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PHARMACOLOGICAL IMPLICATIONS OF ADENOSINE 2A RECEPTOR- DOPAMINE TYPE 2 RECEPTOR HETEROMERIZATION

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PHARMACOLOGICAL IMPLICATIONS OF ADENOSINE 2A RECEPTOR-DOPAMINE TYPE 2 RECEPTOR HETEROmerization

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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# Table of Contents

Acknowledgement .................................................................................................................. ii

List of Figures .......................................................................................................................... vii

List of Abbreviations ................................................................................................................. ix

Abstract ..................................................................................................................................... xiii

Chapter 1 INTRODUCTION ........................................................................................................ 1

1.1 G protein signaling ................................................................................................................. 1

1.2 The A_2A_2R .......................................................................................................................... 4

1.3 The D_2_2R ............................................................................................................................. 6

1.4 Evidence for A_2A_2R-D_2_2R heteromerization ................................................................. 9

1.5 The A_2A_2R-D_2_2R heteromer interface ............................................................................. 11

1.6 Reciprocal antagonistic A_2A_2R-D_2_2R interactions ....................................................... 13

1.7 The Basal Ganglia ............................................................................................................... 17

1.8 The A_2A_2R-D_2_2R heteromer and the significance for Parkinson’s disease .............. 19

1.9 Antiparkinsonain therapeutics targeting the A_2A_2R-D_2_2R heteromer ....................... 21

1.10 Conclusion .......................................................................................................................... 24

1.11 Hypothesis .......................................................................................................................... 25

Chapter 2 MATERIALS AND METHODS .................................................................................. 35

2.1 Molecular biology ............................................................................................................... 35
Literature Cited...........................................................................................................92
Vita............................................................................................................................99
List of Figures

Figure 1.1 The G protein cycle.................................................................26
Figure 1.2 G_i/o- and G_s-coupled receptor signaling..............................27
Figure 1.3 A2AR signaling......................................................................28
Figure 1.4 D2R localization and signaling..............................................29
Figure 1.5 A2AR and D2R co-localization............................................30
Figure 1.6 The A2AR-D2R Heteromer Interface....................................31
Figure 1.7 The A2AR-D2R Heteromer Interface continued.....................32
Figure 1.8 The Indirect Motor Pathway..................................................33
Figure 1.9 Localization of the A2AR and D2R......................................34
Figure 3.1 The GIRK1/2 (G1/2) channel is a suitable reporter for G_i signaling through the D2R.................................................................52
Figure 3.2 A2AR coexpression in various ratios to the D2R inhibits G_i signaling through the D2R.................................................................53
Figure 3.3 A2AR ligands crosstalk to the D2R........................................54
Figure 3.4 A2AR ligands crosstalk to the D2R continued.......................55
Figure 3.5 A2AR(S374A) abolishes crosstalk of A2AR ligands to G_i signaling through the D2R.................................................................56
Figure 3.6 Figure 3.6. A2AR(S374A) is functionally similar to WT A2AR........57
Figure 3.7 A2AR ligands do not agonize or antagonize homomeric D2R G_i Activity........................................................................58
Figure 3.8 Summary D2R Signaling........................................................................................................59

Figure 4.1 The GIRK1/2 (G1/2) channel is a suitable reporter for Gs signaling through the A2AR........................................................................................................69

Figure 4.2 Gs signaling through the A2AR in the presence of PTX.................................70

Figure 4.3 Xenopus oocytes have an endogenous Gi-coupled A1R.................................71

Figure 4.4 The effect of D2R co-expression on Gs signaling through the A2AR..........72

Figure 4.5 D2R ligands crosstalk to the A2AR........................................................................73

Figure 4.6 D2R-9A disrupts the allosteric modulation of the D2R on the A2AR........74

Figure 4.7 Figure 4.7 D2R-9A is functionally similar to WT D2R.................................75

Figure 4.8 D2R ligands do not agonize or antagonize homomeric A2AR Gs activity.....76

Figure 4.9 Summary A2AR Signaling..................................................................................77

Figure 5.1 Optogenetic-microdialysis probe.......................................................................83

Figure 5.2 Schematic displaying timeline of unilateral AAV microinjection and optogenetic-microdialysis experiment.................................................................84

Figure 5.3 Confocal laser microscopy of coronal sections showing the localization of ChR2-EYFP after unilateral AAV microinjection in the motor cortex and ERK phosphorylation immunohistochemistry.........................................................85

Figure 5.4 Effect of optogenetic stimulation in the striatum..............................................86

Figure 5.5 Basal Glutamate Concentration.......................................................................87
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>A₁R</td>
<td>Adenosine A1 receptor</td>
</tr>
<tr>
<td>A₂AR</td>
<td>Adenosine 2A receptor</td>
</tr>
<tr>
<td>A₂BR</td>
<td>Adenosine 2B receptor</td>
</tr>
<tr>
<td>A₃R</td>
<td>Adenosine A3 receptor</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ADO</td>
<td>adenosine</td>
</tr>
<tr>
<td>AGT</td>
<td>SNAP-tag protein</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>ASUL</td>
<td>amisulpride</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>barium chloride</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD4</td>
<td>cluster of differentiation 4</td>
</tr>
<tr>
<td>CGS</td>
<td>CGS 21680</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
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</table>
ChR2 ................................................................. channel rhodopsin 2
CNS ............................................................... central nervous system
CPA ............................................................... N6-cyclopentyladenosine
CREB ............................................................ cAMP response element binding protein
CTX ............................................................... cholera toxin
D1R .............................................................. Dopamine type 1 receptor
D2R .............................................................. Dopamine type 2 receptor
D2L .............................................................. long isoform of the Dopamine type 2 receptor
D2S .............................................................. short isoform of the Dopamine type 2 receptor
D3R .............................................................. Dopamine type 3 receptor
D4R .............................................................. Dopamine type 4 receptor
D5R .............................................................. Dopamine type 5 receptor
DA ............................................................... dopamine
DARPP-32 ..................................................... dopamine- and cAMP-regulated neuronal phosphoprotein
E_K ............................................................... potassium equilibrium potential
EYFP ............................................................. enhanced yellow fluorescent protein
FDA ............................................................... U.S. Food and Drug Administration
FRET ........................................................... fluorescence resonance energy transfer
G1/2 ............................................................. GIRK1/2
Gα .............................................................. G protein alpha subunit
GABAB R2 ....................................................... Gamma-aminobutyric acid B receptor 2
Gβ .............................................................. G protein beta subunit
Gβγ ............................................................. G protein beta gamma dimer
GDP ................................................................. guanine diphosphate
GEF ................................................................. guanine exchange factor
GFP2 ......................................................... green fluorescent protein 2
GIRK .............................................................. G protein-coupled inwardly rectifying potassium channel
GPCR ........................................................... G protein-coupled receptor
Gpe ................................................................. external segment of the globus pallidus
Gpi ................................................................. internal segment of the globus pallidus
GTP ................................................................. guanine triphosphate
Gγ ................................................................. G protein gamma subunit
HEK-293 ......................................................... human embryonic kidney 293
HIV TAT ......................................................... human immunodeficiency virus trans-activator of transcription
HK ................................................................. ND96K solution
i.p ................................................................. intraperitoneal
KO ................................................................. knockout
KW ................................................................. KW-6002
KW-6002 ........................................................ istradefylline
L-DOPA ........................................................ Levodopa
MAPK .......................................................... mitogen-activated protein kinase
MSN .............................................................. medium spiny neuron
PAL .............................................................. paliperidone
PD ................................................................. Parkinson’s disease
PKA ............................................................. protein kinase A
PLA .............................................................. proximity ligation assay
PLC..........................................................phospholipase C
PTX..........................................................pertussis toxin
QUIN..........................................................quinpirole
RGS..........................................................regulator of G protein signaling
Rluc..........................................................Renilla Luciferase
STN..........................................................subthalamic nucleus
TM..........................................................transmembrane domain
VDCC.......................................................voltage dependent calcium channels
WT..........................................................wild-type
YFP..........................................................yellow fluorescent protein
Abstract

PHARMACOLOGICAL IMPLICATIONS OF ADENOSINE 2A RECEPTOR-DOPAMINE TYPE 2 RECEPTOR HETEROMERIZATION

By Candice Nicole Hatcher-Solis, B.A.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

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Professor and Chair, Department of Physiology and Biophysics

G protein-coupled receptors (GPCRs) are heptahelical, transmembrane proteins that mediate a plethora of physiological functions by binding ligands and releasing G proteins that interact with downstream effectors. GPCRs signal as monomers, complexes of the same receptor subtype (homomers), or complexes of different receptor subtypes (heteromers). Recently, heteromeric GPCR complexes have become attractive targets for drug development since they exhibit distinct signaling and cell-specific localization from their homomeric counterparts. Yet, the effect of heteromerization on the pharmacology of many GPCR homomers remains unknown. Therefore, we have undertaken the task to examine the effect of heteromerization on Gs signaling through the adenosine 2A receptor (A2A R) and Gi signaling through the
dopamine type 2 receptor (D₂R) since the A₂AR-D₂R heteromer is an emerging therapeutic target for Parkinson’s disease (PD). We examined the effect of heteromerization on A₂AR and D₂R homomeric signaling using electrophysiology and the *Xenopus laevis* oocyte heterologous expression system. G protein-coupled inwardly rectifying potassium channels (GIRKs) were used as reporters for Gᵢ signaling because activation leads to direct Gbeta-gamma (Gβγ)-mediated stimulation of the GIRK current. We also coupled GIRK channels to Gₛ signaling by overexpressing Gαₛ and signaling through Gαₛβγ. Our electrophysiological assay is innovative because it allows us to optimize the conditions of heteromerization and directly observe GPCR signaling at the G protein level. Our data demonstrate that heteromer formation alone decreases dopamine-elicited Gᵢ signaling through the D₂R and CGS-21680-elicited Gₛ signaling through the A₂AR. Furthermore, this reciprocal antagonism was predominately due to changes in efficacy versus potency. We also examined crosstalk observing that applying agonists or antagonists to the adjacent receptor further modulate this inhibition with the combination of agonists and antagonists relieving inhibition. Mutating the A₂AR-D₂R heteromer interface abrogated all of the aforementioned ligand-induced effects on G protein signaling through the A₂AR-D₂R heteromer. We are currently aiming to validate our results from the oocyte experiments with an *in vivo* model. Our data further elucidate the effect of various ligands on G protein signaling through the A₂AR-D₂R heteromer, which may facilitate future studies that examine A₂AR-D₂R heteromer signaling.
Chapter 1 INTRODUCTION

1.1 GPCR signaling

GPCRs are critical for mediating many physiological and pathophysiological processes and are thus located on almost every cell type. There are over 800 genes encoding GPCRs in the human genome with distinct tissue and subcellular profiles (Lin 2013). GPCRs have an overall conserved structure including an extracellular amino terminus, seven transmembrane (TM) alpha helices, an intracellular carboxy terminus, and loops (3 extracellular and 3 intracellular) connecting the 7 TM helices (Ji, Grossmann et al. 1998). Various stimuli activate GPCRs including hormones, neurotransmitters, odorants, tastants, small molecules, ions, and photons. The majority of GPCR signaling involves transducing extracellular stimulation into intracellular signaling events through G proteins. GPCR-mediated G protein signaling involves the G protein heterotrimer that consists of Gα, Gβ, and Gγ subunits (Lefkowitz 2007). Each subunit has distinctive isoforms including 16 Gα, 5 Gβ, and 14 Gγ isoforms that assemble in distinct combinations with GPCRs (Milligan and Kostenis 2006). Overall, G protein signaling can be functionally grouped into 4 families: Gαi, Gαs, Gαq, and Gα12/13. These G proteins have canonical signaling pathways regulating effector proteins, such as enzymes, ion channels, and other proteins that in turn modify various intracellular
second messengers that ultimately mediate physiological responses (Neves, Ram et al. 2002).

The molecular rearrangements of the Gα and Gβγ subunits of the heterotrimer mediate G protein signaling through GPCRs. In the absence of ligand, the GPCR is in an inactive state and the Gα subunit of the heterotrimer, which contains the guanine nucleotide binding site is bound to guanine diphosphate (GDP) forming an inactive GαGDPβγ heterotrimer complex (Bunemann, Frank et al. 2003). Ligand binding to the GPCR promotes a conformational change in the receptor that is transmitted to the Gα subunit increasing its affinity for guanine triphosphate (GTP) and promoting the exchange of GDP for GTP (Birnbaumer, Abramowitz et al. 1990). The ligand-bound, activated receptor is thus serving as a guanine exchange factor (GEF). The exchange of GDP for GTP is the rate-limiting step in activating the G protein. Next, there is a conformational change allowing the activated GαGTP subunit and the Gβγ subunits to interact with downstream effectors such as adenylyl cyclases (AC), phospholipases, ion channels, and phosphodiesterases (Fig. 1.2), (Hatcher-Solis et al. 2014). Hydrolysis of GTP to GDP by the intrinsic GTPase activity of the Gα subunit promotes a conformational rearrangement of the Gα and Gβγ subunits into the inactive GαGDPβγ heterotrimer complex (Fig. 1.1) (Li, Wright et al. 2007). GTPase activating proteins (GAPs) including regulator of G protein signaling (RGS) proteins accelerate the hydrolysis of GTP to GDP inactivating G proteins (Li, Wright et al. 2007). G protein signaling is also modulated by cholera toxin (CTX) and pertussis toxin (PTX). CTX ADP-ribosylates the Gα subunit of Gs proteins at Arg-201 disrupting intrinsic GTPase activity rendering the Gαs protein constitutively active (Aktories 2011). Whereas, PTX ADP-
ribosylates the Gα subunit of Gi proteins in the c-terminal Cys-351 moiety functionally uncoupling Gi proteins from receptors and reducing their activation (Locht, Coutte et al. 2011).
1.2 The $A_{2A}R$

Adenosine (ADO) is an endogenous nucleoside produced by the metabolism of adenosine triphosphate (ATP) and released along with glutamate from nerve terminals and astrocytes (Fuxe, Marcellino et al. 2010). ADO acts on $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptors (Ferre, Quiroz et al. 2008). These receptors are further categorized based on their ability to stimulate or inhibit the activity of AC. Both the $A_{2A}$ and $A_{2B}$ receptors couple to $G_s$ through residues in the 3rd intracellular loops of the receptors and stimulate the activity of AC. However, the $A_1$ and $A_3$ receptors couple to $G_i$ through residues in the 3rd intracellular loop and carboxy terminus of the receptors and inhibit the activity of AC (Cabello, Gandia et al. 2009). The $A_1$ and the $A_2$ receptors function as the two main adenosine receptors in the central nervous system (CNS) (Ferre, Quiroz et al. 2008). Unlike the widespread distribution of the $A_1$R throughout the brain, the $A_2$ receptor is primarily localized in the striatum and mainly concentrated in the GABAergic striatopallidal neurons (Fuxe, Marcellino et al. 2010). Ultrastructural studies suggest that $A_{2A}$Rs are localized predominantly in the post-synaptic dendrites and dendritic spines of striatal GABAergic neurons even though there is some localization on the pre-synaptic glutamatergic terminals. The predominantly post-synaptic localization of $A_{2A}R$ indicates the significant role adenosine plays in modulating inhibitory output from striatal neurons (Hettinger, Lee et al. 2001).
The $A_2A$R couples to $G_{s/olf}$ with $G_{olf}$ being the more abundant $G_s$ family G protein in the striatum. Via coupling to $G_{s/olf}$ proteins, $A_2A$R stimulates adenylyl cyclase and activates the cAMP-protein kinase A (PKA) signaling pathway. This signaling cascade results in the phosphorylation of dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32), cAMP response element binding protein (CREB), and AMPA receptors. Accumulation of cAMP leads to the increase in PKA activation which phosphorylates DARPP-32 and CREB resulting in the activation of mitogen-activated protein kinases (MAPK), which in turn leads to an increase in expression of different genes such as $c$-fos and preproenkephalin in GABAergic, enkephalinergic striatal neurons (Ferre et al. 2008, Fig.1.3).

In 2004, Canals et al. provided the first evidence of $A_2A$R homodimerization. Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and immunoblotting were employed to demonstrate that in transfected HEK-293 cells the $A_2A$R exists in monomeric and homomeric forms. Using cell surface biotinylation experiments, the authors determined that 90% of $A_2A$Rs exist as homodimers (Canals, Burgueno et al. 2004). Subsequent studies by Gandia and colleagues in 2008 using bimolecular fluorescence complementation (BiFC) provided additional evidence that $A_2A$Rs can form higher order oligomers. The ability of $A_2A$Rs to form homomers has significant implications for the subunit stoichiometry of heteromers that the $A_2A$R forms with various receptors (Gandia, Lluis et al. 2008).
1.3 The D₂R

Dopamine (DA) is essential for modulating a plethora of central nervous system (CNS) functions including mood, feeding, reward, fear, cognition, attention, arousal, and voluntary movement (Fuxe, Marcellino et al. 2010). Most dopaminergic innervation in the mammalian brain is through 4 major pathways named the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular systems. The majority of dopaminergic neurons in brain modulate fast neurotransmission mediated by GABA and glutamate. DA is the endogenous ligand for five different receptors (D₁R, D₂R, D₃R, D₄R, D₅R) (Cabello, Gandia et al. 2009) and is synthesized locally in neurons from its immediate precursor L-DOPA, which is synthesized directly from the amino acid tyrosine. DA receptors can be further subdivided as being Gₛ coupled activators of AC and D₁-like (D₁, D₅), or Gᵢ coupled inhibitors of AC and D₂-like (D₂R, D₃R, D₄R) (Hasbi, O'Dowd et al. 2011). The D₁R and D₂R are found most prominently in GABAergic striatopallidal neurons and are pivotal to motor control through the direct and indirect pathway. The D₁-like receptors are predominantly localized postsynaptically, whereas the D₂-like receptors function presynaptically as autoreceptors on dopaminergic neurons and postsynaptically on dopamine targeting neurons (Boyd and Mailman 2012).

The D₂R has two splice variants the D₂ₛR (short isoform), which functions presynaptically as an autoreceptor and the D₂ₗR (long isoform), which functions postsynaptically with important implications for motor control. These two splice variants differ by 29 amino acids in the 3\textsuperscript{rd} intracellular loop (Beaulieu and Gainetdinov 2011).
Presynaptically localized autoreceptors provide a negative feedback regulating neuronal firing, synthesis, and release of dopamine in response to changing extracellular dopamine levels. Presynaptic D2Rs function to reduce dopamine release in the synapse that ultimate leads to decreased motor activity (Boyd and Mailman 2012). However, stimulation of postsynaptic D2Rs results in increased locomotor activity. Pre- and postsynaptic D2Rs exert the aforementioned functional effects through G<sub>i/o</sub> protein signaling (Beaulieu and Gainetdinov 2011).

G protein signaling through D2Rs regulates ion channels with important implications for neuronal excitability. DA binding to D2Rs induces a conformational change activating the D2R resulting in the liberation of the Gβγ subunits from the G protein heterotrimer. Next, the liberated Gβγ subunits bind to voltage dependent calcium channels (VDCC) reducing calcium (Ca<sup>2+</sup>) influx (Ikeda 1996), (Herlitze, Garcia et al. 1996). Without Ca<sup>2+</sup> influx depolarizing the membrane, the medium spiny neuron (MSN) remains hyperpolarized near the potassium equilibrium potential. The released Gβγ subunits also bind to the amino and carboxy terminals of GIRKs potentiating their activity (Logothetis, Kurachi et al. 1987). Increased potassium efflux also hyperpolarizes neurons reducing excitability (Fig.1.4). The ability of the D2R to inhibit neuronal excitability is opposed by adenosine (ADO) activating the A<sub>2A</sub>R (Ferre, Ciruela et al. 2004).

In 2009, Han and colleagues engineered an obligate D2R homodimer to elucidate G protein signaling through the D2R homomer. Using a Gq signaling based calcium mobilization assay, an obligate D2R homodimer was engineered by expressing a wild-type (WT) D2R protomer incapable of signaling through endogenous Gq proteins in HEK
cells and a second truncated D₂R protomer fused to a chimeric Giq protein unable to signal independently due to steric hindrance, but whose signaling was rescued by the WT D₂R protomer. These obligate D₂R homodimers functioned similarly to the GABA₆R requiring both protomers to efficiently bind ligands and signal through G proteins. This study demonstrated that agonist binding to one D₂R protomer produced activation, but agonist binding to both D₂R protomers inhibited activation. Additionally, agonist binding to one D₂R protomer and an inverse agonist binding to the second D₂R protomer enhanced signaling. These studies suggest that signaling through D₂R homomers is asymmetrical with activation requiring differential conformational changes from each protomer (Han, Moreira et al. 2009).
1.4 Evidence for A2AR-D2R heteromerization

Biochemical and biophysical techniques have provided evidence that the A2AR and D2R form a functional heteromeric complex. In 2002, Hillion et al. demonstrated co-localization of the A2AR and D2R in SH-SY5Y human neuroblastoma cells stably transfected with D2R using double immunofluorescence experiments (Fig. 1.5A). Immunolabeling experiments were also conducted in neuronal primary cultures of rat striatum whereby most neurons exhibited A2AR-D2R immunoreactivity in the dendrites. In the same study, co-immunoprecipitation experiments were conducted in membrane preparations from D2R transfected SH-SY57 neuroblastoma cells and mouse fibroblast Ltk- stably transfected with D2R and transiently co-transfected with the A2AR. Western blots were also utilized to demonstrate co-localization since probing with an antibody targeting the D2R was able to pull down the A2AR-D2R heteromer out of solution (Hillion, Canals et al. 2002).

Furthermore, in 2003 Canals et al. used FRET in HEK-293T cells transfected with D2R-GFP2 and A2AR-YFP to determine if the receptors were close enough in proximity to produce energy transfer. The FRET efficiency was determined to be in the range of 23-25%, while there was significantly lower FRET efficiency in the negative control using D2R-GFP2 and CD4-YFP demonstrating the specificity in energy transfer between D2R-GFP2 and A2AR-YFP. Additionally, BRET saturation curves in HEK-293T cells co-transfected with a constant amount of A2AR-Rluc construct with increasing concentrations of the D2R-YFP plasmid displayed a positive BRET signal for the
transfer of energy. The BRET signal increased as a hyperbolic function of YFP fusion construct (Fig. 1.5B). BRET experiments were repeated in the presence of cyclodextrin to disrupt membrane rafts to preclude the notion that energy transfer was occurring in closely located receptors within plasma membrane microdomains (Canals, Burgueno et al. 2004). A negative control BRET experiment with A2AR-Rluc and GABABR2-YFP lead to an undetectable BRET signal. Moreover, undetectable energy transfer was observed in BRET saturation experiments using the A2AR and a chimeric dopamine receptor type 1 (D1R)–D2R, where the regions of the D2R known to interact with the A2AR (helices 5 and 6, intracellular loop 3) were replaced with the corresponding regions of the D1R, which does not interact with A2AR (Canals, Marcellino et al. 2003, Ferre, Ciruela et al. 2004).

In 2011, Trifilieff and colleagues used the proximity ligation assay (PLA) to detect co-localization of endogenous A2ARs and D2Rs in ex vivo preparations from mouse striatum. These studies were motivated by concerns that A2AR-D2R co-localization may not be physiologically relevant because previous studies demonstrating co-localization were performed by overexpressing the A2AR and D2R in heterologous expression systems. The advantage of PLA is that it can determine the close proximity of endogenous proteins in their native state at the single molecule level with high specificity. PLA performed on murine striatal slices produced a positive signal for co-localization of the A2AR and D2R that was absent when the same probes were used in knock out (KO) A2AR and D2R mice. These experiments were critical for demonstrating the anatomical relevance of A2AR-D2R co-localization in native tissue (Trifilieff, Rives et al. 2011).
1.5 The A_2A-R-D_2R heteromer Interface

Additional experiments have been conducted to determine the A_2A-R-D_2R heteromer interface. Canals et al. (2003) performed rigid body docking simulations on minimized structures of the A_2A-R and D_2R to determine potential A_2A-R-D_2R heteromer interfaces. The two populations of structures obtained had the amino terminal portion of the 3\textsuperscript{rd} intracellular loop of the D_2R approaching the 4\textsuperscript{th} helix and carboxy terminal portion of the A_2A-R. These structures had high docking scores and were consistent with BRET experiments involving the D_1R-D_2R chimera showing that the 3\textsuperscript{rd} intracellular loop of the D_2R is part of the heteromer interface (Canals, Marcellino et al. 2003).

Motivated by the work of Canals et al., Circuela et al. (2004) used biochemical pull down assays and mass spectrometry to demonstrate that heteromerization depends on electrostatic interactions between an arginine-rich epitope of the amino terminal segment of the 3\textsuperscript{rd} intracellular loop of the D_2R and phosphorylated Ser374 in the carboxy terminus of A_2A-R (Fig.1.6). Pull down assays were performed where a GST fusion protein containing the carboxy terminal domain of A_2A-R was able to pull down the whole D_2R solubilized from transfected HEK cells. Furthermore, a peptide of the arginine rich 3\textsuperscript{rd} intracellular loop of the D_2R was able to pull down the carboxy terminus of the A_2A-R as well as the whole A_2A-R solubilized from transfected HEK-293 cells. Mass spectrometry also showed that the arginine rich 3\textsuperscript{rd} intracellular loop of D_2R binds the carboxy terminus of the A_2A-R containing the phosphorylated serine residue (Ciruela,
Burgueno et al. 2004). Likewise, Borroto-Escuela et al. (2010) demonstrated that mutagenesis of Ser374 to alanine decreased A2A-D2R interaction (Figs. 1.6 and 1.7). BRET saturation curves in transfected cells established that A2AR-mediated inhibition of D2R agonist binding and Gi signaling was also abolished by the A2AR(Ser374) to alanine mutation. Thus, an electrostatic interaction between the arginine rich 3rd intracellular loop of the D2R and the phosphorylated Ser374 of the carboxy terminal A2AR could mediate A2AR-D2R heteromerization (Borroto-Escuela, Marcellino et al. 2010).

In 2015, Bonaventura and colleagues used FRET and PLA to demonstrate that TM 5s of the A2AR and D2R are involved in heteromerization. FRET experiments were conducted in HEK-293 cells transfected with the A2AR fused to the YFP Venus amino terminal hemiprotein and the D2R fused to the YFP Venus carboxy terminal hemiprotein. A significant fluorescent signal was obtained indicating fusion of the aminio and carboxy terminal YFP hemiproteins and A2AR-D2R heteromerization. Next, the experimenters used HIV TAT-fused interfering peptides for TM 5 and 7 of each receptor to determine the heteromer interface. Peptides for TM 5, but not TM 7 of the A2AR and D2R were able to disrupt fluorescence complementation. Using sheep striatal brain slices in a PLA, endogenous A2AR-D2R heteromerization was shown in the same study. Similar to the FRET experiments, interfering HIV TAT peptides from TMs 5 and 7 were used in the PLA and TM 5 but not TM 7 abolished the PLA signal. These findings demonstrate that TM 5 of the A2AR and D2R form the heteromer interface as these peptides disrupted fluorescence complementation and the PLA signal (Bonaventura, Navarro et al. 2015).
1.6 Reciprocal antagonistic A$_{2A}$R-D$_{2}$R interactions

The antagonistic interaction involving the ability of the A$_{2A}$R to modulate the effect of D$_{2}$Rs on ligand binding, neurotransmitter release, neuronal excitability, and motor activity is critical for striatal function (Ferre, Quiroz et al. 2008). Several different research groups have demonstrated an antagonistic A$_{2A}$-D$_{2}$R interaction using competitive radioligand-binding experiments in membrane preparations from transfected cell lines, and in human and rat striatum. In these experiments, the application of a selective A$_{2A}$R agonist diminished the capacity of a D$_{2}$R agonist to displace the binding of a discriminatory D$_{2}$R ligand (Ferre, von Euler et al. 1991, Dasgupta, Ferre et al. 1996, Kull, Ferre et al. 1999, Salim, Ferre et al. 2000, Diaz-Cabiale, Hurd et al. 2001).

Subsequent experiments established the antagonistic A$_{2A}$R-D$_{2}$R interaction whereby stimulating the A$_{2A}$R counteracts D$_{2}$R mediated inhibition of neurotransmitter release (Ferre, Quiroz et al. 2008). Ferré et al. (1993) showed the antagonistic A$_{2A}$R-D$_{2}$R interaction experimentally through in vivo microdialysis experiments whereby perfusion of a D$_{2}$R agonist into the dorsal striatum caused a decrease in the extracellular levels of the neurotransmitter GABA in the globus pallidus (Ferre, Snaprud et al. 1993). Conversely, when an A$_{2A}$R agonist was perfused, there were no direct effects, but the action of a D$_{2}$R agonist was blocked (Ferre, Snaprud et al. 1993). In the ventral striatum, D$_{2}$Rs exert a more tonic stimulation through endogenous DA on neurotransmitter release from GABAergic enkephalinergic neurons. Consequently,
ventral striatal perfusion of an A$_{2A}$R agonist increased the extracellular levels of GABA in the ventral pallidum acting as a D$_2$R antagonist (Ferre, O'Connor et al. 1994).

The ability of the A$_{2A}$R to antagonize D$_2$R mediated inhibition of neuronal firing has also been well characterized (Ferre, Quiroz et al. 2008). Striatal output is determined by the firing frequency of inhibitory GABAergic MSNs. Corticospinal inputs to the striatum depolarize GABAergic MSNs from a resting hyperpolarized membrane potential activating D$_2$Rs preventing MSNs from transitioning to a more depolarized membrane potential. Azdad et al. (2009) conducted experiments utilizing perforated patch clamp recordings from brain slices to demonstrate the role of A$_{2A}$R-D$_2$R interactions in regulating the membrane potential transitions of striatopallidal neurons. Application of a D$_2$R agonist hyperpolarized the striatal MSNs and abolished the firing (Azdad, Gall et al. 2009). Ensuing application of an A$_{2A}$R agonist failed to modify neuronal state transitions or firing frequency, but reversed the effect of the D$_2$R activation. The ability of A$_{2A}$R agonists to disinhibit neuronal firing was blocked by A$_{2A}$R antagonists. Furthermore, A$_{2A}$R ligands had no effect on neuronal firing in A$_{2A}$R KO mice (Azdad, Gall et al. 2009).

Similar to neurotransmitter release and neuronal excitability, motor activity is also governed by the antagonistic A$_{2A}$-D$_2$R interaction. Several experiments have revealed the ability of selective A$_{2A}$R ligands to offset or potentiate the motor activation created by D$_2$R agonists (Ferre, Herrera-Marschitz et al. 1991, Rimondini, Ferre et al. 1998, Ferre, Popoli et al. 2001). Ferré et al. (2009) performed behavioral experiments in neurotoxin induced DA depleted rats showing that the A$_{2A}$R agonist CGS 21680 attenuated the effects of a D$_2$R agonist quinpirole on turning behavior (Ferre, Baler et al.
Accordingly, Stromberg et al. 2000 demonstrated that an A\textsubscript{2A}R antagonist MSX-3 significantly potentiated the quinpirole induced turning behavior of DA denervated rats. These experiments argued for a specific A\textsubscript{2A}R-D\textsubscript{2}R interaction in enkephalinergic striatal neurons because MSX-3 did not significantly alter the turning behavior of rats given the D\textsubscript{1}R agonist SKF 38393 (Stromberg, Popoli et al. 2000).

Fewer studies have examined antagonism of the D\textsubscript{2}R on A\textsubscript{2A}R signaling and binding. Signaling through the A\textsubscript{2A}R results in cAMP accumulation, CREB phosphorylation and an increase in c-fos expression. Under physiological conditions, the D\textsubscript{2}Rs exhibit a steady activation that impedes the ability of A\textsubscript{2A}Rs to signal through the cAMP-PKA pathway (Konradi and Heckers 1995). Experimental demonstrations in rodent striatum show that in vivo administration of D\textsubscript{2}R antagonists produces a significant increase in the PKA dependent phosphorylation of DARPP-32 or the AMPA receptor resulting in an increase in the expression of c-fos or preproenkephalin genes. This increase in gene expression depends on the ability of the antagonist to block the D\textsubscript{2}R and release A\textsubscript{2A}R signaling stimulated by endogenous ADO (Pollack and Fink 1995). In the antagonistic A\textsubscript{2A}R-D\textsubscript{2}R interaction at the second messenger level, the D\textsubscript{2}R receptor lacks an independent effect on gene expression, but stimulation of the D\textsubscript{2}R receptor counteracts the effects of A\textsubscript{2A}R receptor stimulation (Svenningsson, Lindskog et al. 2000).

In 2013, Fernandez-Duenas and colleagues developed a novel A\textsubscript{2A}R fluorescent ligand MRS5424 and used real-time FRET along with flow cytometry to examine the effect of the D\textsubscript{2}R on A\textsubscript{2A}R agonist binding. HEK-293 cells were transfected with A\textsubscript{2A}R-CFP and D\textsubscript{2}R-AGT and co-localization of the A\textsubscript{2A}R and D\textsubscript{2}R was visualized using SNAP
tag technology. Real-time FRET experiments demonstrated that binding of the A\textsubscript{2A}R agonist MRS5424 was reduced upon perfusion of the D\textsubscript{2}R agonists pramipexole, rotigotine, and apomorphine. In cells transfected only with the A\textsubscript{2A}R, perfusion of the previously mentioned D\textsubscript{2}R agonists had no effect on MRS5424 binding. Flow cytometry was also conducted with similar results to the real-time FRET experiments where the D\textsubscript{2}R agonists pramipexole, rotigotine, and apomorphine caused a significant reduction in MRS5424 binding to the A\textsubscript{2A}R. These experiments provide evidence for agonists binding to the D\textsubscript{2}R acting as negative allosteric modulators of agonist binding to the A\textsubscript{2A}R (Fernandez-Duenas, Gomez-Soler et al. 2013).
1.7 The Basal Ganglia and the A$_{2A}$R-D$_2$R Heteromer

Essential for the planning and initiation of movement, the basal ganglia are a group of subcortical nuclei consisting of the caudate nucleus, putamen, globus pallidus, substantia nigra, and the subthalamic nucleus (STN). The purpose of the basal ganglia circuit is to process incoming signals from the cortex and to relay an output signal to the cortex through the thalamus to execute voluntary movement. Consisting of the caudate nucleus, putamen, and nucleus accumbens, the striatum is the main input structure of the basal ganglia circuit (Blandini, Nappi et al. 2000). The major neural innervations of the striatum that are relevant to our study consist of excitatory glutamatergic projections from nearly all cortical areas and dopaminergic inputs from the substantia nigra pars compacta (Fig. 1.9(McGeorge and Faull 1989, Blandini, Nappi et al. 2000)). MSNs comprise over 95% of the striatum with GABA being the main neurotransmitter of these neurons (Kemp and Powell 1971, Kita and Kitai 1988). Within the MSNs, GABA can be co-localized with either enkephalin or substance P/dynorphin. Enkephalinergic MSNs contain A$_{2A}$Rs and D$_2$R and project to the external segment of the globus pallidus; whereas, dynorphinergic MSNs contain A$_1$Rs and D1Rs and project to the internal segment of the globus pallidus (Fig. 1.9). The striatal organization of enkephalinergic and dynorphinergic MSNs form the basis for the direct and indirect motor pathways (Blandini, Nappi et al. 2000).

Two main striatal motor pathways regulate the output from the basal ganglia: the direct and indirect pathways. We will focus on the indirect pathway since it involves the
A₂A-R-D₂R heteromer and is implicated in PD (Kull, Ferre et al. 1999). Under physiological conditions, striatal MSNs expressing the A₂A-R-D₂R heteromer receive dopaminergic input from the substantia nigra pars compacta. In the striatum, D₂R signaling inhibits neuronal excitability and reduces inhibitory GABAergic output to the external segment of the globus pallidus (Gpe) (Lang and Lozano 1998, Blandini, Nappi et al. 2000). Through heteromerization, the A₂A-R is critical in modulating neuronal excitability and GABA release by counterbalancing D₂R signaling. Next, the tonically active inhibitory neurons of the Gpe relay to provide tonic, inhibitory GABAergic projections to the STN. Then, the STN conducts transient, glutamatergic excitatory output to the internal segment of the globus pallidus (Gpi). The Gpi subsequently sends a tonic inhibition to the ventrolateral thalamus. Consequently, the ventrolateral thalamus sends a transient excitatory projection to the motor cortex to initiate movement (Fig. 1.8 left, (Lang and Lozano 1998)). Overall, the signaling balance through the A₂A-R-D₂R heteromeric complex is a critical component of the indirect motor pathway regulating the neuronal excitability of striatal GABAergic MSNs.
1.8 The $A_{2A}R$-$D_{2}R$ Heteromer and the significance for Parkinson’s disease

Discovered by James Parkinson in 1817, PD is the second most common neurodegenerative disease affecting 1-2% of people over the age of 60. For most patients, there is a slow progression of the disease between the ages of 50 and 70 followed by death 10 to 20 years later (Lang and Lozano 1998). The etiology of PD remains unclear, but considerable research has led to the notion that genetic predisposition and exposure to pesticides contributes to disease formation. The hallmark of PD is the formation of Lewy bodies (aggregates of $\alpha$-synuclein) and the progressive deterioration of dopaminergic neurons in the substantia nigra pars compacta (Lozano, Lang et al. 1998). After loss of approximately 80% of dopaminergic neurons in the substantia nigra, Parkinsonian symptoms appear including a decrease in spontaneous movement, postural instability, bradykinesia, rigidity, and a resting tremor (Lang and Lozano 1998). These defects in motor function are attributable to increased inhibitory output from the basal ganglia causing decreased thalamic activation of the motor cortex (Lang and Lozano 1998).

In cases of PD, decreased homomeric dopaminergic signaling through the $D_{2}R$ along with antagonism of the $A_{2A}R$ on the residual $D_{2}R$ signaling will increase inhibitory GABAergic projections from the basal ganglia to the ventrolateral thalamus thus diminishing excitatory thalamic output to the motor cortex (Fig.1.8 right, (Kull, Ferre et al. 1999)). The excessive inhibition of thalamocortical projections causes Parkinsonian
symptoms. Currently, there is no cure for PD, but D₂R agonists and A₂A R antagonists are used clinically to manage motor dysfunction (Jenner 2003).
1.9 Antiparkinsonian therapeutics targeting the A$_{2A}$R-D$_2$R Heteromer

Symptomatic management of PD is based on using D$_2$R agonists to mimic DA mediated neurotransmission in the nigrostriatal pathway. Levodopa (L-DOPA), the immediate precursor of DA, is highly effective in managing the early stages of PD (Jenner 2003). However, adverse side effects caused by D$_2$R agonists include nausea, vomiting and hypotension. Chronic side effects include dyskinesia, psychosis and loss of drug efficacy. Furthermore, D$_2$R agonists do not alleviate the Parkinsonian symptom of postural instability or address the neurodegeneration responsible for the advent of motor dysfunction (Jenner 2003). In order to circumvent the problems associated with D$_2$R agonists, researchers have explored the use of A$_{2A}$R antagonists based on the well-characterized antagonistic interaction between the A$_{2A}$R and the D$_2$R.

A$_{2A}$R antagonists that inhibit postsynaptic A$_{2A}$Rs in enkephalinergic MSNs would be beneficial for the treatment of PD through inhibiting the indirect motor pathway and increasing motor activity. However, A$_{2A}$R antagonists that inhibit presynaptic A$_{2A}$Rs that contact dynorphinergic MSNs would decrease glutamate release in the direct motor pathway inhibiting motor activity. Thus, antiparkinsonian therapeutics targeting the A$_{2A}$R should have a higher affinity for postsynaptic versus presynaptic A$_{2A}$Rs. In 2011, Orrú and colleagues used radioligand-binding experiments in stable cell lines containing A$_{2A}$Rs, A$_{2A}$R-D$_2$R heteromers (postsynaptic A$_{2A}$Rs), and A$_{2A}$R-A$_1$R heteromers (presynaptic A$_{2A}$Rs). Among the A$_{2A}$R antagonists tested, SCH 442416 had the lowest
affinity in cell lines containing A\textsubscript{2A}R-D\textsubscript{2}R heteromers whereas istradefylline (KW-6002) had the highest relative affinity in cells containing A\textsubscript{2A}R-D\textsubscript{2}R heteromers. Additional studies demonstrated that KW-6002 reduced motor impairment in neurotoxin induced Parkinsonism animal models (Orru, Bakesova et al. 2011). Therefore, KW-6002 was examined as a promising antiparkinsonian therapeutic as it targets postsynaptic A\textsubscript{2A}R-D\textsubscript{2}R heteromers increasing motor activity.

The majority of information available on the effectiveness of A\textsubscript{2A}R antagonists in PD patients was collected in clinical trials with KW-6002. Phase IIa clinical trials with KW-6002 suggest that the drug has antiparkinsonian actions significantly reducing motor deficits in advanced PD patients. According to the study, KW-6002 had no effect on Parkinsonian symptoms when solely administered. However, when combined with a low dose of L-DOPA, patients experienced improved motor function comparable to an optimal dose of L-DOPA alone. Combining KW-6002 with L-DOPA reduced side effects with patients experiencing milder dyskinesia, dizziness, insomnia and nausea. Only 5% of patients experienced increased anxiety. When KW-6002 was co-administered with L-DOPA, the half-life of L-DOPA was prolonged (Chen, Wang et al. 2013). Despite these promising results, KW-6002 did not receive FDA approval in 2008, but it ignited further studies into A\textsubscript{2A}R antagonists as antiparkinsonian drugs (Armentero et al. 2011). In March of 2013, KW-6002 became the first A\textsubscript{2A}R antagonist approved for treatment of PD in Japan. As of 2014, clinical trials were in progress for the following A\textsubscript{2A}R antagonists: phase I for PBS-509, ST1535, ST4206 and V81444; phase II for tozadenant. Learning more about signaling through the A\textsubscript{2A}R-D\textsubscript{2}R heteromeric complex
will facilitate the development of novel and more efficacious medications for PD (Armentero, Pinna et al. 2011).
1.10 Conclusion

Homomeric G protein receptor signaling has been characterized for many years, but recent data suggest that many GPCRs form oligomeric complexes with distinct signaling properties. The effect of heteromerization on the pharmacology and signaling of many GPCR homomers is still not well understood. Thus, GPCR heteromers provide new therapeutic targets and research into the signaling of heteromers could provide novel therapeutics for important diseases. The $G_{\alpha_S/olf}$ coupled A$_2$AR and the $G_{\alpha_i/o}$ coupled D$_2$R are two distinct GPCRs that heteromerize in the dorsal and ventral striatopallidal GABA pathway (Ferre, Quiroz et al. 2008). The physiological relevance of the A$_2$AR-D$_2$R heteromer is based on receptor antagonism. A$_2$ARs antagonize the inhibitory role of D$_2$Rs on neurotransmitter release, neuronal firing, and locomotor activity. There is exaggerated antagonism of the D$_2$R by the A$_2$AR in Parkinson’s disease patients due to decreased dopaminergic tone in the striatum (Kull, Ferre et al. 1999). Therefore, A$_2$AR antagonists are being explored as possible therapeutics for Parkinson’s disease because they alleviate the A$_2$AR inhibition of the D$_2$R (Ferre, Ciruela et al. 2007). D$_2$Rs also antagonize A$_2$AR agonist binding and at the second messenger level. The A$_2$AR-D$_2$R heteromer is an emerging therapeutic target for dysfunctional dopaminergic signaling and studies examining G protein signaling through the heteromer could yield important insight for drug development.
1.11 Hypothesis

Based on previous studies elucidating reciprocal antagonistic A$_{2A}$R-D$_2$R interactions, we hypothesized that A$_{2A}$R-D$_2$R heteromerization would reduce both D$_2$R-mediated G$_i$ signaling and A$_{2A}$R-mediated G$_s$ signaling. To test our hypothesis, we studied A$_{2A}$R-D$_2$R heteromer cross signaling by expressing the GPCRs as well as reporter channels using the Xenopus oocyte heterologous expression system. Our electrophysiological assay is innovative in that it will allow us to control the conditions of heteromerization to tease apart the differences from homomeric or monomeric signaling. By monitoring the current through the reporter channel, we found that A$_{2A}$R-D$_2$R heteromerization inhibited both D$_2$R-mediated G$_i$ signaling and A$_{2A}$R-mediated G$_s$ signaling even when the adjacent receptor was unliganded and perfusion of specific ligand combinations targeting the A$_{2A}$R-D$_2$R heteromer induced crosstalk and modulated this inhibition. After completing our in vitro experiments in Xenopus oocytes, we transitioned to an in vivo system utilizing a novel optogenetics-microdialysis assay and examined the effects of A$_{2A}$R antagonists on striatal glutamatergic release. We demonstrated that A$_{2A}$R antagonists with a preferred presynaptic profile were able to inhibit the release of glutamate; whereas, A$_{2A}$R antagonists with a preferred postsynaptic profile targeting the A$_{2A}$R-D$_2$R heteromer were not able to inhibit striatal glutamate release.
Figure 1.1 The G protein cycle. Ligand binding to the GPCR promotes a conformational change in the receptor that is transmitted to the Gα subunit increasing its affinity for guanine triphosphate (GTP) and promoting the exchange of GDP for GTP. Next, the activated Gα subunit dissociates from the Gβγ subunits allowing interactions with downstream effectors. Hydrolysis of GTP to GDP by the intrinsic or stimulated GTPase activity of the Gα subunit promotes reassociation of the Gα and Gβγ subunits into the inactive heterotrimer complex. Adopted from Li et al., 2007.
Figure 1.2 $G_{i/o}$- and $G_s$-coupled Receptor Signaling. Receptor stimulation results in the liberation of $G_{\beta\gamma}$ subunits from the G protein heterotrimer that are free to bind to nearby GIRKs potentiating their current. Adapted from Hatcher-Solis et al. 2014.
Figure 1.3 $A_{2A}$R Signaling. Diagram showing the predominantly postsynaptic localization of $A_{2A}$Rs along with $A_{2A}$R signaling.
Figure 1.4 D₂R Localization and Signaling. Diagram showing the pre- and postsynaptic localization of D₂Rs along with D₂R signaling affecting neuronal excitability.
Figure 1.5 A2AR and D2R Co-localization. (A) SH-SY5Y human neuroblastoma cells stably transfected with D2R (a) A2AR immunoreactivity, (b) D2R immunoreactivity, and (c) A2AR-D2R colocalization. Adapted from Hillion et al. 2002. (B) Competition BRET saturation curve of HEK-293 cell co-transfected with A2AR and D2R. A2AR-Rluc and D2R-YFP (squares) or A2AR-Rluc and GABA$_B$R2-YFP (triangle). Adapted from Canals et al. 2003.
Figure 1.6 The $\text{A}_{2\text{A}}\text{R}$-$\text{D}_{2\text{R}}$ Heteromer Interface. (A) WT $\text{D}_{2\text{R}}$ (red) with arginine rich 3\textsuperscript{rd} intracellular loop. $\text{D}_{2\text{R}}$ (blue) with arginine to alanine mutations in the 3\textsuperscript{rd} intracellular loop. (B) BRET saturation curve for $\text{A}_{2\text{A}}\text{R}$-$\text{D}_{2\text{R}}$ (red) and $\text{A}_{2\text{A}}\text{R}$-$\text{D}_{2\text{R}}$(R-A) (blue). Adapted from Circuela et al. 2004.
Figure 1.7 The A2A/R-D2R Heteromer Interface Continued. (A) Snake plot of the WT A2AR. (B) BRET saturation curve of the D2R-Rluc and A2AR-YFP (red), D2R-Rluc and A2AR-YFP(S374A, grey), D2R-Rluc and A2AR(DD)-YFP (white), and D2R-Rluc and A2AR(SDD)-YFP (black). Adapted from Escuela et al. 2010.
Figure 1.8 The Indirect Motor Pathway. Diagram of the indirect motor pathway (left) and the alterations in signaling that occur during PD (right).
Figure 1.9  Pre- and Postsynaptic Localization of $A_{2A}$Rs and $D_2$Rs. $A_{2A}$Rs are co-localized with $D_2$Rs postsynaptically in the dendritic spines of enkephalinergic medium spiny neurons in the striatum. $A_{2A}$Rs are also co-localized with $A_1$Rs presynaptically in glutamatergic afferents to enkephalinergic and dynorphynergic medium spiny neurons. $D_2$Rs are similarly located presynaptically in glutamatergic and dopaminergic afferents projecting to enkephalinergic and dynorphynergic medium spiny neurons. Adapted from Ferre 2008.
Chapter 2 MATERIALS AND METHODS

2.1 Molecular Biology

The following cDNAs used in the *Xenopus* oocyte heterologous expression system were subcloned into the pXOOM oocyte expression vector: mD_{2}R, 3HA-hA_{2A}R, m-PHOS, D_{2}R(9A), A_{2A}R(S374A), Kir 3.2, and βARK. Previous studies in the lab provided the following cDNAs subcloned into the pGEMHE oocyte expression vector Kir 3.1, Gαs, and PTX. Linearized and purified (Pure Link PCR Purification kit, Thermo Fisher Scientific) plasmids were transcribed using an mMESSAGE mMACHINE T7 Transcription kit (Thermo Fisher Scientific). Mutations were performed using a standard Pfu-based mutagenesis protocol and were verified using sequencing (Genewiz).

2.2 Reagents

CGS 21680, KW-6002, SCH44216, N^{6}-cyclopentyladenosine, and paliperidone were purchased from Tocris. Dopamine, amisulpride, adenosine, and quinpirole were purchased from Sigma. SCH44216 used in the microdialysis experiments was kindly provided by the CHDI foundation. CGS 21680, KW-6002, and SCH44216 were dissolved in DMSO at a dilution that was less than 1% DMSO. Dopamine, amisulpride, paliperidone, adenosine, N^{6}-cyclopentyladenosine, and quinpirole were dissolved in
water. SCH44216 used in the microdialysis experiments was dissolved in a mixture of tween-80 detergent and DMSO.

2.3 Oocyte preparation and injection

*Xenopus laevis* (African clawed frog) colonies (*Xenopus* Express) were maintained at the Virginia Commonwealth University according to IACUC approved protocols. The *Xenopus laevis* surgeries conducted in this study were approved by protocol AD20112: Heterologous Expression Studies in *Xenopus laevis* Oocytes. Briefly, *Xenopus* were anesthetized with a buffered Tricaine solution and ovarian tissue was excised unilaterally. Excised oocytes were defolliculated using a collagenase solution following standard protocols (Logothetis, Movahedi et al. 1992). Oocytes were then washed and incubated at 18°C in an OR2 solution with 2 mM Ca²⁺ and Penicillin/Streptomycin antibiotics. Oocytes at stage V or VI of maturation were selected for microinjection of 0.5-2 ng of cRNA suspended in DEPC treated water. Injected oocytes were incubated for 2-3 days at 18°C to allow for optimal protein expression.

2.4 Two-electrode voltage-clamp

Borosilicate glass tubing was pulled using a Flaming-Brown micropipette puller. The micropipettes were then back filled with a 3 M KCl solution that was 1.4% agarose. Acceptable resistances for the micropipettes were between .1 and 1 MΩ. Two-electrode voltage-clamp whole-cell current recordings for the Gi and Gs assay were performed using a GeneClamp 500 amplifier (Axon Laboratories). Oocytes were held at 0 mV (Ek) and the current was monitored using a 800 ms ramp protocol with a command potential
from -80 mV to +80 mV to assess the inward and outward currents. Basal current through the GIRK channel was measured in a ND96K solution (HK, 96 mM KCl, 10 mM HEPES-K, 1 mM MgCl2, and 1.8 mM CaCl2. 3 mM Barium Chloride (Ba^{2+}) in HK was used to block the GIRK current. Only the Barium-sensitive current was used for the statistical analysis. Each data set consisted of 5-10 recordings, each from separate oocytes. Experiments were repeated using oocytes from at least 2 different frogs.

2.5 Experimental Mice

WT C57BL/6 mice (Charles River Laboratories) were housed four per cage and kept on a 12 h light/dark cycle with food and water available ad libitum. All mice used in this study were maintained in accordance with the guidelines of the National Institutes of Health Animal Care and the mouse research conducted to perform this study was approved by the NIDA IRP Animal Care and Use Committee protocol no. 12-BNRN-73.

2.6 Craniotomy surgical procedures

For all craniotomy surgical procedures, mice were anesthetized with a combination of ketamine and xylazine (NIDA Pharmacy) and placed in a stereotaxic apparatus. For optogenetic-microdialysis experiments, mice received unilateral injections of adeno-associated virus (AAV) encoding channel rhodopsin 2 (ChR2) fused to enhanced yellow fluorescent protein (EYFP) under control of the CaMKII neuronal promoter (AAV-CaMKIIa-hChR2(H134R)-EYFP; University of North Carolina core vector facility) in the motor cortex. The coordinates of virus injection were 1.9 mm anterior, 1.6 mm lateral, and 1.8 mm ventral with respect to bregma. See Quiroz, Orru
et al. 2016 for detailed a protocol of virus injection. Approximately, 4 weeks after virus injection a modified microdialysis probe with an embedded light-guiding optic fiber (Quiroz, Orru et al. 2016) was implanted in the striatum. The coordinates for probe implantation were 0.3 mm posterior, 2.7 mm lateral and 4.2 mm ventral with respect to bregma. The optogenetic microdialysis probes were fixed to the skull with dental cement and acrylic resin.

2.7 Optogenetic stimulation

Optical stimulation was delivered by coupling the light guiding port of the implanted optogenetic-microdialysis probe to a 473 nm solid-state laser module driven by an electrical stimulator (Gross S88 Stimulator). Light was administered over a 20 min period in 160 ms trains of 2 ms pulses at 100 Hz with a light intensity of 5-8 mW at the probe tip. The light intensity delivered through the probe was measured using an integrating sphere silicon photodiode power sensor designed for optical power measurements independent of beam shape and divergence (model S144C, Thor labs).

2.8 In vivo Microdialysis

Microdialysis sampling was performed with optogenetic stimulation to analyze the extracellular concentration of glutamate in the striatum of freely moving mice 48 hrs. after probe implantation. An artificial cerebrospinal fluid (aCSF) containing the following in mM (144 NaCl, 4.8 KCl, 1.7 CaCl2 and 1.2 MgCl2) was perfused through the optogenetic-microdialysis probe for the entire duration of the experiment. Perfusion was at a constant rate of 0.5 µL/min and drugs were delivered through intraperitoneal (i.p.)
injection. Sampling was obtained manually at 10 min intervals for 180 minutes. The first 60 min were to examine basal glutamate measurements and the final 120 were to examine extracellular glutamate release in response to drug administration followed by 20 min of optogenetic stimulation. Extracellular glutamate was measured by HPLC coupled to a glutamate oxidase enzyme reactor and electrochemical detector (Eicom). After the microdialysis experiments, the mice were anesthetized with Equithesin (NIDA pharmacy) and perfused transcardially with 0.1 M PBS pH 7.4 followed by 4% formaldehyde in 0.1 M PBS. Brains were stored in the same fixative for 2 hrs and then stored in 30% sucrose in 0.1 M PBS for 48 hrs at 4°C. Brains were then sliced in 40 µm coronal sections using a Leica CM3050S cryostat at -20°C. The brain slices were collected in PBS and stored in antifreeze-buffered solution (20% ethylene glycol, 10% glycerol, and 10% sucrose in PBS) at -80°C until processing. Slices were imaged using confocal fluorescence microscopy acquired with a Zeiss microscope (Examiner Z1) fitted with a confocal laser module (LSM-710, Zeiss).

2.9 Erk 1/2 Immunohistochemistry

Fixed brain slices (see previous section) were stained according to the protocol detailed in Quiroz-Molina et al. 2015. Briefly, the brain slices were rinsed in PBS and incubated in blocking buffer before incubation with rabbit polyclonal anti-phospho-Thr202/Tyr204 ERK1/2 antibody in blocking buffer. Sections were then washed and incubated in biotinylated goat anti-rabbit antibody. The samples were subsequently washed and incubated in ABC reagent and washed again before treatment with DAB, ammonium nickel sulfate, and hydrogen peroxide until development. The samples were
then mounted onto treated glass slides, dehydrated, cleared in xylene, and coverslipped with DPX. Images were acquired using confocal microscopy (see previous section).

2.10 Statistics

All data were expressed as mean ± standard error of the mean (SEM), unless otherwise indicated. Error bars in each figure represent standard error of the mean (SEM). In the oocyte experiments, statistical significance between 2 groups was assessed using an unpaired t-test with an assumption of unequal variance (OriginLab). When multiple groups were analyzed, a one-way ANOVA followed by Tukey’s posthoc test was used (OriginLab). Gi and Gs activities were determined by normalizing the drug-induced current to the basal GIRK current in HK and subtracting out the barium insensitive current. For the microdialysis experiments, extracellular glutamate measurements were normalized to the basal measurements before optogenetic stimulation and drug administration, n=3-6. When multiple groups were analyzed, a one-way ANOVA followed by a Tukey’s posthoc test was used (Prism). The optogenetics-microdialysis experiments were analyzed using a two-way repeated-measures ANOVA followed by a Tukey’s posthoc test (Prism). For all statistical analyses, significance was determined using p<0.05.
Chapter 3  THE EFFECT OF A2AR-D2R HETEROMERIZATION ON G\textsubscript{i} SIGNALING THROUGH THE D2R

3.1 Introduction

The G\textsubscript{i}-coupled D2R and the G\textsubscript{s}-coupled A2AR are co-localized in the striatum on GABAergic MSNs (Fuxe, Ferre et al. 2007). Biochemical pull down assays and biophysical BRET and FRET techniques have provided evidence that the A2AR and D2R form a functional heteromeric complex (Canals, Marcellino et al. 2003). Previous studies determined that the heteromer interface depends on electrostatic interactions between an arginine rich region in the third intracellular loop of the D2R and a phosphorylated serine (Ser374) in the carboxy terminus of the A2AR (Borroto-Escuela, Marcellino et al. 2010). Homomeric D2R and A2AR receptor signaling cascades have been well established, but the implications of receptor heteromerization are not well understood. In MSNs, homomeric D2R signaling through the G\textsubscript{i/o} protein inhibits neuronal excitability. In contrast, the G\textsubscript{s/olf} protein-coupled A2AR is critical in modulating the excitation of striatal neurons by antagonizing the D2R through the formation of a higher order oligomer (Fuxe, Ferre et al. 2007). Thus, through heteromer crosstalk A2AR agonists act as D2R antagonists exerting an excitatory role on the function of MSNs (Ferre, Quiroz et al. 2008). This receptor-mediated antagonism has important implications for the indirect motor pathway circuitry in the basal ganglia. In cases of
Parkinson’s disease, decreased dopaminergic signaling through the D₂R along with antagonism of the A₂AR on residual D₂R signaling through heteromerization increases inhibitory GABAergic projections from the basal ganglia to the ventrolateral thalamus, disrupting excitatory thalamic output to the motor cortex. Thus, A₂AR antagonists are being explored in clinical trials as antiparkinsonian therapeutics to alleviate A₂AR-mediated inhibition on D₂R signaling (Quiroz et al. 2009) The A₂AR-D₂R heteromer is an emerging target for Parkinson’s disease pharmacotherapy and understanding the signaling balance through the heteromer is paramount.

In this study, we examine the effect of A₂AR-D₂R heteromerization on the pharmacology of D₂R signaling. Previous studies lead us to postulate that the A₂AR and D₂R receptor are inversely coupled through heteromeric assembly similar to the serotonin 2A- metabotropic glutamate type 2 receptor heteromer, which was previously characterized by our lab (Fribourg, Moreno et al. 2011). Therefore, we hypothesize A₂AR-D₂R heteromerization induces reciprocal antagonism whereby A₂AR receptor stimulation inhibits D₂R signaling making the combination of D₂R agonists and A₂AR antagonists or inverse agonists promising new therapeutics for Parkinson’s disease because they maximize dopaminergic signaling. In order to test this hypothesis, we have developed electrophysiological assays to study A₂AR-D₂R heteromer cross signaling by expressing the GPCRs together with reporter channels in the Xenopus oocyte heterologous expression system. Our electrophysiological assay is innovative in that it allows us to control the conditions of heteromerization and tease apart the differences from protomer signaling. By monitoring the current through the reporter channel, we anticipate perfusion of specific ligand combinations targeting the A₂AR-D₂R
heteromer will maximize GPCR signaling versus individual drug administration targeting $A_{2\alpha}$Rs or $D_2$Rs.
3.2 Results

G protein-sensitive inwardly rectifying K⁺ (GIRK) channels can serve as reporters for Gᵢ signaling through the D₂R and Gₛ signaling through the A₂AR (Hatcher-Solis, Fribourg et al. 2014). We chose the GIRK1/2 (G1/2) channel heterotetramer as a reporter for G protein signaling because it is co-localized with the A₂AR and D₂R on the postsynaptic site of dopaminergic MSNs. The G1/2 channel can be used as a reporter for Gᵢ or Gₛ signaling because GPCR activation results in liberation of the beta gamma subunits (Gβγ) from the G protein heterotrimer that bind to a cleft formed by cytosolic loops of adjacent subunits of the channel, potentiating its current (Mahajan, Ha et al. 2013). For our electrophysiological assays, we used *Xenopus laevis* oocytes as our heterologous expression system and conducted two-electrode voltage-clamp recordings using a voltage ramp protocol form -80 mV to +80 mV two days following cRNA injection.

Figure 3.1 establishes G1/2 as a suitable reporter for D₂R signaling. Basal G1/2 current in a high potassium (HK) buffer is not sensitive to 1 μM dopamine (DA) without D₂R co-expression (Fig. 3.1A). However, when G1/2 is co-expressed with the D₂R, there is a robust inward DA-elicited current (Fig.3.1B). The DA-elicited current is Gᵢ-mediated as it is blocked by co-expression of pertussis toxin (PTX, Fig. 3.1C). PTX abrogates Gᵢ signaling by catalyzing the ADP-ribosylation of the Gᵢα subunit, physically uncoupling the G protein from the GPCR (Hatcher-Solis, Fribourg et al. 2014). The DA-
elicited current is also Gβγ-mediated as the current is attenuated by co-expression of the pleckstrin homology domain of the beta adrenergic receptor kinase (βARK), a well characterized Gβγ scavenger (Fig. 3.1D). Since βARK has been implicated to bind PIP2 and also GIRK channels directly, we used an additional Gβγ scavenger, myristoylated phosducin (Fig. 3.1E) (Hatcher-Solis, Fribourg et al. 2014). Figure 3.1F is a summary figure establishing the DA-elicited current (-17.23 ± 2.35 µA) as significantly different from the G1/2 basal current (-7.32 ± 0.43 µA). Paired t-tests were performed to compare “Basal” to “Basal + Agonist” currents in the five different expression groups. Significant effects were noted in the “G1/G2 + D2R” expression group, p=0.005. Only the Barium (Ba2+) -sensitive current was analyzed for figure 3.1F as Ba2+ is a blocker of GIRK currents. After characterizing homomeric D2R signaling, we pursued an electrophysiological assay to assess the effect of A2AR-D2R heteromerization on homomeric D2R signaling.

After characterizing Gi signaling through the D2R, we tested the effect of heteromerization. A2ARs and D2Rs are known to have two types of interactions: 1) direct receptor-receptor interactions or heteromerization and 2) downstream signaling from separate populations of homomers converging on effector molecules (Ferre, Agnati et al. 2007). When assessing the effect of A2AR-D2R heteromerization, we blocked the downstream signaling of the adjacent GPCR so changes in homomeric signaling should result from heteromerization. Thus, while examining D2R-mediated signaling through the A2AR-D2R heteromer, we did not co-express Gαs to affect A2AR signaling (Lim, Dascal et al. 1995).
To test the effect of heteromerization on G\(_i\) signaling through the D\(_2\)R, different D\(_2\)R:A\(_{2A}\)R cRNA ratios were used to achieve the level of protein expression for maximal signaling through heteromerization. Downstream A\(_{2A}\)R signaling was blocked by not overexpressing G\(_{\alpha S}\) since the endogenous level of Xenopus G\(_{\alpha S}\) is not sufficient to elicit signaling through the A\(_{2A}\)R (Lim, Dascal et al. 1995). Compared to G\(_i\) signaling through the D\(_2\)R (Fig. 3.2A), there is decreased D\(_2\)R signaling with A\(_{2A}\)R co-expression (D\(_2\)R:A\(_{2A}\)R) in the following ratios: 1:1 (Fig. 3.2B), 1:2 (Fig. 3.2C), and 2:1 (Fig. 3.2D).

Figure 3.2E is a summary figure of the normalized Ba\(^{2+}\)-sensitive, DA-induced current showing A\(_{2A}\)R co-expression significantly reduces G\(_i\) signaling through the D\(_2\)R in all ratios tested. Paired t-tests with a Bonferroni correction were performed to compare normalized G\(_i\) activity with and without A\(_{2A}\)R co-expression (1:0, 2.84 ± 0.14). Significant effects were noted in all the D\(_2\)R:A\(_{2A}\)R expression groups: 1:1 (2.26 ± 0.12), 1:2 (2.06 ± 0.09), and 2:1 (1.95 ± 0.10), p<0.05. In summary, we have established an electrophysiological assay demonstrating that the unliganded A\(_{2A}\)R is a negative allosteric modulator for D\(_2\)R signaling.

Next, we evaluated whether A\(_{2A}\)R ligands could crosstalk to G\(_i\) signaling through the D\(_2\)R. The ratio of D\(_2\)R:A\(_{2A}\)R used was 2:3 (0.5 ng to 0.75 ng) because previous studies established that this ratio is most similar to that seen in the striatum. D\(_2\)R signaling (Figs. 3.3A and 3.4A) and the effect of A\(_{2A}\)R co-expression (Figs. 3.3B and 3.4B) were repeated to compare G\(_i\) activity within the same experiment. The A\(_{2A}\)R agonist CGS 21680 partially removed the inhibition of the unliganded A\(_{2A}\)R on the D\(_2\)R, but did not restore D\(_2\)R signaling to the level observed when the D\(_2\)R is expressed alone (Figs. 3.3C and 3.4C). Perfusion of the A\(_{2A}\)R antagonists KW-6002 (Fig. 3.3D) and ZM
241385 (Fig. 3.4D) resulted in $G_i$ signaling that was not significantly different from that displayed with the $D_2R$. Interestingly, the combination of CGS 21680 and the $A_{2AR}$ antagonists KW-6002 (3.3E) and ZM 241385 (Fig. 3.4E) resulted in $G_i$ signaling through the $D_2R$ that was not significantly different from $G_i$ signaling though the $D_2R$ protomer. Figures 3.3 and 3.4 demonstrate that the addition of $A_{2AR}$ ligands can further modulate the inhibition of the unliganded $A_{2AR}$ on $D_2R$ signaling.

After evaluating $A_{2AR}$-$D_2R$ crosstalk, we sought to determine if the allosteric modulation of the $A_{2AR}$ on the $D_2R$ was due to heteromerization; therefore, we mutated the heteromer interface (Fig. 3.5) and tested the $A_{2AR}$ ligands on homomeric $D_2R$ signaling (Fig. 3.7). We utilized $A_{2AR}(S374A)$ to disrupt heteromerization and test if this mutant affected the ability of $A_{2AR}$ ligands to crosstalk to the $D_2R$. In all ligand combinations tested, co-expressing $A_{2AR}(S374A)$ with the WT $D_2R$ abrogated the effect of the $A_{2AR}$ ligands CGS 21680 (Fig. 3.5C), ZM 241385, (Fig. 3.5D) KW-6002 (Fig. 3.5E), CGS 21680 + ZM 241385 (Fig. 3.5F), and CGS 21680 + KW-6002 (Fig. 3.5G) on $D_2R$ signaling. In figure 3.6, the $A_{2AR}$ mutant $A_{2AR}(S374A)$ (3.6B) was shown to have $G_i$ signaling that was not significantly different from the WT $A_{2AR}$ (Fig. 3.6A). Thus, the difference in function between $A_{2AR}(S374)$ and $A_{2AR}$ was not the reason for the termination of the $A_{2AR}$-mediated effects on $D_2R$ signaling. We also tested all of the $A_{2AR}$ ligands used in previous figures to assess if these ligands exerted independent effects on $D_2R$ signaling. Compared to dopamine-elicited $D_2R$ signaling, neither CGS 21680, ZM 241385, nor KW-6002 had any effect on $D_2R$ signaling. Furthermore, these ligands did not have any effect on basal $D_2R$ signaling. Figures 3.5, 3.6, and 3.7 provide
additional evidence that the antagonism of A$_{2A}$R ligands on D$_2$R signaling was due to heteromerization.
3.3 Discussion

GPCR heteromers are emerging therapeutic targets for a plethora of diseases due to cell-specific localization and distinct pharmacology from their homomeric or monomeric counterparts (Ferre, Quiroz et al. 2008). The A2AR-D2R heteromer is of interest since the reciprocal antagonistic interaction can be exploited to develop novel antiparkinsonian therapeutics (Ferre, Agnati et al. 2007). Signaling through the D2R inhibits the neuronal excitability of striatal enkephalinergic MSNs allowing normal circuitry through the indirect motor pathway to the motor cortex resulting in the initiation of movement. Through heteromerization, the A2AR antagonizes D2R signaling modulating neuronal excitability and GABA release. In cases of Parkinson’s disease, decreased homomeric D2R signaling combined with heteromer-mediated A2AR antagonism on residual dopaminergic signaling increases inhibitory GABAergic projections from the basal ganglia to the ventrolateral thalamus disrupting thalamic output to the motor cortex to initiate movement (Kull, Ferre et al. 1999). Consequently, the A2AR antagonists istradefylline (KW-6002), vipadenant, and tozadenant have been explored for Parkinson’s disease pharmacotherapy in clinical trials (Jenner 2003). Therefore, characterization of A2AR-D2R heteromer signaling could elucidate the molecular mechanism for A2AR antagonists potentiating D2R signaling and being efficacious therapeutics for Parkinson’s disease.
In the current study, we have developed electrophysiological assays to characterize the effect of $\text{A}_2\text{A}-\text{D}_2\text{R}$ heteromerization on signaling through the $\text{D}_2\text{R}$. Two-electrode voltage-clamp was used to measure current through the GIRK1/2 channel, which served as a reporter for G protein signaling through the $\text{D}_2\text{R}$, or $\text{A}_2\text{A}-\text{D}_2\text{R}$ heteromer in the *Xenopus laevis* oocyte heterologous expression system. Our results demonstrate GIRK channels can serve as reporters for $\text{G}_i$-coupled signaling through the $\text{D}_2\text{R}$ (Fig. 3.1). Previous studies revealed $\text{A}_2\text{A}$ antagonism of $\text{D}_2\text{R}$ signaling effects on neurotransmitter release, neural firing, and behavior (Agnati, Ferre et al. 2003, Ferre, Agnati et al. 2007, Azdad, Gall et al. 2009). Consistent with previous findings, our work demonstrates that mere co-injection of the $\text{A}_2\text{A}$ reduces $\text{D}_2\text{R}$ signaling (Fig. 3.2). Our study is the first to demonstrate that the unliganded $\text{A}_2\text{A}$ antagonizes $\text{G}_i$ signaling through the $\text{D}_2\text{R}$. Furthermore, the addition of the $\text{A}_2\text{A}$ ligand agonist CGS 21680 in combination with dopamine relieved the inhibition of the unliganded $\text{A}_2\text{A}$ on $\text{D}_2\text{R}$ signaling even though $\text{G}_i$ signaling was not equivalent to the level when the $\text{D}_2\text{R}$ is expressed alone. In contrast, the combination of $\text{A}_2\text{A}$ antagonists KW-6002 and ZM 241385 with dopamine inhibited $\text{D}_2\text{R}$ signaling similarly to the unliganded $\text{A}_2\text{A}$. The combination of CGS 21680 with either KW-6002 or ZM 241385 resulted in $\text{G}_i$ signaling through the $\text{D}_2\text{R}$ that is similar to that displayed when the $\text{D}_2\text{R}$ is expressed alone (Figs. 3.3, 3.4, and 3.8). Crosstalk was mediated by heteromerization because abrogating the heteromer interface using $\text{A}_2\text{A}(\text{S374A})$ abolished the inhibition of $\text{A}_2\text{A}$ ligands on $\text{D}_2\text{R}$ signaling (Figs. 3.5, 3.6, and 3.8). Our data provide evidence at the molecular level for the combination of agonists and antagonists for the $\text{A}_2\text{A}$ potentially being effective as putative antiparkinsonian therapeutics. These results are
consistent with the findings of Bonaventura et al. 2015, which examined the effect of A$_{2A}$R ligands on D$_2$R signaling at the second messenger and behavioral level. Overall, we demonstrate that the A$_{2A}$R antagonizes G$_i$-mediated D$_2$R signaling through heteromerization at the G protein level and we have developed an assay for the screening of A$_{2A}$R ligands against G$_i$ signaling through the D$_2$R.
Figure 3.1. The GIRK1/2 (G1/2) channel is a suitable reporter or G\(_i\) signaling through the D\(_2\)R. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K\(^+\) solution (HK, solid line) and 1 µM dopamine (DA, dashed line) in oocytes expressing G1 + G2 (3.1A), G1 + G2 + D\(_2\)R (3.1B), G1 + G2 + D\(_2\)R + PTX (3.1C), G1 + G2 + D\(_2\)R + βARK (3.1D), and G1 + G2 + D\(_2\)R + PHOS (3.1E). Barium (3 mM Ba\(^{2+}\), dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. βARK and PHOS are proteins that bind G\(β\)γ, acting as scavengers. Summary figure where each bar represents the mean of n=5-6 experiments with error bars depicting the standard error of the mean (3.1F). Significance was determined using a paired t-test p<0.01.
Figure 3.2. A2AR coexpression in various ratios to the D2R inhibits Gi signaling through the D2R. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K+ solution (HK, solid line) and 1 μM dopamine (DA, dashed line) in oocytes expressing G1 + G2 + D2R (1 ng, 3.2A), G1 + G2 + D2R + A2AR (0.5 ng, 3.2B), G1 + G2 + D2R + A2AR (1 ng, 3.2C), and G1 + G2 + D2R + A2AR (2 ng, 3.2D). Barium (3 mM Ba2+, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-6 experiments with error bars depicting the standard error of the mean (3.2E). Significance was determined using a one-way ANOVA with a Tukey’s posttest, p<0.05.
Figure 3.3. $A_2A$R ligands crosstalk to the $D_2R$. Representative barium-sensitive traces of G1/2 currents obtained in response to a high $K^+$ solution (HK, solid line) and 1 µM dopamine (DA, dashed line); 1 µM dopamine + 10 µM CGS 21680 (DA + CGS, dashed line); 1 µM dopamine + 10 µM KW-6002 (DA + KW, dashed line); or 1 µM dopamine + 10 µM CGS 21680 + 10 µM KW-6002 (DA + CGS + KW, dashed line). Oocytes expressed G1 + G2 + D2R (0.5 ng, 3.3A), or G1 + G2 + D2R + $A_2A$R (0.75 ng, 3.3B, 3.3C, 3.3D, 3.3E). Barium (3 mM Ba$^{2+}$, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (3.3F). Significance was determined using a one-way ANOVA with a Tukey’s posttest, $p<0.05$. 

![Graphs showing current traces and bar charts for different conditions](image)
Figure 3.4. A$_{2A}$R ligands crosstalk to the D$_2$R continued. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K$^+$ solution (HK, solid line) and 1 µM dopamine (DA, dashed line); 1 µM dopamine + 10 µM CGS 21680 (DA + CGS, dashed line); 1 µM dopamine + 10 µM ZM 241385 (DA + ZM, dashed line); or 1 µM dopamine + 10 µM CGS 21680 + 10 µM ZM 241385 (DA + CGS + ZM, dashed line). Oocytes expressed G1 + G2 + D$_2$R (0.5 ng, 3.4A), or G1 + G2 + D$_2$R + A$_{2A}$R (0.75 ng, 3.4B, 3.4C, 3.4D, 3.4E). Barium (3 mM Ba$^{2+}$, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (3.3F). Significance was determined using a one-way ANOVA with a Tukey’s posttest, p<0.05.
Figure 3.5. $A_{2A}\text{R}(S374A)$ abolishes crosstalk of $A_{2A}\text{R}$ ligands to $G_i$ signaling through the $D_2\text{R}$. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K$^+$ solution (HK, solid line) and 1 μM dopamine (DA, dashed line); 1 μM dopamine + 10 μM CGS 21680 (DA + CGS, dashed line); 1 μM dopamine + 10 μM ZM 241385 (DA + ZM, dashed line) 1 μM dopamine + 10 μM KW-6002 (DA + KW, dashed line); 1 μM dopamine + 10 μM CGS 21680 + 10 μM ZM 241385 (DA + CGS + ZM, dashed line); or 1 μM dopamine + 10 μM CGS 21680 + 10 μM KW-6002 (DA + CGS + KW, dashed line). Oocytes expressed G1 + G2 + D2R (0.5 ng, 3.5A), or G1 + G2 + D2R + $A_{2A}\text{R}(S374A, 0.75$ ng, 3.5B, 3.5C, 3.5D, 3.5E, 3.5F, 3.5G). Barium (3 mM Ba$^{2+}$, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (3.5H). Significance was determined using a one-way ANOVA with a Tukey’s posttest, p<0.05.
Figure 3.6. A$_2$AR(S374A) is functionally similar to WT A$_2$AR. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K$^+$ solution (HK, solid line) and 10 µM CGS 21680 (CGS, dashed line) in oocytes expressing G1 + G2 + G$_{as}$ + A$_2$AR (3.6A), or G1 + G2 + G$_{as}$ + A$_2$AR(S374A, 3.6B). Barium (3 mM Ba$^{2+}$, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (3.6C). Significance was determined using a paired t-test, p<0.05.
Figure 3.7. A$_{2A}$R ligands do not agonize or antagonize homomeric D$_2$R G$_i$ activity. Summary figure of oocytes expressing G1 + G2 + D$_2$R where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean. Oocytes were perfused with 1 µM Dopamine (DA), 10 µM CGS 21680 (CGS), 10 µM ZM 241385 (ZM), 10 µM KW-6002 (KW), 1 µM DA + 10 µM CGS, 1 µM DA + 10 µM ZM, or 1 µM DA + 10 µM KW. Significance was determined using a one-way ANOVA with a Tukey’s posttest.
**Figure 3.8 Summary D<sub>2</sub>R Signaling.** Perfusion of DA to an oocyte injected with cRNA for the D<sub>2</sub>R, GIRK1, and GIRK2 leads to channel activation resulting in robust agonist-induced current (left panel, 3.1B). Co-expression of the unliganded A<sub>2A</sub>R with blocked downstream signaling results in a decrease of the DA-elicited current through the D<sub>2</sub>R (second panel, 3.4B). Application of the A<sub>2A</sub>R agonist CGS-21680 results in a relief of the inhibition of the unliganded A<sub>2A</sub>R. Application of the A<sub>2A</sub>R antagonists KW-6002 or ZM241385 results in inhibition similar to that seen with the unliganded A<sub>2A</sub>R. Application of A<sub>2A</sub>R agonists and antagonists results in a relief of inhibition similar to that seen with perfusion of CGS-21680 alone (third panel). Disrupting the heteromer interface using A<sub>2A</sub>R(S374A) abrogates all crosstalk.
Chapter 4 THE EFFECT OF HETEROMERIZATION ON Gs SIGNALING THROUGH THE A2AR

4.1 Introduction

The Gαs/olf-coupled A2AR and the Gαi/o-coupled D2R are two distinct GPCRs that heteromerize in the striatopallidal GABA pathway (Ferre, Quiroz et al. 2008). Previous studies determined that heteromerization depends on electrostatic interactions between an arginine rich region in the third intracellular loop of the D2R and a phosphorylated serine (Ser374) in the c-terminus of the A2AR (Borroto-Escuela, Marcellino et al. 2010). The physiological relevance of the A2AR-D2R heteromer is based on receptor antagonism. A2ARs antagonize the inhibitory role of D2Rs on neurotransmitter release and neuronal firing. There is exaggerated antagonism of the D2R by the A2AR in Parkinson’s disease patients due to decreased dopaminergic tone in the striatum (Kull, Ferre et al. 1999). Therefore, A2AR antagonists are being explored as possible therapeutics for Parkinson’s disease because they alleviate the A2AR inhibition of the D2R (Fuxe, Ferre et al. 2007). A plethora of studies have explored the antagonism of the A2AR on the D2R, but few studies have examined the antagonism of the D2R on A2AR signaling. Fernández-Dueñas et al. 2013 provided evidence for D2R agonists (e.g. apomorphine, rotigotine, and pramipexole) antagonizing A2AR agonist
binding, but this study did not examine the effect of D₂R ligands on A₂A-R signaling (Fernandez-Duenas, Gomez-Soler et al. 2013). Therefore, we aim to further understand A₂A-R signaling through the A₂A-R-D₂R complex. We will study A₂A-R-D₂R heteromer cross signaling through the Gₛ-coupled A₂A-R by expressing the GPCRs as well as reporter channels using the Xenopus oocyte heterologous expression system. Our electrophysiological assay is innovative in that it will allow us to control the conditions of heteromerization to tease apart the differences from protomer signaling. Based on previous studies, we hypothesize that D₂R agonists will inhibit Gₛ signaling through the A₂A-R and specific ligand combinations targeting the A₂A-R-D₂R heteromer will be more effective in maximizing GPCR signaling than individual drug administration.
4.2 Results

Lim et al. 1995 demonstrated in *Xenopus* oocytes that GIRK channels could couple to the β2 adrenergic receptor when G\textsubscript{as} is over expressed with the channel. Using a similar approach, we determined G1/2 as a suitable reporter for G\textsubscript{s} signaling through the A\textsubscript{2A}R when G\textsubscript{as} is over expressed (Lim, Dascal et al. 1995). Figures 4.1A-C serve as negative controls establishing that the G1/2 channel can only couple to G\textsubscript{as} with current elicited by the A\textsubscript{2A}R agonist CGS-21680 (CGS) when the channel is co-expressed with both the A\textsubscript{2A}R and G\textsubscript{as}. Figure 4.1D shows 10 µM CGS induces an inward current mediated by G\textsubscript{s} signaling through the A\textsubscript{2A}R when G\textsubscript{as}, G1/2, and A\textsubscript{2A}R are co-expressed validating G1/2 as a reporter for G\textsubscript{s} signaling. Figure 4.1E demonstrates the CGS-elicited current is Gβγ-mediated, as it is abolished by the Gβγ scavenger βARK. Myristoylated phosducin was also used as a specific Gβγ scavenger (Fig. 4.1F). Figure 4.1G is a summary figure of the Ba\textsuperscript{2+}-sensitive current showing the CGS-induced current (-11.58 ± 0.98 µA) is significantly different from G1/2 basal current (-4.80 ± 0.30 µA). Paired t-tests were performed to compare “Basal” to “Basal + Agonist” in the six different expression groups. Significant effects were noted in the “G1 + G2 + G\textsubscript{as} + A\textsubscript{2A}R” expression group, p=0.02. Signaling through the A\textsubscript{2A}R was also examined with PTX co-injection. Compared to CGS-elicited signaling through the A\textsubscript{2A}R (Fig. 4.1D), addition of PTX reduced the basal G1/2 current and the CGS-induced current (Figs. 4.2B,C). Paired t-tests were performed to compare “Basal” (-7.05 ± 0.66 µA) to “Basal (+ PTX)” current (-4.16 ± 0.61 µA) and “Agonist” (-3.78 ± 0.43 µA) to
“Agonist (+ PTX)” (-2.82 ± 0.41 µA) current in the two different treatment groups, p<0.05. A2AR-mediated Gαs signaling persisted in the presence of PTX confirming Gs signaling is independent of Gi signaling in our assay. In summary, we have established electrophysiological assays using G1/2 for signaling through the A2AR.

Adenosine (ADO), the nonselective endogenous ligand for the A2AR, was not used in our assay for A2AR signaling because Xenopus oocytes have an endogenous Gαi-coupled adenosine type 1 receptor (A1R, Fig. 4.3D). In oocytes only injected with G1/2 cRNA, ADO elicited a robust agonist-induced current (Fig. 4.3A). Furthermore, N6-cyclopentyladenosine (CPA), a selective A1R agonist, stimulated agonist-induced current (Fig. 4.3C) while CGS, a selective A2AR agonist, did not (Fig. 4.3B). Figure 4.3D is a summary figure of the Ba2+-sensitive current showing the ADO-induced current (-7.79 ± 0.58 µA) and the CPA-induced current (-11.36 ± µA) are significantly different from the G1/2 basal current for the “ADO” (-3.19 ± 0.14 µA) and “CPA” (-5.46 ± 0.50 µA) treatment groups. Paired t-tests were performed to compare “Basal” to “Basal + Agonist” in the three different treatment groups. Significant effects were noted in the “ADO” and “CPA” expression groups, p<0.01.

After characterizing protomeric signaling through the A2AR, we tested the effect of heteromerization. A2ARs and D2Rs are known to have two types of interactions: 1) direct receptor-receptor interactions or heteromerization and 2) downstream signaling from separate populations of homomers converging on effector molecules (Ferre et al. 2007). When assessing the effect of A2AR-D2R heteromerization, we blocked the downstream signaling of the adjacent GPCR so changes in signaling should result from
heteromerization. $A_{2A}R$-mediated signaling through the $A_{2A}R$-$D_2R$ heteromer was evaluated with PTX co-expression to abrogate $D_2R$ signaling.

Our lab is the first to examine the functional implications of $A_{2A}R$-$D_2R$ heteromerization on $A_{2A}R$ signaling using electrophysiology (Fig. 4.4). We tested the effect of heteromerization on $G_s$ signaling through the $A_{2A}R$ using different $A_{2A}R$:D$_2$R cRNA ratios to achieve the level of protein expression for maximal cross-signaling. PTX was co-expressed to block downstream D$_2$R signaling. Compared to $G_s$ signaling through the $A_{2A}R$ (Fig. 4.4A), there is decreased $A_{2A}R$ signaling with $D_2R$ co-expression ($A_{2A}R$:D$_2$R) in the following ratios: 1:1 (Fig. 4.4B), 1:2 (Fig. 4.4C), and 2:1 (Fig. 4.4D). Figure 4.4E is a summary figure of the normalized Ba$^{2+}$-sensitive, CGS-induced current showing $D_2R$ co-expression significantly reduces $G_s$ signaling through the $A_{2A}R$ in all ratios tested. A one-way ANOVA with a Tukey’s posttest was performed to compare normalized $G_s$ activity with and without $D_2R$ co-expression (1:0, 2.12 ± 0.23). Significant effects were noted in all the $A_{2A}R$:D$_2$R expression groups: 1:1 (1.64 ± 0.08), 1:2 (1.45 ± 0.08), and 2:1 (1.39 ± 0.05), p<0.05. Thus, we have established an electrophysiological assay demonstrating that the $D_2R$ antagonizes $A_{2A}R$ signaling.

Next, we evaluated whether $D_2R$ ligands could crosstalk to $G_s$ signaling through the $A_{2A}R$. The ratio of $D_2R$:A$_{2A}R$ used was 2:3 (0.5 ng to 0.75 ng) because previous studies have established that this ratio is most similar to that seen in the striatum. $A_{2A}R$ signaling (Fig. 4.5A) and the effect of $D_2R$ co-expression (Fig. 4.5B) were repeated to compare $G_s$ activity within the same experiment. Perfusion of the $D_2R$ agonist quinpirole (Fig. 4.5C) or the $D_2R$ antagonists paliperidone (Fig. 4.5D) and amisulpride (Fig. 4.5E) resulted in $G_s$ signaling that was significantly decreased from that displayed with co-
expression of the unliganded D$_2$R. The combination of the D$_2$R ligands quinpirole and paliperidone inhibited G$_s$ signaling through the A$_{2A}$R at similar levels to that seen with either ligand alone (4.5F). In contrast, the combination of the D$_2$R ligands quinpirole and amisulpride (4.5G) resulted in G$_s$ signaling through the A$_{2A}$R that was not significantly different from protomeric levels. Figure 4.5 demonstrates that the addition of D$_2$R ligands can further modulate the inhibition of the unliganded D$_2$R on A$_{2A}$R signaling.

After evaluating A$_{2A}$R-D$_2$R crosstalk, we sought to determine if the allosteric modulation of the D$_2$R on the A$_{2A}$R was due to heteromerization; therefore, we mutated the heteromer interface (Fig. 4.6) and tested the D$_2$R ligands on protomeric A$_{2A}$R signaling (Fig. 4.8). We mutated arginines 217, 219, 220, 222, 267, 268, and 269 along with lysines 218 and 221 in the third intracellular loop of the D$_2$R to alanine to disrupt heteromerization and termed the mutant D$_2$R(9A). We utilized D$_2$R(9A) to disrupt heteromerization and test if this mutant affected the ability of D$_2$R ligands to crosstalk to the A$_{2A}$R. In all ligand combinations tested, co-expressing D$_2$R(9A) with the WT A$_{2A}$R abrogated the effect of the D$_2$R ligands quinpirole (Fig. 4.6C), paliperidone (Fig. 4.6D), amisulpride (Fig. 4.6E), quinpirole + paliperidone (Fig. 4.6F), and quinpirole + amisulpride (Fig. 4.6G) on A$_{2A}$R signaling. In figure 4.7, the D$_2$R mutant D$_2$R(9A, Fig. 4.7B) was shown to have G$_i$ signaling that was not significantly different from the WT A$_{2A}$R (Fig. 3.6A). Thus, the difference in function between D$_2$R(9A) and D$_2$R was not the reason for the termination of the D$_2$R-mediated effects on A$_{2A}$R signaling. We also tested all of the D$_2$R ligands used in previous figures to assess if these ligands exerted independent effects on A$_{2A}$R signaling. Compared to CGS 21680-elicited A$_{2A}$R signaling, neither quinpirole, paliperidone, nor amisulpride had any effect on protomeric
$A_{2A}$R signaling. Furthermore, these ligands did not have any effect on basal $A_{2A}$R signaling. Figures 4.6, 4.7, and 4.8 provide additional evidence that the antagonism of $A_{2A}$R ligands on $D_2$R signaling was due to heteromerization.
4.3 Discussion

The A<sub>2A</sub>R-D<sub>2</sub>R heteromer is an emerging therapeutic target for Parkinson’s disease because of the established antagonism of the A<sub>2A</sub>R on the D<sub>2</sub>R. In Parkinson’s disease, there is a loss of dopaminergic innervation to the striatum and the A<sub>2A</sub>Rs antagonize residual D<sub>2</sub>R signaling. Therefore, most studies have focused on elucidating the antagonism of the A<sub>2A</sub>R on the D<sub>2</sub>R targeting A<sub>2A</sub>R antagonists as antiparkinsonian therapeutics (Jenner 2003). The A<sub>2A</sub>R antagonist KW-6002 is currently used in Japan to treat Parkinson’s disease and several other A<sub>2A</sub>R antagonists are in clinical trials (Chen, Wang et al. 2013). Compared to the extensive research characterizing A<sub>2A</sub>R antagonism of the D<sub>2</sub>R, much less is known of the D<sub>2</sub>R-mediated antagonism of the A<sub>2A</sub>R. Various studies have detailed the antagonism of the D<sub>2</sub>R on the A<sub>2A</sub>R at the second messenger level through the canonical G<sub>i</sub> signaling pathway inhibiting adenylate cyclase, cAMP accumulation, PKA phosphorylation of downstream effectors, and gene expression of c-fos and preproenkephalin (Ferre, Quiroz et al. 2008). However, this negative allosterism at the second messenger level is not heteromer dependent. Fewer studies have focused on the therapeutic potential of D<sub>2</sub>R agonists that crosstalk to the A<sub>2A</sub>R through heteromerization inhibiting A<sub>2A</sub>R ligand binding and signaling. In 2013, Fernández-Dueñas and colleagues used real-time FRET and flow cytometry in transfected cells to provide evidence for antiparkinsonian D<sub>2</sub>R agonists antagonizing A<sub>2A</sub>R agonist binding. More studies are needed to examine the effect of D<sub>2</sub>R ligands on A<sub>2A</sub>R signaling through heteromerization as this could lead to novel antiparkinsonian therapeutics targeting the D<sub>2</sub>R.
Expanding on the Fernández-Dueñas study, we developed an electrophysiological assay to examine the effect of A2AR-D2R heteromerization on A2AR signaling at the G protein level. We used *Xenopus laevis* oocytes as our heterologous expression system and the GIRK1/2 channel as a reporter for A2AR signaling by over expressing Gαs (Fig. 4.1). In our assay, co-expression of the unliganded D2R inhibits A2AR signaling (Fig. 4.4). Our study is one of the first to demonstrate that the unliganded D2R antagonizes Gαs signaling through the A2AR. Furthermore, the addition of the D2R agonist quinpirole or the D2R antagonists paliperidone and amisulpride further inhibited signaling through the A2AR (Fig. 4.5 and Fig. 4.9). Our work provides evidence for both D2R agonists and antagonists diminishing Gαs signaling through the A2AR. The combination of the D2R ligands quinpirole and paliperidone inhibited A2AR signaling similarly to each ligand when administered alone. In contrast, co-administration of quinpirole and amisulpride resulted in Gαs signaling through the A2AR that is similar to protomeric levels (Fig. 4.5). Crosstalk was mediated by heteromerization because abrogating the heteromer interface using D2R(9A) abolished the inhibition of D2R ligands on A2AR signaling (Figs. 4.6, 4.7, and 4.9). Overall, we have demonstrated that the D2R antagonizes Gαs-mediated A2AR signaling through heteromerization at the G protein level and we have developed an assay for the screening of D2R ligands against Gαs signaling through the A2AR.
Figure 4.1 The GiRK1/2 (G1/2) channel is a suitable reporter for Gs signaling through the A2AR. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K+ solution (HK, solid line) and 10 μM CGS-21680 (CGS, dashed line) in oocytes expressing G1 + G2 (4.1A), G1 + G2 + Gas (4.1B), G1 + G2 + A2AR (4.1C), G1 + G2 + Gas + A2AR (4.1D), G1 + G2 + Gas + A2AR + βARK (4.1E), and G1 + G2 + Gas + A2AR + PHOS (4.1F). Barium (3 mM Ba²⁺, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-6 experiments with error bars depicting the standard error of the mean (4.1G). Significance was determined using a paired t-test p<0.03.
Figure 4.2 Gs signaling through the A2AR in the presence of PTX. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K+ solution (HK, solid line) and 10 µM CGS-21680 (CGS, dashed line) in oocytes expressing G1 + G2 + Gas + A2AR (4.2A) and G1 + G2 + Gas + A2AR + PTX (4.2B). Barium (3 mM Ba2+, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-6 experiments with error bars depicting the standard error of the mean (4.2C). Significance was determined with a paired t-test, p<0.05.
Figure 4.3 *Xenopus* oocytes have an endogenous G\(_i\)-coupled A\(_1\)R. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K\(^+\) solution (HK, solid line) and 10 µM adenosine (ADO, dashed line) the nonselective endogenous ligand for adenosine receptors (4.3A), 10µM CGS-21680 (CGS, dashed line) the selective agonist for the A\(_{2A}\)R (4.3B), and 10 µM N6-cyclopentyladenosine (CPA, dashed line) the selective agonist for the A1R (4.3C). Barium (3 mM Ba\(^{2+}\), dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-6 experiments with error bars depicting the standard error of the mean (4.3D). Significance was determined using a paired t-test p<0.01.
Figure 4.4 The effect of D$_2$R co-expression on G$_s$ signaling through the A$_{2A}$R. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K$^+$ solution (HK, solid line) and 10 µM CGS-21680 (CGS, dashed line) in oocytes expressing G1, G2, the A$_{2A}$R, PTX and D$_2$R in the following ratios: 1:0 (4.4A), 1:1 (4.4B), 1:2 (4.4C), and 2:1 (4.4D). Barium (3 mM Ba$^{2+}$, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure of normalized G$_s$ activity (Agonist + Basal Current/ Basal Current) where each bar represents the mean of n=4-6 experiments with error bars depicting the standard error of the mean (4.4E). Significance was determined using a one-way ANOVA with a Tukey’s posthoc test, p<0.05.
Figure 4.5 \(D_2R\) ligands crosstalk to the \(A_{2A}R\). Representative barium-sensitive traces of G1/2 currents obtained in response to a high K\(^+\) solution (HK, solid line) and 10 \(\mu\)M CGS 21680 (CGS, dashed line); 10 \(\mu\)M CGS 21680 + 1 \(\mu\)M quinpirole (CGS + QUIN, dashed line); 10 \(\mu\)M CGS 21680 + 10 \(\mu\)M paliperidone (CGS + PAL, dashed line); or 10 \(\mu\)M CGS 21680 + 1 \(\mu\)M quinpirole + 10 \(\mu\)M paliperidone (CGS + QUIN + PAL, dashed line); or 10 \(\mu\)M CGS 21680 + 1 \(\mu\)M quinpirole + 10 \(\mu\)M amisulpride (CGS + ASUL, dashed line); or 10 \(\mu\)M CGS 21680 + 1 \(\mu\)M quinpirole + 10 \(\mu\)M amisulpride. Oocytes expressed G1 + G2 + G\(\alpha_s\) + PTX+ \(A_{2A}R\) (0.75 ng, 4.5A), or G1 + G2 + \(D_2R\) + PTX+ G\(\alpha_s\) + \(A_{2A}R\) (0.75 ng, 4.5B, 4.5C, 4.5D, 4.5E, 4.5F, 4.5G). Barium (3 mM Ba\(^{2+}\), dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (4.5H). Significance was determined using a one-way ANOVA with a Tukey’s posthoc test, p<0.05.
Figure 4.6 D<sub>2</sub>R-9A disrupts the allosteric modulation of the D<sub>2</sub>R on the A<sub>2A</sub>R. We mutated arginines 217, 219, 220, 222, 267, 268, and 269 along with lysines 218 and 221 in the third intracellular loop of the D<sub>2</sub>R to alanine to disrupt heteromerization and termed the mutant D<sub>2</sub>R D<sub>2</sub>R(9A). Representative barium-sensitive traces of G1/2 currents obtained in response to a high K<sup>+</sup> solution (HK, solid line) and 10 µM CGS 21680 (CGS, dashed line); 10 µM CGS 21680 + 1 µM quinpirole (CGS + QUIN, dashed line); 10 µM CGS 21680 + 10 µM paliperidone (CGS + PAL, dashed line) 10 µM CGS 21680 + 10 µM amisulpride (CGS + ASUL, dashed line); 10 µM CGS 21680 + 1 µM quinpirole + 10 µM paliperidone (CGS + QUIN + PAL, dashed line); or 10 µM CGS 21680 + 1 µM quinpirole + 10 µM amisulpride (CGS + QUIN + ASUL, dashed line). Oocytes expressed G1 + G2 + Gas + PTX + A<sub>2A</sub>R (0.75 ng, 4.6A), or G1 + G2 + D<sub>2</sub>R(9A) + PTX + Gas + A<sub>2A</sub>R(0.75 ng. 4.6B, 4.6C, 4.6D, 4.6E, 4.6F, 4.6G). Barium (3 mM Ba<sup>2+</sup>, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (4.6H). Significance was determined using a one-way ANOVA with a Tukey’s posttest, p<0.05.
Figure 4.7 D₂R-9A is functionally similar to WT D₂R. Representative barium-sensitive traces of G1/2 currents obtained in response to a high K⁺ solution (HK, solid line) and 1 µM dopamine (DA, dashed line) in oocytes expressing G1 + G2 + D₂R (A), or G1 + G2 + D₂R(9A) (B). Barium (3 mM Ba²⁺, dotted line) inhibited G1/2 and allowed for subtraction of G1/2 independent currents. Summary figure where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean (C). Significance was determined using a paired t-test, p<0.05.
Figure 4.8 D₂R ligands do not agonize or antagonize homomeric A₂AR Gₛ activity. Summary figure of oocytes expressing G1 + G2 + Gas + A₂AR where each bar represents the mean of n=5-8 experiments with error bars depicting the standard error of the mean. Oocytes were perfused with 10 µM CGS 21680 (CGS), 1 µM Quinpirole (QUIN), 10 µM Paliperidone (PAL), 10 µM Amisulpride (ASUL), 10 µM CGS + 1 µM QUIN, 10 µM CGS + 10 µM PAL, or 10 µM CGS + 10 µM ASUL. Significance was determined using a one-way ANOVA with a Tukey’s posthoc test.
Figure 4.9 Summary A$_{2A}$R Signaling. Perfusion of CGS to an oocyte injected with cRNA for the A$_{2A}$R, PTX, Gαs, GIRK1, and GIRK2 leads to channel activation resulting in robust agonist-induced current (left panel). Co-expression of the unliganded D$_2$R with blocked downstream signaling results in a decrease of the CGS-elicited current through the A$_{2A}$R (second panel). Application of the D$_2$R agonist Quinpirole results in increased inhibition compared to the unliganded D$_2$R. Application of the D$_2$R antagonists KW-Paliperidone or Amisulpride also results in increased inhibition compared to that seen with the unliganded D$_2$R. Co-application of the D$_2$R agonist Quinpirole and antagonist amisulpride results in a relief of inhibition (third panel). Disrupting the heteromer interface using D$_2$R(9A) abrogates all crosstalk.
Chapter 5 OPTOGENETIC-MICRODIALYSIS ASSAY TO ASSESS THE PRESYNAPTIC PROFILE OF A\textsubscript{2}A\textsubscript{R} ANTAGONISTS

5.1 Introduction

Smooth motor function is achieved through counterbalancing the activating and inhibiting influences of the direct and indirect pathways of the striatum. Dopaminergic innervation of the striatum results in motor activation by stimulating the direct pathway via D\textsubscript{1}Rs and inhibiting the indirect pathway via D\textsubscript{2}Rs. Adenosine is also an important neuromodulator in the striatum as stimulation or inhibition of postsynaptic A\textsubscript{2}A\textsubscript{R}s results in the blockade or potentiation of D\textsubscript{2}R signaling (Quiroz, Lujan et al. 2009). Striatal presynaptic A\textsubscript{2}A\textsubscript{R}s in glutamatergic terminals also affect motor activity by influencing glutamate release in the direct pathway. A\textsubscript{2}A\textsubscript{R} antagonists that inhibit postsynaptic A\textsubscript{2}A\textsubscript{R}s would be beneficial for the treatment of PD by inhibiting the indirect motor pathway and increasing motor activity. However, A\textsubscript{2}A\textsubscript{R} antagonists that inhibit presynaptic A\textsubscript{2}A\textsubscript{R}s would decrease glutamate release in the direct motor pathway inhibiting motor activity. Thus, antiparkinsonian therapeutics targeting the A\textsubscript{2}A\textsubscript{R} should have a higher affinity for postsynaptic versus presynaptic A\textsubscript{2}A\textsubscript{R}s. In 2011, Orrú and colleagues used \textit{in vitro} radioligand-binding and \textit{in vivo} locomotor activity experiments to determine the preferential presynaptic or postsynaptic action of A\textsubscript{2}A\textsubscript{R} antagonists. Among the A\textsubscript{2}A\textsubscript{R} antagonists tested, SCH 442416 acted predominantly on presynaptic
$A_2AR$s; whereas, istradefylline (KW-6002) acted primarily on postsynaptic $A_2AR$s. In this study, we have utilized the optogenetic-microdialysis technique of Quiroz-Molina et al. 2016 to determine the presynaptic or postsynaptic preference of $A_2AR$ antagonists. We used optogenetics to directly stimulate the striatal MSNs of the direct pathway to induce glutamate release and microdialysis to determine if $A_2AR$ antagonists affect extracellular glutamate concentrations. We anticipate $A_2AR$ antagonists that are potent inhibitors of glutamate release act primarily presynaptically.
5.2 Results

We used an optogenetic-microdialysis probe (Fig. 5.1) implanted in the striatum of mice expressing AAV ChR2-EYFP to measure extracellular glutamate release in response to light-induced activation of the cation ChR2 (Fig. 5.2). Optogenetic stimulation resulted in rapid increases in the extracellular glutamate concentration above basal levels (~300%). Termination of the optogenetic stimulation resulted in slow decay of the extracellular glutamate concentration to basal levels. Administration of the A$_{2A}$R antagonist SCH 442416 via i.p. injection before optogenetic stimulation prevented increases in the extracellular glutamate concentration (Fig. 5.4). SCH 442416 inhibited glutamate release indicating that this drug acts preferentially on presynaptic A$_{2A}$Rs. However, i.p. injection of the A$_{2A}$R antagonist KW-6002 preceding optogenetic stimulation had no significant effect on extracellular glutamate concentration demonstrating that KW-6002 acts preferentially on postsynaptic A$_{2A}$Rs (Fig. 5.4). Figure 5.5 is a control experiment showing that the basal levels of glutamate in the mice across all treatment groups (vehicle, SCH 442416 0.1 mg/kg, and KW-6002 1 mg/kg) obtained prior to optogenetic stimulation are not significantly different as determined by a one-way ANOVA followed by a Tukey’s posthoc test (Prism). Thus, differences in basal glutamate concentrations cannot account for the differences observed after optogenetic stimulation. Erk 1/2 phosphorylation was also examined as an indicator of neuronal activation. Robust ERK 1/2 labeling was present in the striatum of mice subjected to laser stimulation while sparse labeling was observed in the motor cortex, the site of
virus injection (Fig. 5.3). These experiments provide further evidence that optogenetic stimulation in the striatum, the site of probe implantation, resulted in neuronal activation and glutamate release.
5.3 Discussion

The A$_{2A}$R-D$_{2}$R heteromer is an emerging therapeutic target for Parkinson’s disease based on receptor antagonism. A$_{2A}$R antagonists that act postsynaptically through the indirect motor pathway produce motor activation by potentiating D$_{2}$R signaling through A$_{2A}$R-D$_{2}$R interactions. However, A$_{2A}$R antagonists that act presynaptically through the direct motor pathway decrease motor activity by inhibiting glutamate release. Therefore, it is essential to identify the presynaptic or postsynaptic preference of A$_{2A}$R antagonists to determine their therapeutic potential as antiparkinsonian drugs. In this study, we have utilized an *in vivo* optogenetic-microdialysis assay with the advantage of direct stimulation of glutamatergic terminals. Optogenetic stimulation resulted in increases the extracellular concentration of glutamate that were abrogated by pre-administration SCH 442416 (Fig. 5.4) Our results are consistent with the findings of Orrú and colleagues, who determined the potent activity of SCH 442416 on presynaptic A$_{2A}$Rs using *in vitro* radioligand binding and *in vivo* locomotor experiments. Future studies will involve assaying more A$_{2A}$R antagonists especially the postsynaptic acting KW-6002 to see if this compound is able to inhibit glutamate release in our assay. Our assay is useful for screening the presynaptic activity of A$_{2A}$R antagonists that are potential antiparkinsonian therapeutics.
Figure 5.1 Optogenetic-microdialysis probe. Image of the hand made microdialysis-optogenetics probes with the semi-permeable microdialysis membrane, the microdialysis inlet and outlet, and the fiber optic (left). Illuminated microdialysis tube with laser stimulation (right).
Figure 5.2 Schematic displaying timeline of unilateral AAV microinjection and optogenetic-microdialysis experiment. WT C57BL/6 mice received unilateral injections of adeno-associated virus (AAV) encoding channel rhodopsin 2 (ChR2) fused to enhanced yellow fluorescent protein (EYFP) under control of the CaMKII neuronal promoter. The coordinates of virus injection were 1.9 mm anterior, 1.6 mm lateral, and 1.8 mm ventral with respect to bregma to inject the virus into the motor cortex. Approximately, 4 weeks after virus injection a modified microdialysis probe with an embedded light-guiding optic fiber was implanted in the striatum. The coordinates for probe implantation were 0.3 mm posterior, 2.7 mm lateral and 4.2 mm ventral with respect to bregma in the striatum. Adapted from (Guo, Xiong et al. 2015).
Figure 5.3 Confocal laser microscopy of coronal sections showing the localization of ChR2-EYFP after unilateral AAV microinjection in the motor cortex and ERK phosphorylation immunohistochemistry. A Motor Cortex, scale bar 50 uM B Inset C Localization of probe in striatum, scale bar 50 uM D Inset
Figure 5.4 Effect of optogenetic stimulation in the striatum. Time course of extracellular concentrations of glutamate in the striatum with i.p. injection of Vehicle, the $A_2A$R antagonist SCH 442416 0.1 mg/kg, or the $A_2A$R antagonist KW-6002 1 mg/kg. Negative time values represent basal glutamate before stimulation. Optogenetic stimulation was for 20 minutes and is represented by the blue bar. Results are expressed as mean ± SEM of percentages of glutamate normalized to basal values before stimulation, n=3-6. Results were analyzed using a two-way repeated-measures ANOVA with a Tukey’s posthoc test (Prism), * p≤0.05, ** p≤0.01, **** p≤0.0001.
Figure 5.5 Basal Glutamate Concentration. Bar graph summarizing the basal levels of glutamate obtained prior to optogenetic stimulation. Results are expressed as mean ± SEM of glutamate concentration, n=3-6. There was no significant difference between basal values in the three treatment groups (vehicle, SCH 442416 0.1 mg/kg, and KW-6002 1 mg/kg) as determined by a one-way ANOVA followed by a Tukey’s posthoc test (Prism).
Chapter 6 CONCLUDING REMARKS

We investigated A₂A R and D₂R interactions using an electrophysiological, *in vitro* assay with *Xenopus* oocytes as the heterologous expression system and an *in vivo*, optogenetic-microdialysis assay in freely moving mice. Examining A₂A R-D₂R heteromerization in a heterologous expression system allowed us to manipulate the conditions of heteromerization by using interfering mutants. Our electrophysiological assay also provided a membrane-delimited, direct readout of GPCR signaling at the G protein level versus downstream second messengers. The *Xenopus laevis* heterologous expression system also permitted titration of A₂A R and D₂R expression. Similarly, there were many advantages to incorporating an *in vivo* assay into our studies. Using an *in vivo* assay allowed us to use native neurons in the mouse striatum to explore A₂A R and D₂R interactions. Through optogenetics, we were able to directly stimulate striatal neurons and observe glutamate release. The optogenetic-microdialysis experiments also provided physiological relevance to studies evaluating antiparkinsonian therapeutics. Both our *in vivo* and *in vitro* assays provided a multitude of technical advantages that allowed key insights into A₂A R-D₂R interactions.

In chapter 3, we utilized an electrophysiological assay with GIRK channels as reporters for Gᵢ signaling through the D₂R to determine the effect of A₂A R-D₂R heteromerization on Gᵢ signaling through the D₂R. The following conclusions can be drawn from chapter 3 of this investigation. We determined that GIRK1/2 could serve as
a reporter for Girt signaling through the D2R as the liberated βγ subunits from D2R activation resulted in channel potentiation. Next, we co-expressed the A2AR along with the D2R and found that the unliganded A2AR antagonizes dopamine-elicited Girt signaling through the D2R in various ratios of overexpression of the two receptors. Our lab was the first to uncover the effect of the unliganded A2AR on Girt signaling through the D2R. We further perfused ligands to the A2AR to examine crosstalk. Perfusion of the A2AR agonist CGS 21680 partially relieves the inhibition of the unliganded A2AR on D2R signaling. Whereas, perfusing the A2AR antagonists ZM 241385 and KW-6002 both inhibited dopamine-elicited signaling through the D2R. The combinations of A2AR agonists (CGS 21680) and antagonists (KW-6002, ZM 241385) restored dopamine-elicited Girt signaling through the D2R. We also utilized mutagenesis to examine whether the aforementioned crosstalk was due to heteromerization. We discovered that the ability of A2AR ligands to crosstalk to the D2R is mediated by A2AR-D2R heteromerization as disrupting the heteromer interface using A2AR(S374A) relieves the inhibition of the A2AR on the D2R. As a control, we tested all of the A2AR ligands used to ensure they were specific for the A2AR and did not have any activity on the D2R.

Chapter 4 of this investigation focused on using an electrophysiological assay with GIRK channels as reporters for Gs signaling through the A2AR to determine the effect of A2AR-D2R heteromerization on Gs signaling through the A2AR. We demonstrated that GIRK 1/2 can also serve as a reporter for Gs signaling through the A2AR when G_{αs} is overexpressed and channel activation is mediated by the liberated βγ subunits from A2AR activation. Additionally, the unliganded D2R antagonizes CGS 21680-elicited Gs signaling through the A2AR in various ratios of overexpression of the
two receptors. Our results were the first to determine the effect of the unliganded D$_2$R on Gs signaling through the A$_{2A}$R. Next, we examined crosstalk and found that perfusion of the D$_2$R agonist quinpirole further inhibits G$_s$ signaling through the A$_{2A}$R compared to the unliganded D$_2$R. Furthermore, both of the D$_2$R antagonists amisulpride and paliperidone inhibit G$_s$ signaling through the A$_{2A}$R similarly to quinpirole. The combination of the quinpirole and amisulpride, but not quinpirole and paliperidone restored CGS 21680-elicited signaling through the A$_{2A}$R to homomeric levels. We also determined that the ability of D$_2$R ligands to crosstalk to the A$_{2A}$R was mediated by heteromerization as disrupting the heteromer interface using D$_2$R(9A, R217A, R219A, R220A, R222A, R267A, R268A, R269A, K218A and K221A) relieves the inhibition of the D$_2$R on the A$_{2A}$R. We also determined that quinpirole, amisulpride, and paliperidone were specific for the D$_2$R and did not any extraneous effect on the homomeric A$_{2A}$R. Our in vitro assays elucidated signaling through the A$_{2A}$R-D$_2$R heteromer and could provide insight for future studies examining A$_{2A}$R-D$_2$R heteromerization.

In chapter 5, we utilized a recently developed optogenetic-microdialysis technique to examine the presynaptic profile of A$_{2A}$R antagonists on glutamatergic afferents to the striatum. We used optogenetics to directly stimulate the striatal MSNs of the direct pathway to induce glutamate release and microdialysis to determine if A$_{2A}$R antagonists affect extracellular glutamate concentrations. An optogenetic-microdialysis probe was implanted in the striatum of mice expressing AAV ChR2-EYFP to measure extracellular glutamate release in response to light-induced activation of the cation ChR2. Optogenetic stimulation resulted in rapid increases in the extracellular glutamate concentration above basal levels (~300%). Termination of the optogenetic stimulation
resulted in slow decay of the extracellular glutamate concentration to basal levels. Administration of the A$_{2A}$R antagonist SCH 442416 before optogenetic stimulation prevented increases in the extracellular glutamate concentration. However, administration of the A$_{2A}$R antagonist KW-6002 before optogenetic stimulation did not have an effect on presynaptic glutamate release. SCH 442416 inhibited glutamate release indicating that this drug preferentially acts on presynaptic A$_{2A}$Rs whereas the inability of KW-6002 to affect presynaptic glutamate release indicates that this drug preferentially acts on postsynaptic A$_{2A}$Rs. Erk 1/2 phosphorylation was also examined as an indicator of neuronal activation. Robust ERK 1/2 labeling was present in the striatum of mice subjected to laser stimulation while sparse labeling was observed in the motor cortex, the site of virus injection. Our optogenetic-microdialysis assay is useful for evaluating the therapeutic potential of antiparkinsonian drugs. Futures studies for the in vitro experiments will include dose-response curves to evaluate how different concentrations of A$_{2A}$R and D$_2$R ligands affect A$_{2A}$R-D$_2$R heteromer crosstalk. We are also currently developing an optogenetic-locomotor activity assay to examine the postsynaptic profile of A$_{2A}$R antagonists.


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PUBLICATIONS


ABSTRACTS


