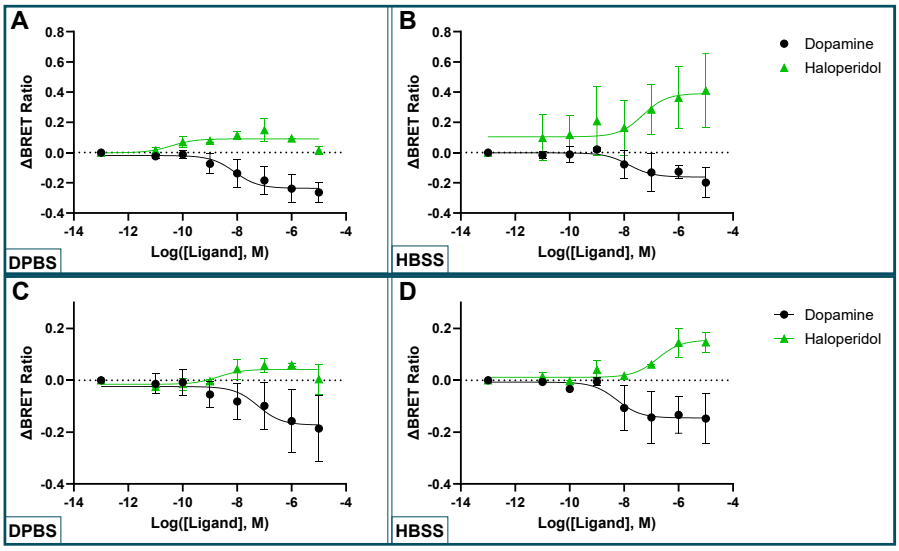


Figure 13: The impact of using DPBS (A,C) vs. HBSS (B,D) on the response elicited by the agonist dopamine and the inverse agonist haloperidol. HEK293 cells expressing the hD_2R **(A,B)** or hD_3R **(C,D)** along with $G\alpha_{oA}$ and the components of the BRET TRUPATH assay, as detailed in the methods, exposed to the two compounds and receptor activation was measured as a decrease in BRET ratio. Responses are presented as a change from baseline. Data presented is the average of two independent experiments performed in triplicate ±SEM (n=2).

It is abundantly clear from the above data that the use of DPBS resulted in greater consistency between individual repeats than HBSS did. Taking into consideration the greater haloperidol responses for both receptors, it is possible that the HBSS used does indeed have the effect of increasing measured constitutive activity in dopamine receptors, giving it a greater tendency to shift into its 'active formation'.

Inorganic Salts	DPBS		HBSS	
	Conc. (mg/L)	mM	Conc. (mg/L)	mM
Calcium Chloride ($CaCl_2$) (anhyd.)	~	~	140	1.26
Magnesium Chloride ($MgCl_2$-$6H_2O$)	~	~	100	0.49
Magnesium Sulphate ($MgSO_4$-$7H_2O$)	~	~	100	0.41
Potassium Chloride (KCl)	200	2.67	400	5.33
Potassium Phosphate monobasic (KH_2PO_4)	200	1.47	60	0.44
Sodium Bicarbonate ($NaHCO_3$)	~	~	350	4.17
Sodium Chloride (NaCl)	8000	137.93	8000	137.93
Sodium Phosphate dibasic (Na_2HPO_4) (anhyd.)	2160	8.06	48	0.34

Table 14: Components of Dulbecco's phosphate-buffered saline (DPBS) compared to Hank's balanced salt solution (HBSS)[60,61]**.** All concentrations are according to the '1X' solutions.

When investigating the specific constituents of the two buffers used, it becomes apparent that the DPBS used has a higher concentration of sodium ions when compared to HBSS (~146 mM for DPBS vs. ~142.5 mM for HBSS), which in turn contained magnesium where DPBS did not. If, as discussed, the interaction of sodium ions with the receptor decreases likelihood of constitutive activity from that receptor, then this could help provide an explanation for the increased basal readings when using a buffer with fewer sodium ions.

Though it should be noted that this difference in the presence of sodium ions is only relatively minor, so the possibility of some other compounding factor playing a role, such as the presence of magnesium ions resulting in an

interaction with the receptor, causing a shift towards their active conformations, cannot be dismissed.

What is interesting is that there doesn't seem to be much of a change in the magnitude of the dopamine responses between DPBS and HBSS for either receptor; the difference is almost entirely from the haloperidol. In the case of constitutive activity, it might be expected that an increase in inverse agonist or antagonist response would go hand-in-hand with a decrease in agonist response; therefore, these results are not necessarily consistent with a 'typical' case of high basal activity.

3.1.3 BRET1 mG_o assay

In both previous assays looking at the level of G protein activation, there is a constant and significant level of constitutive activity. It is possible that, for both the βγ-release and TRUPATH assays, such constitutive activity can be detected perhaps due to amplification of the signal through multiple G protein activation cycles. Thus, the next assay attempted was the 'mini-G' assay, involving the use of a modified G protein construct (mG_o), which lacks the GTPase domain. This means they can be recruited to the receptor but will not undergo multiple activation cycles.

Therefore, the mini-G assay can be used to measure receptor activation and G protein activation, without measuring any *subsequent* G protein activation.

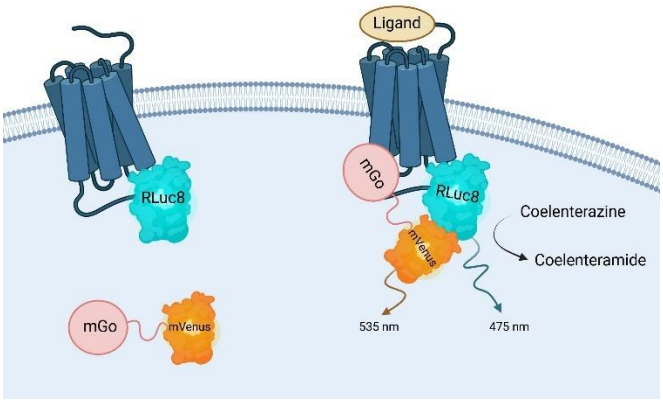


Figure 14: Representation of the mG_o assay. Upon ligand binding to the RLuc8-tagged receptor, the mVenus-labelled modified Gα subunit is recruited to the receptor, resulting in an increase in BRET ratio.

Given the influence of receptor expression observed in the assays above, it was deemed necessary to try to optimise the signal window from the very first experiment.

The effect of varying receptor cDNA mass:

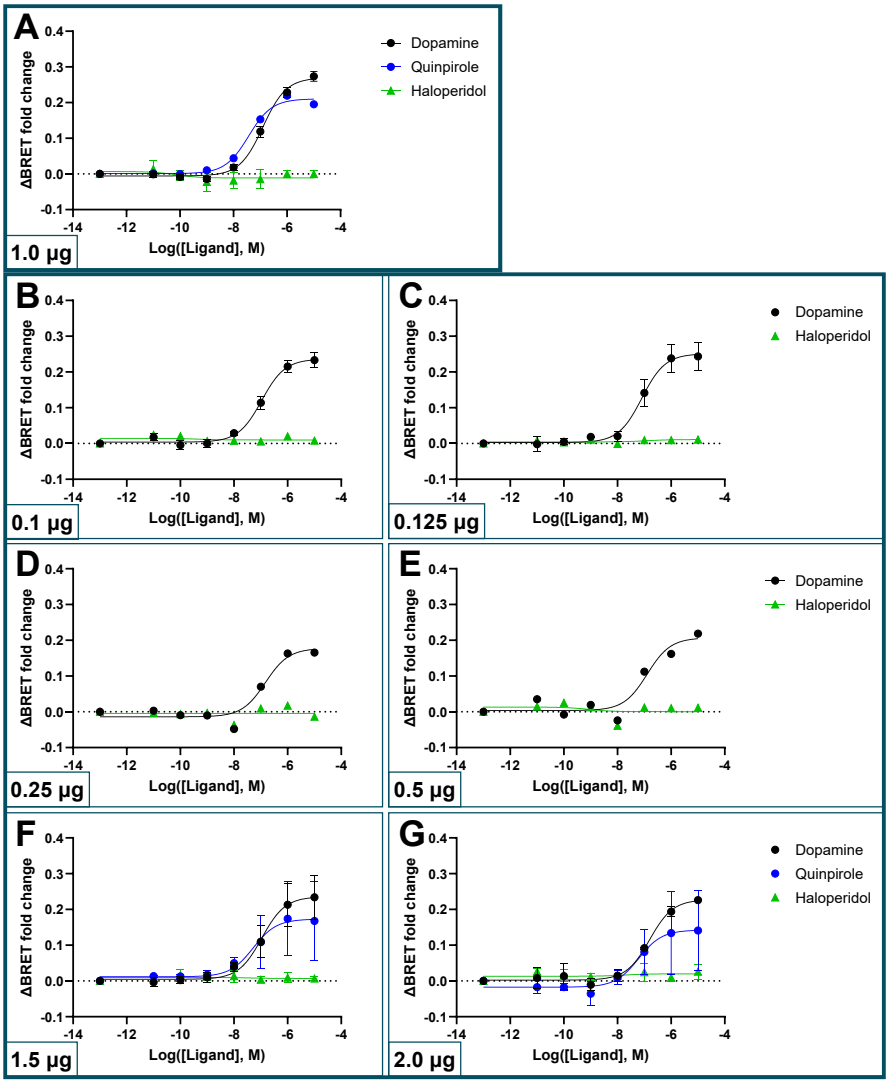


Figure 15: The effect of altering mass of hD$_2$R cDNA used in transfections. HEK293 cells transiently transfected with varying masses of DNA encoding the hD$_2$R (**(A)** 1.0 µg, **(B)** 0.1 µg, **(C)** 0.125 µg, **(D)** 0.25 µg, **(E)** 0.5 µg, **(F)** 1.5 µg, **(G)** 2.0 µg) along with the mG$_o$ and the components of the BRET mG$_o$-recruitment assay as detailed in the methods exposed to the two agonists dopamine and quinpirole, and the inverse agonist haloperidol. mG$_o$ recruitment was measured as an increase in BRET ratio. Responses are normalised to the control vehicle-only response, then presented as a change from baseline. Data presented is the average of two independent experiments performed in triplicate (n=2), with the exception of **(A)**, which is the average of nine independent experiments performed in triplicate ±SEM (n=9, Table 15).

Each experiment performed for graphs **(B)-(G)** in figure 15 was run simultaneously with transfected cells containing 1.0 µg of cDNA on the same 96-well assay plate for direct on-the-day comparison, in order to avoid variations resulting from aged cells or experimental errors on a particular day. These data were then collated to produce graph **(A)**, resulting in a reported total n=9.

Receptor cDNA mass	Dopamine		Quinpirole	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
1.0 µg ('standard')	6.898 ± 0.158	0.270 ± 0.180	7.438 ± 0.707	0.211 ± 0.545
0.1 µg	6.968 ± 0.176	0.236 ± 0.169	~	~
0.125 µg	7.091 ± 0.359	0.251 ± 0.364	~	~
0.25 µg	6.828 ± 0.608	0.177 ± 0.538	~	~
0.5 µg	6.892 ± 0.822	0.207 ± 0.701	~	~
1.5 µg	6.960 ± 0.613	0.236 ± 0.538	7.283 ± 1.587	0.173 ± 0.084
2.0 µg	6.804 ± 0.335	0.228 ± 0.032	7.214 ± 1.578	0.142 ± 0.092

Table 15: Potencies and E_{max} of ligands at varying hD_2R cDNA masses used in transient transfections. Data is derived from figure 15.

There does not seem to be great variation in data procured from assays with different masses of hD_2R cDNA transfected into the cells, though error values, as displayed in table 15, do appear to increase, occasionally drastically. Quinpirole appears to display higher potency at D_2R than dopamine, though with a lower E_{max}, indicative of partial agonistic behaviour.

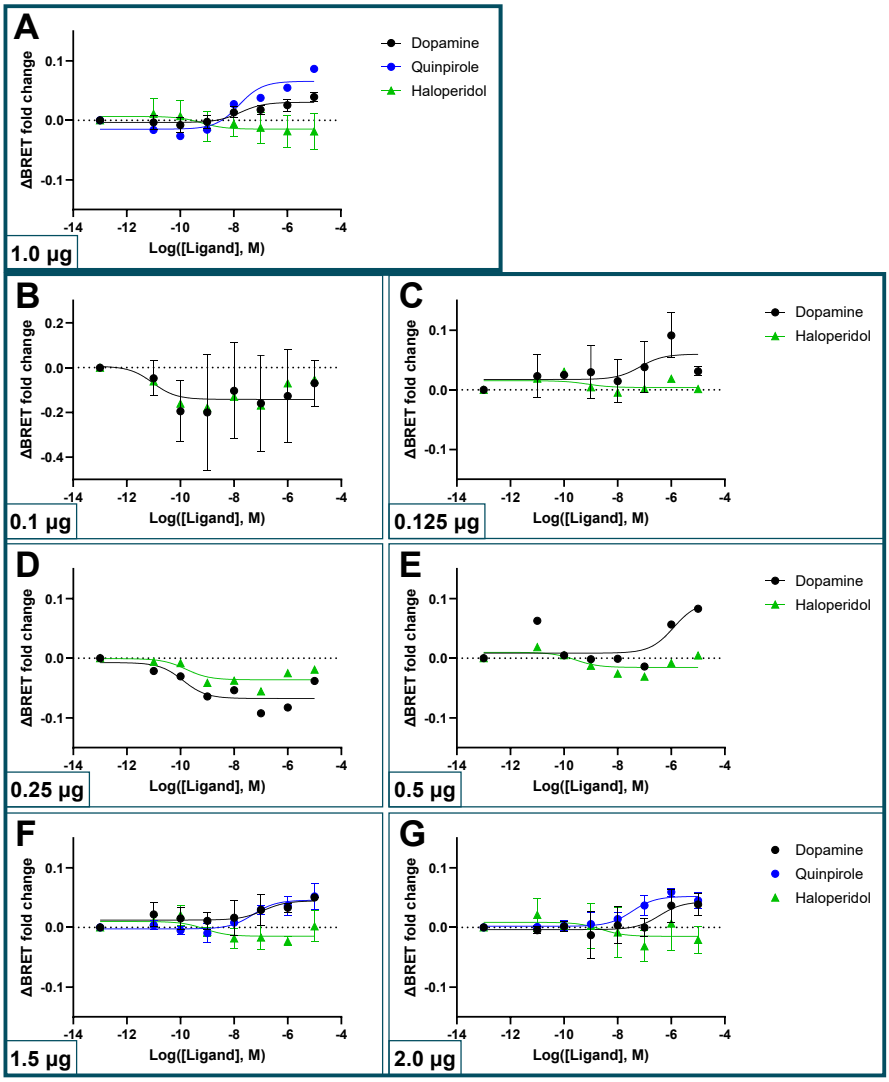


Figure 16: The effect of altering mass of hD$_3$R cDNA used in transfections. HEK293 cells transiently transfected with varying masses of DNA encoding the hD$_3$R (**(A)** 1.0 µg, **(B)** 0.1 µg, **(C)** 0.125 µg, **(D)** 0.25 µg, **(E)** 0.5 µg, **(F)** 1.5 µg, **(G)** 2.0 µg) along with the mG$_o$ and the components of the BRET mG$_o$-recruitment assay as detailed in the methods exposed to the two agonists dopamine and quinpirole, and the inverse agonist haloperidol. mG$_o$ recruitment was measured as an increase in BRET ratio. Responses are normalised to the control vehicle-only response, then presented as a change from baseline. Data presented is the average of two independent experiments performed in triplicate (n=2), with the exception of **(A)**, which is the average of nine independent experiments performed in triplicate ±SEM (n=9, Table 16).

There appeared to be a great degree of variability in response window upon changing the mass of hD$_3$R cDNA transfected into cells. In the case of the 0.1

µg transfection in particular, responses were so inconsistent that the dopamine response appeared to decrease with concentration, behaving as an antagonist, and the haloperidol response was deemed 'too unstable' for analysis by pharmacological software.

Receptor cDNA mass	Dopamine		Quinpirole	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
1.0 µg ('standard')	7.749 ± 1.737	0.030 ± 0.020	7.809 ± 1.442	0.066 ± 0.035
0.1 µg	ND	ND	~	~
0.125 µg	ND	ND	~	~
0.25 µg	ND	ND	~	~
0.5 µg	5.911 ± 3.764	0.095 ± 0.076	~	~
1.5 µg	ND	0.045 ± 0.024	7.293 ± 1.091	0.046 ± 0.018
2.0 µg	6.442 ± 2.705	0.043 ± 0.033	7.462 ± 0.871	0.052 ± 0.015

Table 16: Potencies and E_{max} of ligands at varying hD_3R cDNA masses used in transient transfection. Data is derived from figure 16. (ND = parameter could not be determined).

Data presented in figure 16 and table 16 are frequently unstable, with high error values throughout. Therefore, it can be concluded that there is little benefit to be gained overall from altering the mass of hD_3R cDNA used in the transient transfection process.

Dopamine vs. Quinpirole:

At D_2R, dopamine behaves as a full agonist, with quinpirole behaving as, seemingly, an efficacious partial agonist. Conversely, dopamine at D_3R appears to behave as a partial agonist in comparison to quinpirole's full agonism; dopamine producing an E_{max} of 0.030 (± 0.020), and quinpirole producing 0.066 (± 0.035). While there have been reports of quinpirole's behaving as a full agonist for both D_2R and D_3R, it was not possible to find any precedence of dopamine being identified as a partial agonist for D_3R at the time of writing this thesis[62].

With this in mind, it was decided that experiments moving forward would make use of quinpirole as the control agonist in place of dopamine; the benefits of achieving the largest window and maximal possible efficacy and responses at D_3R were determined to outweigh any potential downsides of only using a partial agonist for D_2R activation. Further, as displayed in tables 15 and 16,

quinpirole produced a higher potency than dopamine at both receptors, once again improving chances of producing the optimal assay window.

The effect of varying mG_o-mVenus cDNA mass:

As varying the mass of receptor cDNA in transfection mixes proved unhelpful in increasing consistency between experimental repeats, it was important to determine the impact of varying the mass of modified G protein DNA, i.e. would changing the ratio of G protein to receptor help any in reducing constitutive activity whilst maintaining ideal levels of receptor expression?

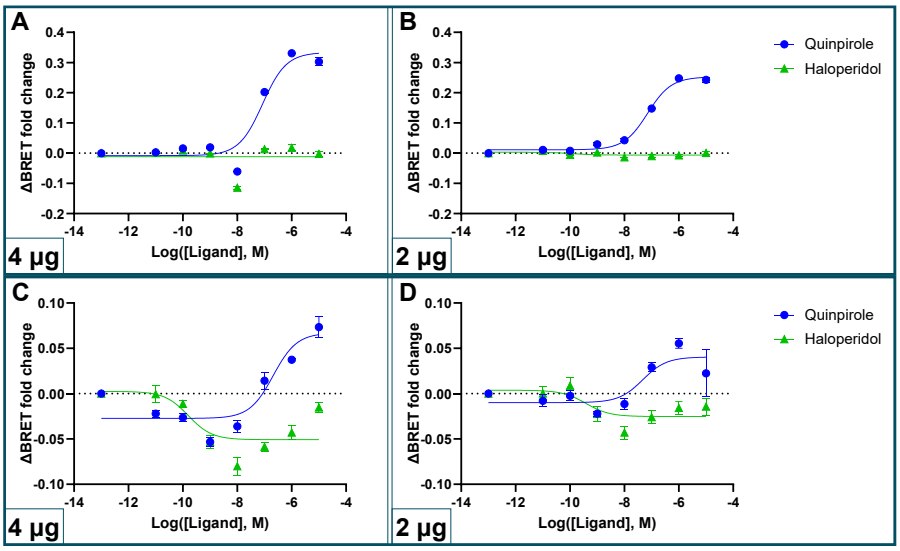


Figure 17: The effect of decreasing mass of mG_o cDNA used in transfection on the response of the agonist quinpirole and the inverse agonist haloperidol. HEK293 cells expressing the hD_2R **(A,B)** or hD_3R **(C,D)** along with either 4 µg of mG_o cDNA or 2 µg and the components of the BRET mG_o assay (**(A)**: hD_2R + 4 µg mG_o; **(B)**: hD_2R + 2 µg mG_o; **(C)**: hD_3R + 4 µg mG_o; **(D)**: hD_3R + 2 µg mG_o), as detailed in the methods, exposed to the two compounds, and mG_o recruitment was measured as an increase in BRET ratio. Responses were normalised to control vehicle-only responses, then presented as a change from baseline. Data presented is the average of two independent experiments performed in triplicate ±SEM (n=2, Table 17).

Receptor	Quinpirole (pEC_{50})	
	4 µg mG_o cDNA ('standard')	2 µg mG_o cDNA
D_2R	7.069	7.146
D_3R	6.680	7.326

Table 17: Quinpirole potency with varying mG_o cDNA mass. Data is derived from figure 17 (n=2).

For both receptors, a reduction in mG_o cDNA in the transient transfections resulted in a decreased assay window but generally had little effect on the measured potency of quinpirole, with the exception of a minor leftward shift, as can be observed in table 17. While it does appear that there was a slight decrease in haloperidol response at D_3R with the reduction to 2 µg of mG_o cDNA, it was determined that the detriment from the overall decrease in the measurement window would outweigh any benefits of potentially reducing the general constitutive activity of the system.

With the above data collected, it was concluded that this assay was now functional and robust enough that G protein recruitment to both the D_2R and D_3R could be measured with reasonable accuracy and consistency.

As outlined in the introduction to this thesis, the overall aim of this project was to investigate the proposed ability of some antipsychotics to behave antagonistically at the G protein, blocking its signalling, whilst activating β-arrestin 2 signalling pathways. Thus, with a functional G protein assay, it was necessary to develop a functional assay to determine the recruitment of β-arrestin 2 to the receptors.

3.1.4 BRET1 β-arrestin 2 recruitment assay

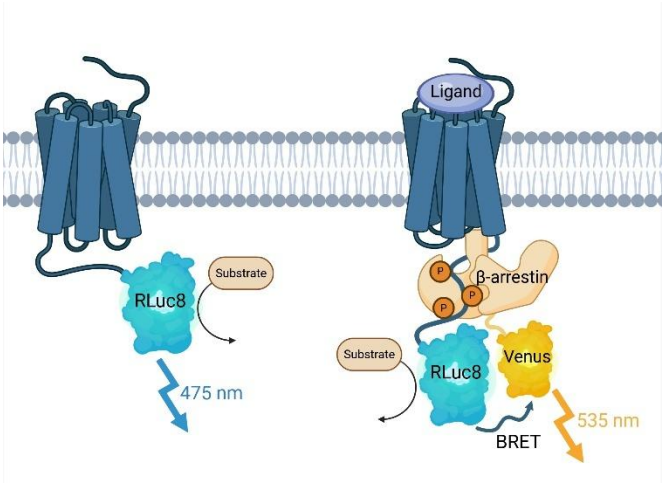


Figure 18: Representation of β-arrestin 2 recruitment assay. Upon ligand activation, if β-arrestin 2 is recruited to the receptor, its mVenus tag increases in proximity to the RLuc8 tag on the receptor, allowing BRET to take place.

Necessity of GRK2 over-expression:

It has been well established that binding of arrestin to GPCRs following activation is driven by the phosphorylation of serine/threonine residues on the receptor's C-terminal tail, or C-tail, which increases the affinity of arrestin for the receptor; though it is worth noting that D_2R has been demonstrated to lack such a C-tail and instead appears to interact with β-arrestin 2 via a phosphorylated intracellular loop 3 (ICL3)[63], and while the recruitment of arrestin to the D_3R has been observed, it is less well characterised[64].

Further, there is some evidence suggesting that the interaction of β-arrestins with the core of the receptor, otherwise referred to as the transmembrane bundle, can occur independently of phosphorylation in the case of many GPCRs[65,66].

Regardless of the point of binding and the strict 'necessity' of phosphorylation, however, phosphorylated residues on the GPCR have been shown to potentiate and greatly increase the likelihood of β-arrestin 2 recruitment.

As receptor phosphorylation increases the affinity of β-arrestin 2 for the receptor, and G protein receptor kinase 2 (GRK2) is considered largely

responsible for this phosphorylation of D_2R and D_3R[67], it was necessary to determine whether native GRK2 levels within the HEK293-T cells would be sufficient to produce reliable data, or if it would be necessary to introduce further GRK2 DNA into the cells to induce GRK2 over-expression.

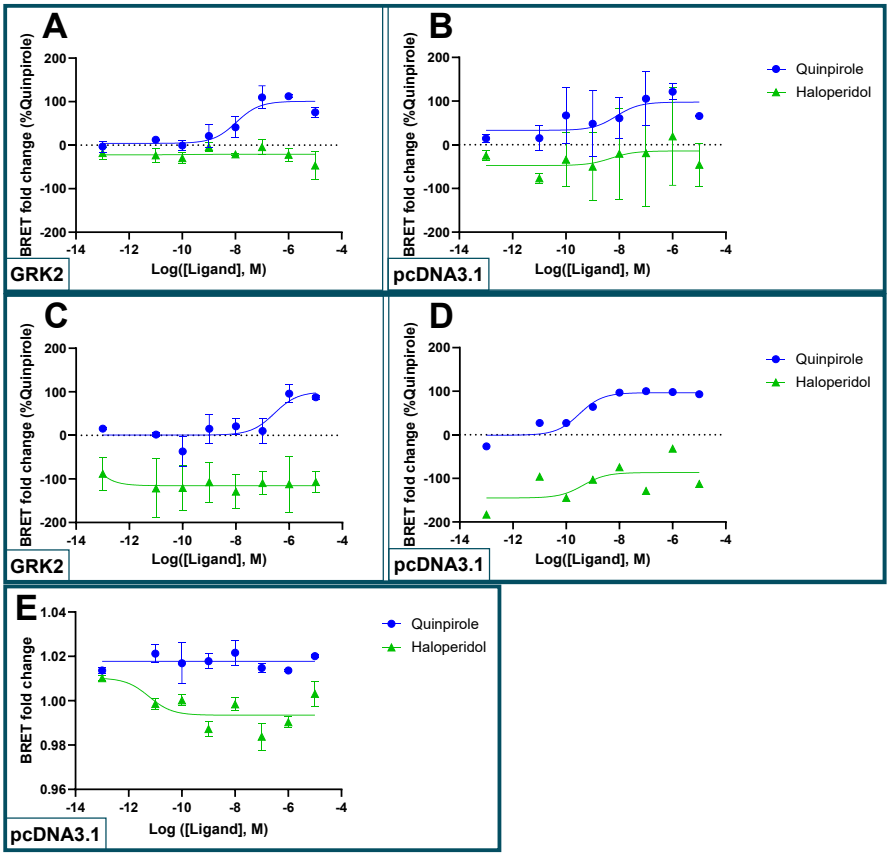


Figure 19: Effect of GRK2 overexpression on β-arrestin 2 recruitment to the D_2R (A,B) or the D_3R (C-E) modulated by the agonist quinpirole and the inverse agonist haloperidol. (**(A,C)**: 2 µg GRK2 DNA; **(B,D,E)**: 2 µg pcDNA3.1 DNA in place of GRK2.) HEK293 cells expressing the hD_2R or hD_3R along with β-arrestin 2 and the components of the BRET β-arrestin 2 recruitment assay, as detailed in the methods, exposed to the two compounds, and β-arrestin 2 recruitment was measured as an increase in BRET ratio. Responses are normalised to the baseline pre-ligand-addition, then to the control vehicle response, and finally to the maximal response of quinpirole. Data presented is the average of two independent experiments performed in triplicate ±SEM (n=2), with the exception of **(E)**, which is a representative single independent experiment ±SEM (n=1).

As is clear in the data in figure 19, relying on native HEK293 GRK2 levels resulted in wildly variable and inconsistent data – including, in one repeat,

entirely unusable data which could not even be rationally normalised and grouped.

So, with the determination that GRK2 over-expression was, indeed, required, this was now considered a functional and relatively robust assay to accompany the mG_o.

3.2 Behaviour of ligands at D_2R vs. D_3R

3.2.1 Ligand effect on G protein activation

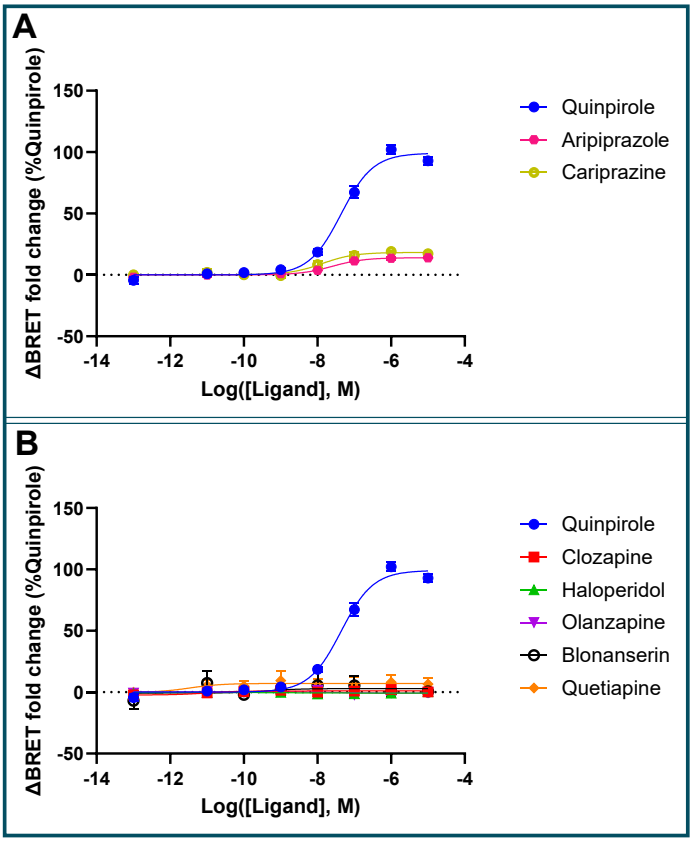


Figure 20: The ability of the partial agonists quinpirole, aripiprazole, and cariprazine (A), and the antagonists clozapine, haloperidol, olanzapine, blonanserin, and quetiapine, (B) to modulate G protein activation at the D_2R. HEK293 cells expressing the hD_2R along with mG_o and the components of the BRET mG_o assay, as detailed in the methods, exposed to the eight compounds, and mG_o recruitment was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole and presented as a change from baseline. Data presented is the average of four experiments performed in triplicate ±SEM (n=4, Table 18).

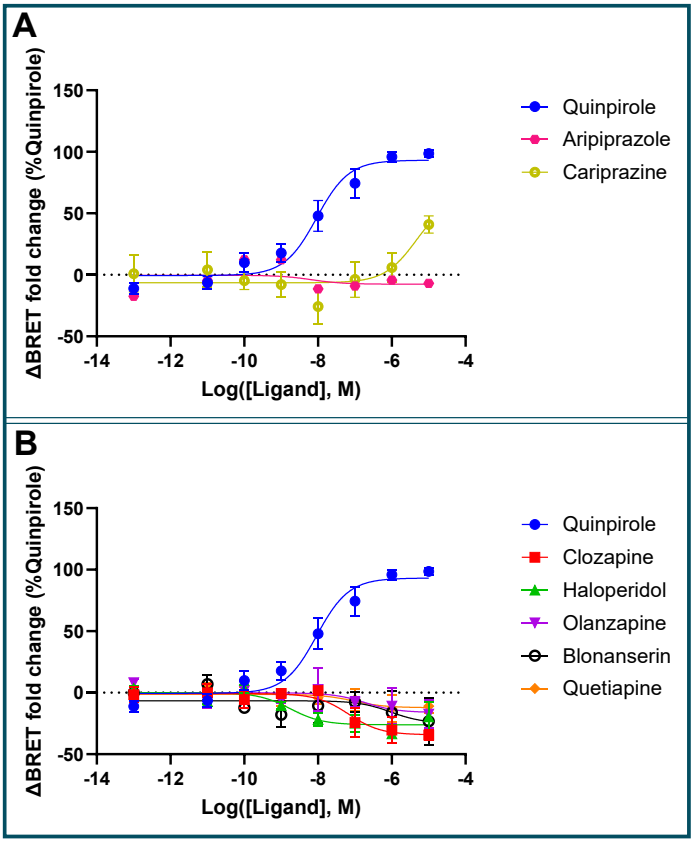


Figure 21: The ability of the partial agonists quinpirole, aripiprazole, and cariprazine (A), and the antagonists clozapine, haloperidol, olanzapine, blonanserin, and quetiapine, (B) to modulate G protein activation at the D_3R. HEK293 cells expressing the hD_3R along with mG_o and the components of the BRET mG_o assay, as detailed in the methods, exposed to the eight compounds, and mG_o recruitment was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole and presented as a change from baseline. Data presented is the average of four experiments performed in triplicate ±SEM (n=4, Table 18).

Ligand	D_2R	D_3R
	pEC_{50}	pEC_{50}
Quinpirole	7.358 ± 0.131	8.037 ± 0.413
Aripiprazole	7.598 ± 0.288	ND
Cariprazine	7.870 ± 0.294	5.332 ± 1.458
Clozapine	ND	7.167 ± 0.741
Haloperidol	ND	8.751 ± 0.951
Olanzapine	ND	ND
Blonanserin	ND	ND
Quetiapine	ND	ND

Table 18: Potencies of ligands used at both the D_2R and D_3R. Data is derived from figures 20 and 21. (ND = parameter could not be determined).

The responses for each ligand at each receptor will be discussed below.

Quinpirole:

As expected, quinpirole produced consistent agonistic responses which could then be used for comparison of all other ligand responses at both receptors. Though it displayed slightly higher error and variability at D_3R (± 0.413 vs. ± 0.131 at D_2R), and marginally higher potency there too (9.13 nM vs. 43.85 nM at D_2R), there was overall little difference between quinpirole's effect at D_2R and D_3R.

Aripiprazole:

Despite aripiprazole being a reported partial agonist for both the D_2R and D_3R, these data show such an effect taking place only at the D_2R. At D_3R, aripiprazole demonstrated a response more similar to what would be expected from an antagonist or inverse agonist, resembling the antagonist olanzapine (discussed below).

Cariprazine:

At both receptors, cariprazine behaved as a partial agonist, though to differing degrees. At the D_2R, its response is almost identical to that of aripiprazole, with a pEC_{50} value of 7.598 (± 0.288) and similar magnitude; at the D_3R, however, cariprazine displays a significantly lower observed potency and a far greater efficacy – its pEC_{50} being 5.332 (± 1.458), as per table 18, and at a maximum

concentration of 10 μM, reaching approximately 60% of the maximal response displayed by quinpirole.

Clozapine:

The response of clozapine at both receptors was as would be expected from such a well-documented and commonly prescribed dopamine receptor antagonist. At the D_2R, there was no discernible response in these data, and stimulation of the receptor by clozapine resulted in a flat line, which is indicative of a receptor system with minimal constitutive activity, and is especially compelling when compared to the response elicited by haloperidol (the control inverse agonist in this research).

A similar conclusion can be reached regarding its effect on the D_3R, where it displays clear antagonism (and potential inverse agonism), though with lower potency than quinpirole and haloperidol, indicating a significant level of basal activity from D_3R – once again, as expected.

Haloperidol:

As previously stated, haloperidol behaves as expected, producing a flat line at the D_2R and a slight inverse agonistic response at the D_3R, once again highlighting the varying degrees of constitutive activity between the two receptors.

Olanzapine, blonanserin, and quetiapine:

These three ligands all produced similar responses: potential antagonism at the D_2R, and potential inverse agonism at the D_3R, with a lower observed potency than clozapine and haloperidol. It is also worth noting that these ligands did not seem to induce the same degree of antagonism at D_3R as haloperidol and clozapine did.

3.2.2 Ligand effect on β-arrestin 2 recruitment

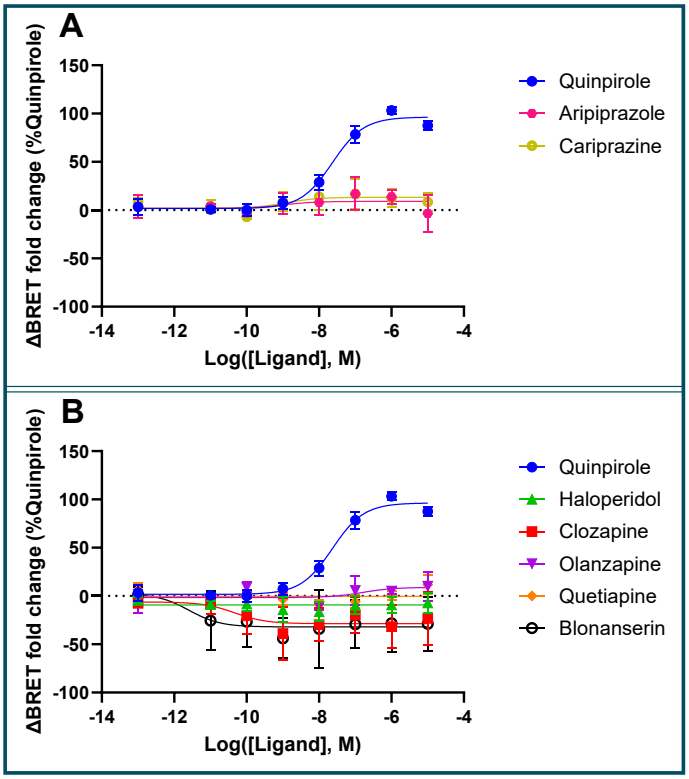


Figure 22: The ability of the partial agonists quinpirole, aripiprazole, and cariprazine (A), and the antagonists clozapine, haloperidol, olanzapine, blonanserin, and quetiapine, (B) to modulate β-arrestin 2 recruitment at the D_2R. HEK293 cells expressing the hD_2R along with β-arrestin 2 and the components of the BRET β-arrestin 2 recruitment assay, as detailed in the methods, exposed to the eight compounds, and β-arrestin 2 recruitment was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole and presented as a change from baseline. Data presented is the average of six experiments performed in triplicate ±SEM (n=6, Table 19).

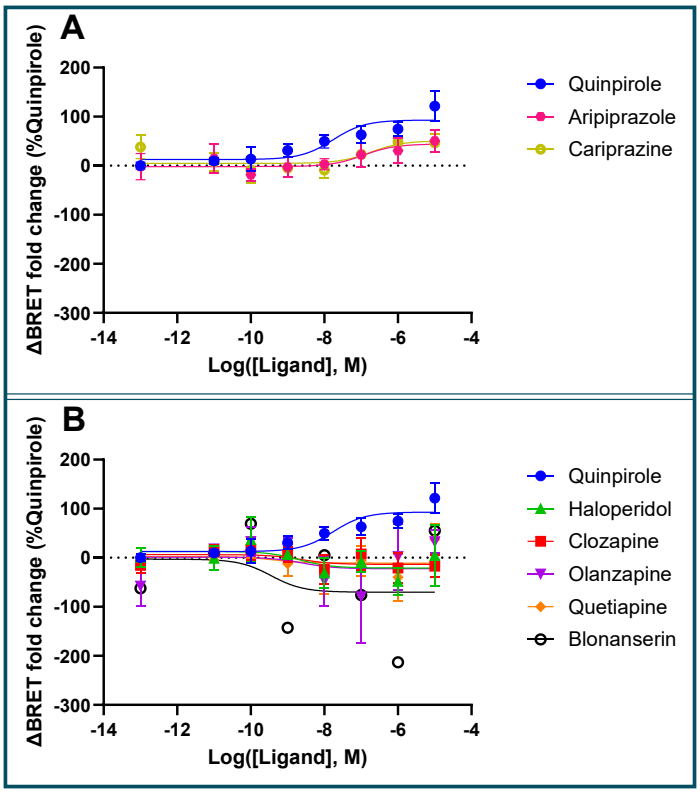


Figure 23: The ability of the partial agonists quinpirole, aripiprazole, and cariprazine (A), and the antagonists clozapine, haloperidol, olanzapine, blonanserin, and quetiapine, (B) to modulate β-arrestin 2 recruitment at the D_3R. HEK293 cells expressing the hD_3R along with β-arrestin 2 and the components of the BRET β-arrestin 2 recruitment assay, as detailed in the methods, exposed to the eight compounds, and β-arrestin 2 recruitment was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole and presented as a change from baseline. Data presented is the average of six experiments performed in triplicate ±SEM (n=6, Table 19).

Ligand	D_2R	D_3R
	pEC_{50}	pEC_{50}
Quinpirole	7.632 ± 0.271	7.763 ± 1.900
Aripiprazole	ND	6.942 ± 2.794
Cariprazine	ND	6.728 ± 1.757
Clozapine	ND	ND
Haloperidol	ND	8.678 ± 4.772
Olanzapine	ND	ND
Blonanserin	ND	ND
Quetiapine	ND	ND

Table 19: Potencies displayed by ligands used at both D_2R and D_3R. Data is derived from figures 22 and 23. (ND = parameter could not be determined)

The responses produced by each ligand at each receptor will be discussed below.

Quinpirole:

Quinpirole again induced an agonist response which could be taken as reference for a full agonistic response, though with a less significant difference in potency: 23.33 nM at D_2R compared to 17.26 nM at D_3R.

The relative similarity between quinpirole's observed potency would suggest that there is a similar degree of signal amplification in both the mG_o and β-arrestin 2 assays, so in cases where (for example) a ligand behaved antagonistically at the G protein but inverse agonistically for β-arrestin 2, this can be interpreted as constitutive β-arrestin 2 recruitment.

Aripiprazole and cariprazine:

At the D_2R, both aripiprazole and cariprazine produced responses that could potentially suggest partial agonism, though as these curves were so minor and inconsistent that analysis software was unable to produce a reliable pEC_{50} value, it is unlikely that this response is significant. This would suggest that, while both ligands have a partial agonistic effect on the activation of D_2R's associated G protein, they have little to no impact on the recruitment of β-arrestin 2 to the receptor.

At the D_3R, unlike what was seen in the mG_o assay, both ligands produced responses consistent with those expected from partial agonists, with lower potency and efficacy in comparison to the full agonist quinpirole.

Clozapine and haloperidol:

At both the D_2R and D_3R, clozapine and haloperidol displayed antagonistic responses, though clozapine's behaviour at D_2R may be better described as inverse agonism, indicating constitutive activity but to a lesser extent than in the mG_o assay. Their potencies, while not dissimilar from those observed in said previous assay, were slightly higher here, though their behaviours did not differ much between assays, suggesting an antagonist/inverse agonistic effect on both G protein activation and β-arrestin 2 recruitment.

Olanzapine:

Interestingly, despite olanzapine's antagonistic behaviour at the D_2R in the mG_o assay, it appears to potentially display partial agonism when observing the recruitment of β-arrestin 2. It is important to note, however, that the minor magnitude of olanzapine's response makes it difficult to draw concrete conclusions on this.

Blonanserin:

Blonanserin stimulation resulted in highly variable data at both receptors, making it difficult to interpret. What little can be seen though is consistent with what would be expected from an antagonist, inhibiting both G protein activation and β-arrestin 2 recruitment for both dopamine receptors.

Quetiapine:

Quetiapine produced no discernible response at the D_2R in either the mG_o or the β-arrestin 2 recruitment assay. When compared to other known antagonists used in this research, this would suggest that quetiapine has an antagonistic effect on the activation of the G protein at D_2R and for the receptor's recruitment of β-arrestin 2. These lack of responses would be consistent with extensive previous literature documenting quetiapine as a D_2R antagonist.

Quetiapine's impact on D_3R was more difficult to determine. It has a clear antagonistic effect on G protein activation, but the highly variable data produced by the β-arrestin 2 recruitment assay is less conclusive and will instead be discussed further in subsequent sections.

3.3 Investigating claims of biased agonism

3.3.1 Effect of quetiapine on G protein activation vs. β-arrestin 2 recruitment at $D_{2/3}R$-RLuc8

In order to validate the results produced by Schamiloglu *et al* (2023) (see section 1.3.3), RLuc8-tagged $D_{2/3}R$ was used alongside mVenus-tagged β-arrestin 2 to directly measure proximity between them following ligand addition to determine the recruitment of β-arrestin 2 to the receptor, as explored in section 3.2.2. This data was then used for direct comparison to quetiapine's response in the mG_o assay, used to determine G protein activation.

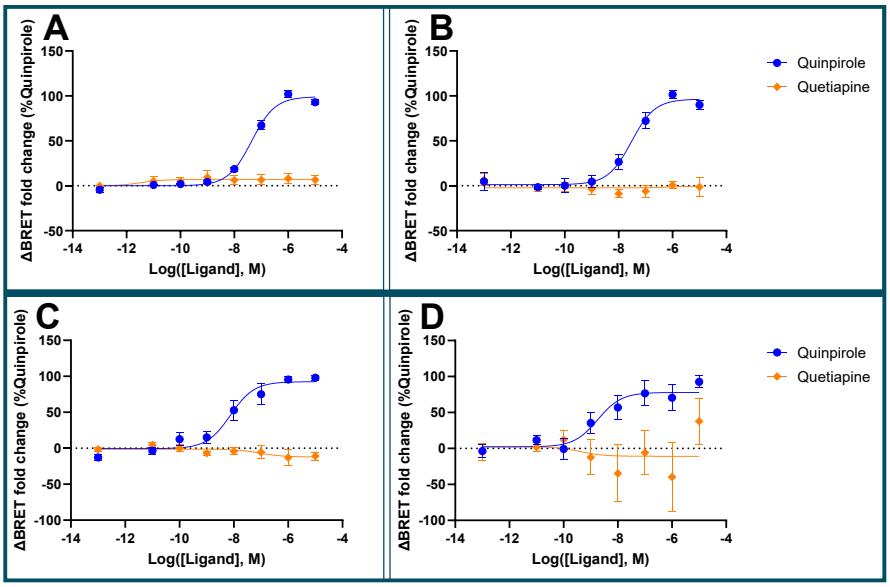


Figure 24: Action of quetiapine upon D_2R (A,B) and D_3R (C,D), and its effect upon recruitment of mG_o and β-arrestin 2 in comparison to quinpirole. HEK293 cells expressing the hD_2R (**(A)** mG_o recruitment; **(B)** β-arrestin 2 recruitment) or hD_3R (**(C)** mG_o recruitment;

(D) β-arrestin 2 recruitment) along with mG_o or β-arrestin 2 and their relevant assay components, as detailed in the methods, exposed to the two compounds and G protein or β-arrestin 2 recruitment was measured as an increase in BRET ratio. Responses are normalised to the maximal response of quinpirole, acting as a control agonist response. Data presented is the average of four independent experiments performed in triplicate ±SEM (n=4) in the case of mG_o recruitment assays, and the average of six (n=6) in the case of β-arrestin 2 recruitment assays. (Table 20).

Receptor	pEC_{50}: mG_o		pEC_{50}: βarr2	
	Quinpirole	Quetiapine	Quinpirole	Quetiapine
D_2R	7.358 ± 0.131	ND	7.528 ± 0.305	ND
D_3R	8.124 ± 0.460	ND	8.739 ± 0.843	ND

Table 20: pEC_{50} of quinpirole and quetiapine at $D_{2/3}R$ in regard to mG_o recruitment and β-arrestin 2 recruitment. Data is derived from figure 24. (ND = parameter could not be determined).

Quinpirole is, once again, used as a control agonist for both G protein and β-arrestin 2 recruitment.

To reiterate, at D_2R, as expected, quetiapine seems to have no discernible impact on either mG_o or β-arrestin 2 recruitment.

D_3R, however, is another matter entirely. For G protein recruitment, there is distinct antagonistic (potentially inverse agonistic) behaviour from quetiapine, which is generally consistent between repeats, and is indicative of minor constitutive activity. But for β-arrestin 2 recruitment, results vary wildly from one day to the next despite quinpirole's response remaining relatively uniform, and while an overall pattern of mild antagonism by quetiapine emerges, it is nearly impossible to draw any conclusions from such data.

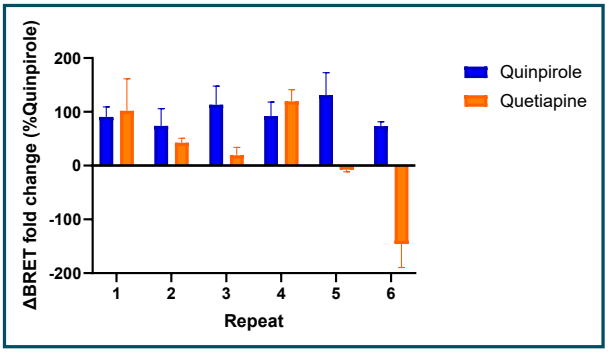


Figure 25: Variations and inconsistencies of effects of 10 µM quinpirole or quetiapine on the recruitment of β-arrestin 2 to the D_3R. Data is derived from figure 24 and demonstrates the varying responses of six experimental repeats ±SEM.

These wild inconsistencies in the quetiapine-stimulated D_3R β-arrestin 2 recruitment data, as demonstrated in figure 25, could possibly indicate that quetiapine acts as a protean agonist – when the observed behaviour of a ligand changes depending on the overall constitutive activity of a given receptor system. In a generally quiescent system, a ligand is able to behave as a partial agonist, inducing partial activation of the system. In a highly active receptor system, such as those made up by dopamine receptors, the ligand will behave as an antagonist and act to reduce overall receptor activity levels.

Given D_3R's high apparent level of constitutive activity, the fact that this behaviour is seemingly unique to quetiapine and was not observed in any of the other investigated ligands, and all previous work listed in this paper endeavouring to develop an assay and experimental environment resulting in the lowest possible basal activity, protean agonism seems a plausible explanation; these differences between repeats could be the result of basal activity levels varying from one day to the next, though further work would need to be done to confirm this.

3.4 Is β-arrestin 2 facilitating receptor internalisation?

3.4.1 CAAX-nGreen and FYVE-mVenus – receptor proximity as a measurement of internalisation

In order to determine the implications of biased agonism, it is important to investigate the internalisation of both D_2R and D_3R.

It has previously been reported that β-arrestin 2 recruitment at D_2R facilitates receptor internalisation[68], and that biased β-arrestin 2 agonism can lead to reduced D_3R availability and presence at the cell surface membrane[49] – presumably via the internalisation, and subsequent degradation, of the receptor.

In order to corroborate these claims, CAAX and FYVE assays were used to approximate receptor internalisation.

CAAX-neonGreen acts as a plasma membrane marker and so, when expressed alongside $D_{2/3}R$-RLuc8, can be used to infer the overall proximity of the receptor to the cell surface membrane.

Similarly, FYVE-mVenus acts as an early endosomal marker, thereby allowing the approximation of receptor proximity to the early endosome.

When data from these two assays are combined, it is possible to draw conclusions on the likelihood of receptor internalisation – i.e. if a marked decrease in CAAX-receptor BRET ratio is observed alongside an increase in FYVE-receptor BRET ratio, that may be indicative of the receptor moving away from the plasma membrane and towards the early endosome, i.e. being internalised.

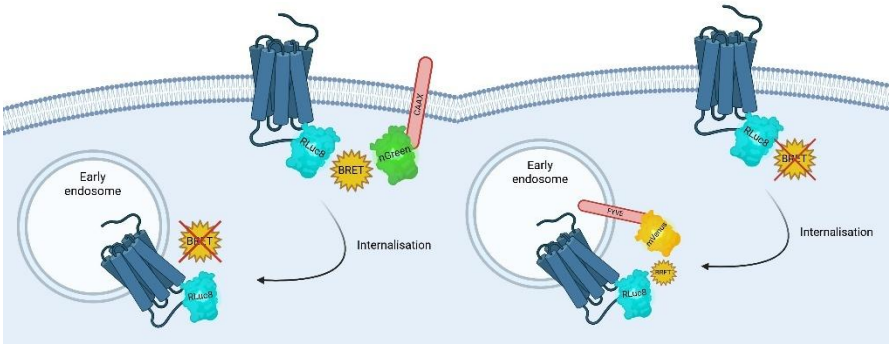


Figure 26: Representation of CAAX and FYVE assays. (Left) CAAX-neonGreen is a membrane marker embedded in the cell surface membrane; proximity of the receptor to CAAX produces a measurable BRET response and can be interpreted as proximity of the receptor to the plasma membrane. **(Right)** FYVE-mVenus is a membrane marker embedded in the early endosomal membrane; proximity of the receptor to FYVE produces a measurable BRET response and can be interpreted as proximity of the receptor to the early endosome.

3.4.2 D_2R

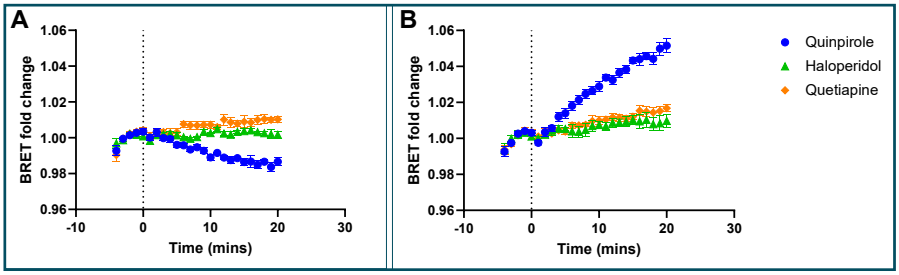


Figure 27: Internalisation of D_2R following ligand stimulation based on proximity to membrane markers CAAX (A) or FYVE (B). HEK293 cells expressing the hD_2R along with CAAX or FYVE and the components of the BRET CAAX/FYVE assay, as detailed in the methods, were exposed to the three compounds at the time point '0 minutes', and readings were taken at a rate of one cycle per minute for a duration of twenty minutes. **(A)** Movement of the D_2R away from the cell surface membrane was measured as a decrease in BRET ratio. **(B)** Movement of the D_2R toward the early endosome was measured as an increase in BRET ratio. Responses are normalised to the baseline reading, then to the control vehicle-only response. Data presented is the average of five independent experiments performed in triplicate ±SEM (n=5).

These data support conclusions drawn from earlier findings indicating that quetiapine is likely not facilitating β-arrestin 2 recruitment at the D_2R as there is very little difference between quetiapine's response and the response

produced by haloperidol, a known inverse agonist which has been shown to inhibit receptor internalisation.

D_2R-quinpirole response provides a model for internalisation:

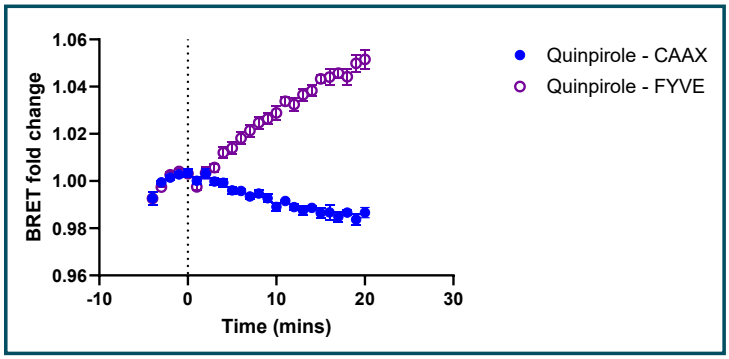


Figure 28: Effect of quinpirole on receptor proximity to both plasma membrane and early endosome. Visualisation of the quinpirole- CAAX and FYVE responses alongside one another. Data is collated from figure 27 **(A)** and **(B)**.

At D_2R, quinpirole provides what could almost be described as a textbook internalisation response; the clear decrease in CAAX ratio alongside an increase in FYVE is highly indicative of a receptor moving away from the plasma membrane and toward the early endosome, being internalised. However, there is a difference in the magnitude of the changes in CAAX vs. FYVE; these disproportionate responses could suggest that, while still being internalised, some of that receptor is being recycled to the membrane almost immediately, resulting in a lower net movement of receptor away from CAAX.

When considered in comparison to quinpirole, quetiapine's complete and utter lack of response (and similarities to haloperidol's effect as an inverse agonist) is a strong indicator that quetiapine does not recruit β-arrestin 2 to the receptor, and does not facilitate receptor internalisation, whether through β-arrestin 2-dependent pathways or otherwise.

3.4.3 D_3R

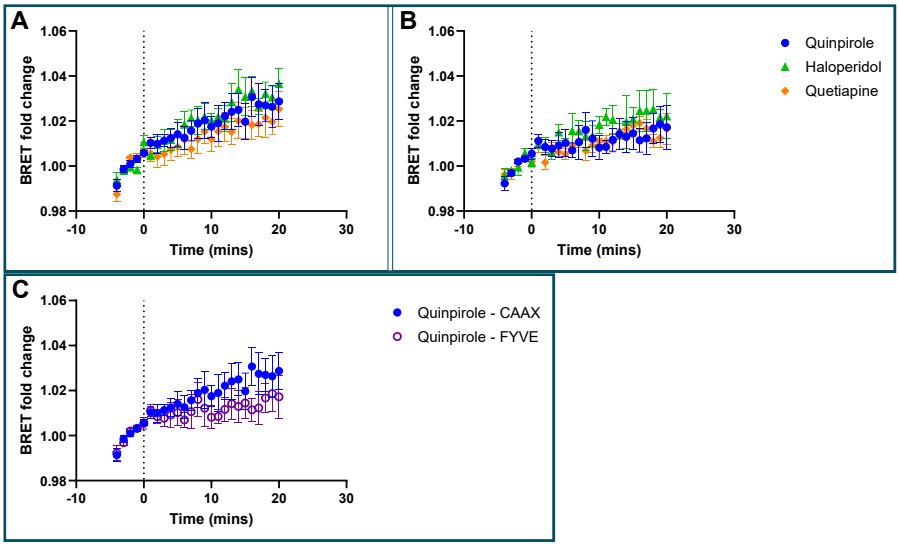


Figure 29: Internalisation assay of D_3R following ligand stimulation based on proximity to membrane markers CAAX (A) or FYVE (B); (C) quinpirole- CAAX and FYVE responses pictured together. HEK293 cells expressing the hD_3R along with CAAX or FYVE and the components of the BRET CAAX/FYVE assay, as detailed in the methods, were exposed to the three compounds at the time point '0 minutes', and readings were taken at a rate of one cycle per minute for a duration of twenty minutes. **(A)** Movement of the D_3R away from the cell surface membrane was measured as a decrease in BRET ratio. **(B)** Movement of the D_3R toward the early endosome was measured as an increase in BRET ratio. **(C)** D_3R quinpirole-CAAX/FYVE responses visualised together. Responses are normalised to the baseline reading, then to the control vehicle-only response. Data presented is the average of five independent experiments performed in triplicate ±SEM (n=5).

The responses at D_3R are difficult to unpick, especially in comparison to D_2R's response.

The minor increase in both the CAAX and FYVE BRET ratios would suggest there is *some* movement of receptor to the early endosome, though the overall movement of receptor to the membrane is greater in comparison. Further, the small window for even quinpirole's full agonist response is even more unhelpful.

Ultimately, given the above data, there are two potential explanations for what has been observed:

Nothing is happening; there are some new receptors being recruited to the plasma membrane, though not many, and there is little to no receptor internalisation occurring. We may then conclude that β-arrestin 2 recruitment does not appear to facilitate receptor internalisation at the D_3R.

So much is happening that it is difficult to measure; the receptor is being internalised – potentially at the same rate as in D_2R – but is being recycled and returned to the plasma membrane so quickly that there is almost no discernible net movement in the current assay set up.

It is very difficult to conclude with any confidence what exactly is taking place from this data alone. Though once again, it is possible to conclude that quetiapine is unlikely to be recruiting β-arrestin 2, as supported by earlier data, and is unlikely to be facilitating receptor internalisation, as was observed at D_2R. This is in line with the longstanding knowledge that quetiapine does not appear to differ especially from other second-generation antipsychotics in terms of clinical efficacy, likelihood of patients to continue with treatment, or in occurrence of side effects[69].

4.0 Concluding statements and future works

The initial aims of this research were to develop functional assays for the measurement of G protein activation and β-arrestin 2 recruitment to both the D_2R and the D_3R, to investigate the behaviour of several common antipsychotics and analyse the differences in interactions between the two receptors, and to verify claims of biased agonism displayed by quetiapine at the D_3R.

By attempting the βγ-release, TRUPATH, and mG_o assays, and altering them as necessary to investigate the impact and importance of certain experimental factors and conditions, a final functional method was optimised, allowing the measurement of G protein activation at $D_{2/3}R$ through the measurement of luminescence, and subsequent calculation of BRET ratio, produced with increasing proximity between an mVenus-tagged modified $G\alpha_o$ subunit and the RLuc8-tagged receptor. Not only did this assay display lower observed levels of constitutive activity in comparison to the TRUPATH method, but the use of said modified G protein construct (mG_o), which lacks the GTPase domain, made it possible to measure receptor and G protein activation without encountering any subsequent G protein activation, as the construct could be recruited to the receptor but would not undergo multiple activation cycles. Thereby, any problematic assay sensitivity and signal amplification, as observed in the βγ-release assay, could be avoided.

Similarly, a functional method for the assessment of β-arrestin 2 recruitment was reached in the early stages of this project, utilising an mVenus-tagged β-arrestin 2 and an RLuc8-tagged receptor, and measuring their proximity as a BRET readout. It was established that the over-expression of GRK2 was necessary to promote receptor phosphorylation, facilitating the recruitment of β-arrestin 2 to the receptor.

Following the investigation of the behaviour of various antipsychotics and their effect upon both G protein activation and β-arrestin 2 recruitment, this project also demonstrated that, while the antipsychotic quetiapine does indeed have an antagonistic effect upon G protein activation at the D_3R, it appears to

have negligible impact on the recruitment of β-arrestin 2 to the receptor, contrary to claims. In fact, the antipsychotic olanzapine at the D_2R, likely insignificant though the response was, displayed behaviour more consistent with biased agonism than quetiapine did at the D_3R.

This discrepancy could have occurred for various reasons.

First, this research made use of HEK293-T cells, as opposed to neuronal cells specifically, so comparison between data sets and between *in vitro* and *in vivo* behaviour is not necessarily accurate. Similarly, where cell lines stably expressing receptor components were used by Schamiloglu *et al*, all experiments presented in this thesis were performed in transiently transfected cells, increasing the possibility of unintended and unanticipated variations between individual transient transfections.

Secondly, the use of a β-arrestin 2 recruitment assay in place of an immunoblot for ERK phosphorylation also poses difficulties in comparability as, though the direct measurement of proximity between β-arrestin 2 and the $D_{2/3}R$ provided a clear picture of the impact of ligands on β-arrestin 2 recruitment, no investigation was done into the downstream consequences of this recruitment or any ensuing signalling cascades.

Calculating ERK phosphorylation could provide more clarity on these signalling pathways, though when used as a measurement of β-arrestin 2 recruitment it relies on the idea that all β-arrestin 2 recruitment led to ERK phosphorylation and that all ERK phosphorylation was a consequence of β-arrestin 2 recruitment to the D_3R. This is especially significant given how generally unclear and debated the specific role and level of involvement β-arrestin 2 plays in the D_3R-ERK signalling pathway currently is.

There has been recent suggestion that ERK phosphorylation in general is facilitated by the recruitment of β-arrestin 2 and is, for some GPCRs, a necessity[51]. However, earlier research suggested that ERK activation following ligand stimulation at the $D_{2/3}Rs$ in particular does *not* involve either β-arrestin or receptor sequestration, instead occurring via signalling by the Gα subunit in the case of D_2R-mediated ERK activation, and the βγ pathway of the

G_i protein for D_3R, involving the transactivation of the epidermal growth factor receptor in HEK293-T cells[70]. Other recent research suggests that β-arrestin-mediated signalling cannot occur whatsoever in the absence of active G proteins, and in cells lacking the $G\alpha_s$ subunit, arrestins alone were not sufficient to initiate endosomal ERK activity in β_2ARs[71], though no research investigating the impact on ERK phosphorylation of the removal of G protein subunits from cells expressing D_2R or D_3R could be identified at the time of writing this thesis.

Furthering discrepancies from Schamiloglu *et al*, comparison of quetiapine's responses to those displayed by the control full agonist quinpirole in both the mG_o and β-arrestin 2 assays and the CAAX/FYVE internalisation assays makes clear from the data collected that there is no net receptor internalisation occurring following stimulation by quetiapine at either receptor. However, as stated earlier, given that the response elicited by quinpirole at the D_3R is similarly unclear to quetiapine's, there are several possible explanations to consider.

It may be that there are some new receptors being recruited to the plasma membrane following their synthesis, though likely very few, and little to no receptor sequestration is taking place. If this is true, the conclusion could be drawn that β-arrestin 2 recruitment to the D_3R is unlikely to facilitate receptor internalisation.

On the other hand, it may be that D_3R is indeed undergoing internalisation at a rate comparable to D_2R following quinpirole stimulation, but receptor – potentially either recycled or freshly synthesised – is joining the cell surface membrane at such a rate that the proximity assays utilised in this research are unable to discern any net movement of receptor. If this is the case, further research would be needed before any reliable conclusions can be drawn.

Potential avenues for future research:

Based on the findings presented in this thesis, it is clear that further experimentation is needed to better clarify and understand quetiapine's effect upon receptor internalisation at the D_3R, i.e. is the receptor being broken down,

is it being recycled, is it being sent back to the golgi and repackaged or modified?

This could be achieved through the use of additional intracellular markers, along with the strategic blockade of several receptor synthesis and trafficking processes.

For example, a mutation to dynamin – a GTPase involved in endocytosis (see section 1.1.3) – dynamin K44E, resulting in a GTP binding deficiency, can lead to the inability to internalise receptors, and clathrin-coated pits can fail to deeply invaginate[72]. The use of this mutation could allow the comparison of previous uncertain data to that of a system where it is *known* that no internalisation is taking place.

A marker for the recycling endosome such as Rab11[73] would also be of great interest. As some of the uncertainty regarding the internalisation assay results came from the possibility that a large number of receptors were being recycled back to the cell surface membrane almost immediately following internalisation, being able to estimate proximity of D_3R to the recycling endosome could provide some clarity.

Similarly, a golgin marker could allow the estimation of new receptor synthesis through the measurement of receptor proximity to the golgi. If this led to the conclusion that there is a significant movement of newly synthesised receptor to the plasma membrane, it may then be necessary to block said movement; this could be done using brefeldin A, a compound which can indirectly inhibit protein transport from the endoplasmic reticulum to the golgi complex through the prevention of the COP-I coat's association to the golgi membrane[74]. This could allow the comparison of data presented in this thesis to data produced by a system where there is reasonable certainty that there is little or no newly synthesised receptor reaching the cell surface membrane.

Significance statement:

Over the course of this research project, a reliable assay method for the measurement of dopamine D_3 receptor activation was developed, and it was

determined that the antipsychotic quetiapine is unlikely to facilitate the recruitment of β-arrestin 2 to the D_3R to any significant degree.

The development of the functioning mG_O BRET method could help streamline future research into dopamine receptor function; this will be key in the future of various pharmacological studies into schizophrenia as well as other disorders related to dysfunctions of dopaminergic neurotransmission such as ADHD or psychostimulant use disorders and addiction.

The conclusions reached regarding quetiapine's inability to facilitate β-arrestin 2 recruitment help to further understanding of the mechanism of action of certain second-generation antipsychotics, as well as highlighting the potential for future research into the cause of certain downstream effects such as the ERK phosphorylation observed by Schamiloglu *et al* (2023), initially thought to function through quetiapine inducing β-arrestin 2 recruitment to dopamine receptors.

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6.0 Addressing corrections

6.1 Introduction

1. A brief introduction to drug-receptor interactions more generally was provided prior to 1.1.1, giving more context and aiming to provide a sense of scale of drug action. I believe the significance of GPCRs as drug targets specifically is emphasised in the new 1.1.2 section.

2. 1.1.1 was renamed to better suit its contents

3. Sections 1.1.2 and 1.1.3 were switched for earlier highlighting of significance

4. References were provided for 1.2.1

5. Text was changed as requested in 1.2.3

6. Text was changed as requested in 1.2.3 2nd paragraph

7. References were provided in 1.2.3

8. A diagram of the brain was included, highlighting the overall dopaminergic signalling pathways, with the key edited to highlight those thought to be impacted by schizophrenia

9. A definition of protean agonism was provided within 1.3.1 Biased agonism

6.2 Methods

10. Addressed and justified the use of HEK cells over a more neuron-like cell line; time constraints, parity with other published works, etc.

11. Made the reporting of manufacturers and suppliers consistent

12. Provided a brief explanation of general BRET function. No diagrams or other explanation was provided as I feel the specifics are covered in the introductory sections for each assay in the results and discussion section

13. More information and explanation were provided regarding the collation of data and the calculation of ‘n’ numbers; where possible, assays included wells using the ‘standard’ methods for direct on-the-day comparison with altered methods

6.3 Results and discussion

14. The key for (then) Figure 8 was modified as requested

15. (Then) Figure 9 was enlarged as requested, along with clarifying labels being added to necessary panels on what was previously Figure 9 and others where it was deemed helpful

16. Provided clarity on the use of ±SEM error bars and addressed the benefits and limitations of using said error bars to determine assay viability. The initial issue faced was regarding the wild inconsistency in assay results from one day to the next; wide margins of error on normalised collated data indicated a lack of consistency in results and, therefore, likely low reproducibility. I believe that, given the timescale of my research, this was an appropriate assessment to make

17. Provided clarity on units used for data provided in what was, at the time of first submission, paragraph 2 of page 35

18. Altered figures for consistency on ordering of altered DNA masses

19. Addressed disparity in 'n' numbers in certain figures. As stated above, assays of (e.g.) altered DNA mass were run directly alongside the 'standard' mass of DNA as a control and for direct on-the-day comparison in order to minimise the impact of variations in data from one day to the next

Additionally, a brief clarification on the specific location of sodium ions binding to class A GPCRs was included. As the binding site of Na^+ is found on the extracellular domain of class A GPCRs, I believe it is unlikely that the receptor function would be meaningfully altered by intracellular Na^+ concentration and so would not expect significant differences in receptor behaviour within HEK cells compared to more neuron-like cells. However, I cannot comment on the impact of variations in buffer components on HEK and neuronal cells as a whole or the potential downstream effects of any changes on the functionality of dopamine receptors.

This is a hypothesis which would require further research to confirm or deny.

6.4 Concluding statements and future works

20. A significance statement was provided at the end of this section, aiming to address potential uses for these findings.

6.5 Bibliography

Authors were listed in full, and references and their associated numbers were altered as appropriate to reflect changes throughout this thesis