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A NOVEL INTERACTION BETWEEN THE A2A ADENOSINE RECEPTOR
AND RAN BINDING PROTEIN 9 (RBP9)
AND THE ROLE OF RBP9 IN GPCR FUNCTION

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by

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Abstract

Several lines of evidence describe an involvement of the A2a adenosine receptor (A2aR), a seven transmembrane, G protein-coupled receptor (GPCR) in Parkinson’s disease (PD). Caffeine, an antagonist of this receptor has been shown to have a protective effect on the development of PD.

Interacting proteins of GPCRs assist in their signaling and regulation. In this work, the yeast two-hybrid assay was used to screen for novel interacting proteins for the A2aR, in order to discover a means of regulating the receptor’s signaling. Several candidate interacting proteins for the A2aR tail were identified. This work characterizes the interaction between the A2aR and one interactor, Ran Binding Protein 9 (RBP9).

RBP9 has been described as a scaffolding protein which interacts with at least three GPCRs. The functional role of RBP9 is still under investigation, but several regulatory roles have been described for its interaction with GPCRs, including mediating internalization, phosphorylation, and receptor expression.

Here, the A2aR-RBP9 interaction is confirmed through GST-pulldown and Co-immunoprecipitation experiments conducted in A2aR-transfected HEK-293 cells and SHSY5Y cells. Furthermore, the two proteins are shown to co-localize in both cell lines.

The possibility that RBP9 is a promiscuous interactor of GPCR proteins was also investigated. Co-immunoprecipitation experiments demonstrate an interaction between RBP9 and the D2 dopamine receptor (D2R), mu, kappa, and delta opioid receptors (MOR, KOR, and
Because RBP9 interacts with several prominent GPCRs, it is possible that it holds a common function in regulation of these receptors.

Through RBP9 siRNA knockdown experiments, a regulatory role for RBP9 in the overall expression of three GPCRs, namely the A2aR, MOR, and D2R in stably-transfected HEK-293 cells is demonstrated. Specifically, knockdown of RBP9 results in a down-regulation of the A2aR, and an up-regulation of the D2R and MOR. RBP9-dependent up-regulation of D2R expression is confirmed in SHSY5Y neuroblastoma cells.

It appears evident that RBP9 is a promiscuous interactor of GPCRs, and has a regulatory role in their overall expression levels. Further studies may expand our understanding of RBP9’s function, and the mechanism by which it regulates GPCR expression, providing a novel pharmaceutical target in GPCR signal regulation.
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Abbreviations

A2aR- Adenosine receptor subtype A2a
Ab- antibody
ADP- Adenosine Diphosphate
AMP- Adenosine Monophosphate
ATP- Adenosine Triphosphate
ATP6V1G1- ATPase, H+ transporting lysosomal 13kD, V1 subunit G1
β-actin- beta- Actin
β-gal- beta- galactosidase
BLAST- Basic Local Alignment Search Tool
BRET- Bioluminescence Resonance Energy Transfer
BZW2- Basic Leucine Zipper and W2 domains 2
cAMP- cyclic AMP
CHD- Chromodomain helicase DNA binding domain
CHD1- Chromodomain helicase DNA binding domain 1
CHD3- Chromodomain helicase DNA binding domain 3
CNS- Central Nervous System
Co-IP- Co-immunoprecipitation
CRA- CT11-RBP9 domain
C-tail- C-terminal tail
CTLH- C-terminal to LiSH domain
D1R- D1 Dopamine Receptor
D2LR- D2 Long Dopamine Receptor
D2SR- D2 Short Dopamine Receptor
D2R- D2 Dopamine Receptor
DA- Dopamine
DMEM- Dulbecco’s Modified Eagle Medium
DNA- Deoxyribonucleic acid
DOR- Delta Opioid Receptor
ECL- Enhanced Chemiluminescence
EMBOSS- The European Molecular Biology Open Software Suite
EST- Expressed Sequence Tag
FBS- Fetal Bovine Serum
For- Forward Primer
FRET- Fluorescence Resonance Energy Transfer
GAPDH- Glyceraldehyde 3-Phosphate Dehydrogenase
GDP- Guanosine Diphosphate
GPCR- G protein-coupled Receptor
G protein- Guanine Nucleotide-Binding Regulatory Protein
GRF- Guanine-Nucleotide Releasing Factor
GRK- G protein-Coupled Receptor Kinase
GST- Glutathione-S-Transferase
GST-PD- Glutathione-S-Transferase Pulldown
GTP- Guanosine Triphosphate
HEK- Human Embryonic Kidney
HRP- Horseradish Peroxidase
ICL1- Intracellular Loop 1
ICL2- Intracellular Loop 2
ICL3- Intracellular Loop 3
IP- Immunoprecipitation
IP3- Inositol Trisphosphate
LiSH- Lissencephaly type-1 like Homology domain
KOR- Kappa Opioid Receptor
MapK- Mitogen-Activated Protein Kinase
mGluR2- Metabotropic Glutamate Receptor 2
mGluR8- Metabotropic Glutamate Receptor 8
MOR- Mu Opioid Receptor
MPTP- 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
mRNA- messenger Ribonucleic Acid
NOR- Nociceptin/ Orphanin Receptor
NT-siRNA- Non-Targeting small interfering RNA
PBS- Phosphate Buffered Saline
PCR- Polymerase Chain Reaction
PD- Parkinson’s Disease
PI3K- Phosphoinositide 3-Kinase
PKC- Protein Kinase C
PVDF- Polyvinylidene Fluoride Membrane
RBP9- Ran Binding Protein 9
Rev- Reverse Primer
RINT1- RAD50 Interactor 1
SDS- Sodium Dodecyl Sulfate
SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM- Standard Error of the Mean
SH2- Src Homology 2 domain
SH3- Src Homology 3 domain
SHSY5Y- Neuroblastoma cell line
siRNA- Small interfering RNA
SKIV2L2- Superkiller Viralicidic Activity 2-like 2
SPRY- Spla and Ryanodine Receptor domain
TBST- Tris Buffered Saline with Tween
USP-11- Ubiquitin Specific Peptidase 11
Y2H- Yeast 2-Hybrid
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Chapter One: Literature Review

1.1 G protein-coupled receptors

1.1.1 Overview of structure and signaling

G-protein coupled receptors (GPCRs) are cell-surface receptor proteins, widely expressed in the nervous system and periphery. Acting through several transduction pathways, GPCRs are responsible for the majority of signal transduction across the plasma membrane (Gutkind 2000; Millar and Newton 2010).

Several hundred GPCRs have been identified to date, classified by sequence analysis into families. The largest, with 672 family members, is the rhodopsin/beta-adrenergic family, of which the A2a adenosine receptor is a member (Strader et al. 1995; Millar and Newton 2010). In all GPCRs, activation of the receptor is initiated by ligand binding, followed by signal transduction, often mediated by a heterotrimeric guanine nucleotide-binding regulatory protein (G-protein). Though sequence homology between receptors diverges, the tertiary structure of all GPCRs is very similar.

GPCRs structurally consist of seven helical transmembrane regions, connected both extracellularly and intracellularly by hydrophilic loops, an extracellular amino-terminal region and an intracellular carboxyl-terminal tail. The degree of sequence homology between GPCRs is highest in the transmembrane regions, with extracellular and intracellular portions having greater variability (Strader et al. 1994). Crystal structures have been solved for rhodopsin, the \( \beta_1 \) and \( \beta_2 \)-adrenergic receptors, and the A2a adenosine receptor, confirming the high degree of
transmembrane similarity in the tertiary structure of these GPCRs (Palczewski et al. 2000; Jaakola et al. 2008; Park et al. 2008; Rasmussen et al. 2007; Scheerer et al. 2008; Warne et al. 2008; Cherezov et al. 2007). The three-dimensional structures also confirm altered positioning of GPCR loop regions, particularly of the second extracellular loop (reviewed in Millar and Newton 2010). Greater variance in intracellular portions allow for receptor-specific interactions with intracellular proteins, which is believed to be one of the reasons GPCRs are associated with such a diverse range of functions throughout the central nervous system (CNS) and periphery (Bockaert et al. 2010).

GPCR-mediated signaling cascades begin with activation of the receptor by ligand binding. Binding of an agonist to its GPCR triggers a conformational change to the active conformation of the receptor (Strader et al. 1994). A predicted re-positioning of transmembrane 6, in response to ligand binding, allows alterations in ionic interactions, which result in the receptor’s activation of one or more second messenger systems (Scheerer et al. 2008; Li et al. 2004; Millar and Newton 2010). As indicated in the name of the receptors, all GPCRs are classically known to achieve downstream signaling via coupling to one or more heterotrimeric G proteins. Several intracellular regions are thought to be important for G protein coupling, including a highly conserved glutamic/aspartic acid, arginine, tyrosine (E/DRY) sequence at the amino-terminal of the second intracellular loop, with areas of the third intracellular loop appearing to regulate specificity of receptor-G protein binding (Ballesteros et al. 1998; Scheerer et al. 2008; reviewed in Olah and Stiles 2000; Millar and Newton 2010). Briefly, G proteins are made up of alpha (α), beta (β), and gamma (γ) subunits. When a receptor is activated by an agonist, its conformational change allows the Gα protein to exchange GDP for
GTP, which results in the dissociation of the $\alpha$-subunit from the $\beta$ and $\gamma$-subunits. Both $G\alpha$ and $G\beta/\gamma$ can then activate separate signaling cascades (Strader et al. 1994). Depending on the subtype of the $G\alpha$ subunit, it is commonly responsible for activation ($G\alpha_s$, $G\alpha_{olf}$) or inhibition ($G\alpha_i$) of cyclic amp, as well as activation of other second messengers, and can result in phosphorylation of other proteins, changes in intracellular $Ca^{2+}$ levels, alterations in gene expression, and more (Strader et al. 1994; Gutkind 2000; Bockaert et al. 2010).

However, the classical view of GPCR signaling, where the focus was on $G$ protein-mediated cascades, has been expanded upon greatly in the past two decades. Many GPCRs are also known to activate Mitogen-activated protein kinase (MapK) pathways, although the mechanism of activation is extremely complex, and may vary according to GPCR, as well as cell or tissue type. The existence of the MapK protein was initially discovered (Cooper and Hunter 1981; Cooper and Hunter 1983) and its classical model of signaling has been thoroughly examined in the family of receptor tyrosine kinases (for review, see Gutkind, 2000). Briefly, binding of a growth factor to its receptor tyrosine kinase is known to initiate phosphorylation of several substrates, including the receptor itself. Adaptor proteins, which contain structural domains that are involved in protein-protein interactions (SH2 and SH3 domains are common examples), can then bind the receptor and mediate the exchange of GDP for GTP on Ras, a small GTPase (Schlessinger 1993; van der Geer et al. 1995). This initiates a cascade of phosphorylation on a MapK kinase kinase (Raf is an example), which activates a MapK kinase (Mek is an example), and ends in phosphorylation of a MapK. In mammalian cells, p44 and p42 (also known as Erk1 and Erk2) are thought to be the two major MapKs. MapKs then phosphorylate substrates of their own, including enzymes and nuclear proteins, resulting in the
alteration of gene expression and ultimately, regulation of cell growth and proliferation (Davis 1993).

The GPCR-mediated MapK pathway is not so clearly understood. Not only can downstream GPCR targets interact with other receptor networks, but there are several documented mechanisms by which GPCRs can independently lead to activation of MapKs (for review, see Gutkind 2000; Musnier et al. 2010). Although the Gα subunit can lead to MapK activation; often, the Gβ/γ heterodimer has been shown to initiate GPCR-mediated MapK activation. Phospholipase C, Phosphoinositide 3-kinase (PI3K), and Ras- guanine-nucleotide releasing factor (GRF), and others, have all been shown to be intermediaries between activation of the G protein subunit and activation of the MapK phosphorylation cascade (Berstein et al. 1992, Hawes et al. 1996, Mattingly and Macara 1996, Gutkind 2000; Musnier et al. 2010). Many proteins have intermediate roles in these pathways, and it is thought that various scaffolding proteins may play a role in regulating specificity of signal transmission, dependent on particular GPCR and cell or tissue type.

These classical views of GPCR signaling are well supported, but current research is investigating several relatively novel mechanisms by which GPCR signaling can be modulated. It is becoming apparent that a receptor can assume several different conformations, mediated by different ligands, and that a specific receptor conformation allows selectivity in its interaction with an intracellular signaling complex (Millar and Newton 2010). The specific intracellular signaling components then allow for a specific intracellular signal. Variations in intracellular GPCR interacting proteins can affect GPCR expression, trafficking, post-translational
modifications, and more (Millar and Newton 2010). Research into the mechanisms by which GPCR signaling can be modulated is a rapidly growing field, to expand upon our classical understanding of GPCR signaling.

Termination of GPCR signaling occurs when receptors are internalized or degraded. Internalization, or endocytosis, of GPCRs has been well-studied and shown to involve GPCR kinases (GRKs) and arrestins (Freedman and Lefkowitz 1996). Agonist binding of the receptor stabilizes it in its active conformation, allowing it to be recognized by GRKs, which then phosphorylate the receptor on specific serine/threonine residues. This initiates the recruitment of arrestins, which are in turn responsible for recruitment of elements of the clatharin coat. Receptors are then internalized in a clathrin-coated pit and further processed for recycling to the membrane or degradation (reviewed by Gurevich and Gurevich 2006).

1.1.2 Implication in disease and regulation by intracellular proteins

GPCRs are known to be involved in countless physiological, and more clinically-relevant, pathological processes, including neurological disorders such as Parkinson’s disease, drug addiction, schizophrenia, depression and anxiety disorders, and many more. GPCRs are estimated to be the targets of 30-50% of drugs currently on the market (Robas et al. 2003; Flower 1999). Though many treatments exist for these diseases, it would hardly be an exaggeration to claim that most pharmacological agents have significant limitations, and that new targets and mechanisms for improved drug treatment are constantly being sought.
It has become increasingly evident that GPCRs do not act in isolation; rather a growing body of evidence supports the idea that other receptors and intracellular proteins interact with and regulate the GPCR life cycle and downstream signaling processes (Klinger et al. 2002; Milligan 2005; Kabbani and Levenson 2007; Ferre et al. 2008; Jin et al. 2010). Several cases have been shown where GPCRs form heterodimers and exert synergistic or antagonistic effects on one another. For example, Hillion and colleagues show that the A2a adenosine receptor and D2 dopamine receptor co-localize and co-immunoprecipitate, and upon agonist stimulation at either GPCR, these receptors are co-desensitized and co-internalized (2002). Additionally, the binding of intracellular proteins, such as GRKs and arrestins, is well-documented to be involved in GPCR internalization, recycling, and degradation (reviewed by Freedman and Lefkowitz 1996; Gurevich and Gurevich 2006). Furthermore, intracellular scaffolding proteins have been shown to affect endocytosis, as well as signaling of GPCRs. For example, overexpression of scaffolding proteins Ran Binding Protein 9 and Spinophilin, both documented to interact with the mu opioid receptor, have separately been shown to affect the receptor’s internalization (Talbot et al. 2009; Charlton et al. 2008, respectively). In addition, knockout of Spinophilin in mice demonstrated an increase in measures of mu opioid receptor signaling (Charlton et al. 2008).

In summary, a picture has emerged where GPCRs are no longer viewed as isolated mediators of intracellular signaling; rather, acting in concert with a cohort of regulatory proteins, to achieve downstream affects. These regulatory proteins are increasingly becoming targets for pharmaceutical research, in an effort to treat diseases known to involve specific GPCRs.
1.2 Adenosine Receptors

1.2.1 Subtypes and their distribution

There are four known receptors for the endogenous nucleoside adenosine. All are 7-transmembrane, G protein-coupled receptors. The A1 and A3 subtypes couple to $G_{\alpha_i}$ proteins, thereby inhibiting adenylyl cyclase (Akbar et al. 1994, Freund et al. 1994, Jockers et al. 1994, Gerwins and Fredholm 1995, Freissmuth et al. 1991). A2a and A2b couple to $G_{\alpha_s}$ proteins, stimulating adenylyl cyclase (Olah 1997, Pierce et al. 1992); A2a also couples to the $G_{\alpha_{olf}}$ subtype in the striatum (Kull et al. 2000). Additionally, all four receptors have been implicated in stimulation of IP$_3$ (Akbar et al. 1994, Freund et al. 1994, Gerwins and Fredholm 1995, Offermanns and Simon 1995, Gao et al. 1999, Linden et al. 1999, Palmer et al. 1995), and A1 has also been shown to increase arachidonate and choline, as second messangers (Freissmuth et al. 1991).

Of the adenosine receptors, the A1 and A2a subtypes have the highest expression in the nervous system. A1 is primarily distributed in the cerebral cortex, hippocampus, thalamus, cerebellum, brain stem, and spinal cord. A2a is primarily present in the medium spiny GABAergic neurons of the striatum, as well as the nerve terminals of glutamatergic corticostriatal projections. A2b is ubiquitously expressed at moderate to low levels throughout the nervous system, but has highest expression in colon and bladder. A3 also has low expression in the nervous system, in the hippocampus and cerebellum, though its main distribution is testis and mast cells. Adenosine receptor distribution is further detailed in reviews by Fredholm et al. (2000) and Benarroch (2008).
1.2.2 Ligands

Adenosine, the endogenous ligand for the four adenosine receptors, structurally consists of a purine ring connected to a ribose sugar. Production of adenosine occurs through a variety of pathways. Adenine nucleotides are extracellularly converted to adenosine by ectoenzymes (Dunwiddie et al. 1997). AMP, ADP, and ATP are all rapidly metabolized to adenosine, and cyclic AMP can also be converted to adenosine, although this occurs at a much slower rate (Dunwiddie et al. 1997).

Adenosine acts at one of the four G protein-coupled receptors, through which it has been shown to generate widespread effects in the nervous system and periphery. For example, it has been shown that adenosine accumulation and release occurs in response to hypoxia and tissue damage, and by acting through its receptors, adenosine mediates a protective response (Linden 2005). Seizure prevention, neuroprotection during ischemia or traumatic injury, involvement in Parkinson’s and Huntington’s disease, as well as sleep induction, are all functions that have been attributed to adenosine’s actions in the nervous system (reviewed in Benarroch 2008).

Caffeine is a non-specific antagonist at all four adenosine receptors, though with a lesser affinity for the A3 receptor subtype (Fredholm et al. 2001, Solinas et al. 2005). Present in coffee, teas, chocolate and other foods and beverages, caffeine is the most widely consumed psychoactive agent world-wide, with about 90% of the US population regularly using caffeine-containing substances (Frary et al. 2005). Acting at different CNS adenosine receptor subtypes, caffeine is known to have psychostimulant effects on motor activity, to increase arousal, and
has been shown to have a protective effect on the development of Parkinson’s disease (reviewed in Ferre 2008). The involvement which adenosine and the A2aR have in Parkinson’s disease will be detailed in the following section.

1.2.3 The A2a Receptor and its involvement in Parkinson’s disease

Parkinson’s disease (PD) is a neurodegenerative disorder associated with decreased motor control (Parkinson 1817). The motor symptoms associated with PD include resting tremor, rigidity, slowness of movement, and postural imbalance (reviewed in Abou-Sleiman et al. 2006). Over the past decade, more recognition has been given to the fact that there are a number of non-motor symptoms associated with PD, which include depression, cognitive deficits, dementia, sleep disorders, fatigue, autonomic dysfunction, and repetitive disorders (reviewed by Borek et al. 2006). Average age of PD diagnosis is 57 years. It generally lasts about 10 years, with patients expiring from complications such as infection or aspiration pneumonia. Parkinson’s disease is characterized by a degeneration of cells in a tract running from the substantia nigra to the striatum (the dopaminergic nigrostriatal pathway). Degeneration of these cells results in a depletion of dopamine (DA) in the striatum, causing the motor symptoms associated with the disease (Bernheimer et al. 1973). Prevailing pharmacological treatment for Parkinson’s disease involves the administration of L-dopa, a DA precursor which can cross the blood brain barrier. L-dopa is subsequently converted to DA, in order to compensate for dopamine being depleted through degeneration of the nigrostriatal tract.
As mentioned above, expression of the adenosine A2a receptor (A2aR) is highest in medium spiny GABAergic neurons in the striatum, the brain region where DA is being depleted in PD. In these cells, A2aR and dopamine D2 receptors (D2Rs) are co-expressed and form functional heteromers, and it is thought that ligands binding to both receptors functionally converge at this heteromer, creating a single cellular response (reviewed in Fuxe et al. 2010). The A2aR has been implicated in Parkinson’s disease because caffeine has been shown to have a protective effect on the development of this disease in several different types of studies. This effect is attributed to caffeine’s known antagonism of the A2aR.

Caffeine intake is well-documented as being inversely related to the development of Parkinson’s disease in humans. Specifically, in men, coffee, total caffeine, and caffeine from non-coffee sources, have all been associated with lower risk of PD (Ross et al. 2000). This result was observed, even after control for other factors, such as age, gender, smoking, and intake of milk and sugar. Ascherio et al. (2001) and Benedetti et al. (2000) found similar results in men, showing a linear inverse relationship between caffeine intake and development of PD. Ascherio and colleagues also reported a lower risk of PD in women with moderate coffee intake (1-3 cups/day) or women in the third quintile of caffeine consumption (2001).

Furthermore, a meta-analysis of the studies which report an inverse relationship between coffee drinking and PD was conducted by Hernan et al. (2002), summarizing eight case-control studies and five cohort studies, conducted between the years 1968 and 2001, in four different countries. These authors discuss possible confounding variables to the observed
inverse relationship, but conclude that there is a reliable 30% lower incidence of PD among coffee drinkers than non-coffee drinkers.

Additionally, several lines of evidence have demonstrated an involvement of the A2aR and caffeine in animal models of Parkinson’s disease. The compound 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) was found to cause dysfunction of motor abilities similar to that seen in PD, along with a corresponding loss of dopaminergic neurons in the substantia nigra (Langston and Ballard 1983; Hantraye et al. 1993). MPTP has since been widely-used to model PD in various animal systems (reviewed in Betarbet et al. 2002). Caffeine has been shown to reduce MPTP-induced DA-ergic neurodegeneration (Chen et al. 2001; Boehmler et al. 2009). Furthermore, A2a receptor blockade, or knockout of the A2aR, reduced dopaminergic neurotoxicity in the MPTP model of PD (Chen et al. 2001), supporting the theory that caffeine’s protective effect in PD is mediated through the A2a receptor. The MPTP model of PD is widely used; however, there are several limitations to using MPTP as a model for human PD. Among these limitations, MPTP results in a relatively sudden onset of symptoms, not characteristic of PD in humans, through exposure to a toxin which is not believed to be directly related to the development of PD in humans. Additionally, no animal model of PD demonstrates the progressive degeneration seen over time in humans with this disease.

Alternate animal models for PD, which use pesticides such as paraquat and maneb, induce oxidative stress, and demonstrate a degeneration of dopaminergic cells in the substantia nigra, as well as motor impairment (Ossowska et al. 2005; Somayajulu-Nitu et al. 2009). These models may have greater relevance to pathogenesis of PD in humans, because exposure to
pesticides, and related environmental factors are risk factors for a higher incidence of PD (Ascherio et al. 2006). Caffeine has also been shown to have a protective effect in pesticide models of PD. Specifically, caffeine demonstrates a protective effect against paraquat and maneblack-induced dopaminergic neuron loss in mice (Kachroo et al. 2010; Morelli and Simola 2010).

As mentioned above, it has been shown that A2aRs and D2Rs form functional heteromers in the striatum, and undergo co-aggregation, co-internalization, and co-desensitization in response to treatment with agonists of either receptor (Hillion et al. 2002). Formation of this heterodimer has been confirmed using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) techniques in cotransfected living HEK-293T cells (Canals et al. 2003; Kamiya et al. 2003). One possibility is that caffeine is acting at this heteromer, enhancing striatal D2R signaling, and through this mechanism, achieving its protective effect on the development of Parkinson’s disease (reviewed in Fuxe et al. 2005).

1.2.4 A2a Receptor Interacting Partners

Only a handful of interacting partners, with modulatory effects on receptor regulation and signaling, have been identified for the A2aR to-date. As mentioned above, the A2aR and D2R have been shown to interact; furthermore, this heteromer is a potential target for treatment in Parkinson’s disease (Ferre et al. 1991; Hillion et al. 2002; Canals et al. 2003; Kamiya et al. 2003). Additionally, three other GPCRs, demonstrating a synergistic or
antagonistic relationship with the A2aR have been documented, namely the A1 adenosine receptor (Ciruela et al. 2006), Cannabanoid CB-1 receptor (Carriba et al. 2007), and metabotropic glutamate receptor 5 (Ferré et al. 2002). Only three other regulatory interacting proteins have yet been identified for the A2aR. These include alpha-actinin, which is thought to be important for agonist-induced A2aR internalization (Burgueno et al. 2003), ARNO, shown to be required for A2aR-mediated MapK pathway stimulation (Gsandtner et al. 2005), and the de-ubiquitinating enzyme USP4 (Milojevic et al. 2006). Interestingly, all three of the intracellular proteins shown to interact with the A2aR bind to regions within the C-terminal tail (C-tail) of the receptor. In addition, the binding sites required for formation of A2aR-D2R heteromers have been localized to the third intracellular loop of the D2R and the C-tail of the A2aR (Ciruela et al. 2004). Furthermore, mutation of a phosphorylated serine residue (374), located within the A2aR C-terminal tail, significantly decreased formation of A2aR-D2R heteromers, as indicated by FRET/BRET experiments (Borroto-Escuela et al. 2010).

In the past ten years, the C-terminal tail of GPCRs has been recognized as a major player in forming interactions with transmembrane and intracellular proteins (Bockaert et al. 2003; Bockaert et al. 2004). Structurally, the A2aR has a relatively long C-tail, consisting of 122 amino acids. For comparison, the A1 adenosine receptor C-tail has only 34 amino acids, and the Beta-Adrenergic receptor has 84. The uniquely longer length of the A2aR C-tail may result in a greater propensity for interactions with intracellular proteins in this region of the receptor (Zezula and Freissmuth 2008). Zezula and Freissmuth also discuss the possibility that the A2aR C-tail has greater flexibility than that of other GPCRs, because it lacks a palmitoylated cysteine residue, common to rhodopsin-like GPCRs, which is thought to confer stability to the tail region,
holding it in an α-helical conformation (2008). The increased length, and possibly greater flexibility, of the A2aR C-tail may make it more accessible for interactions with intracellular and transmembrane proteins.
1.3 Ran Binding Protein 9

Though Ran Binding Protein 9 (RBP9) was first isolated as a 55kD protein that bound to Ran, a small nuclear GTP-ase, in a yeast-two-hybrid assay (Nakamura et al. 1998), the same group later identified a longer 90kD version of the protein, which is produced from the same gene. The 90kD version has been shown to immunoprecipitate with a protein complex of over 670kD, which does not appear to contain much Ran (Nishitani et al. 2001). Different localizations have also been reported for the two isoforms of the protein. The 55kD RBP9 was shown in centrosomes; whereas the 90kD version was initially shown to have nuclear and perinuclear localization (Nakamura et al. 1998; Nishitani et al. 2001) and has since also been shown to have membrane association (Denti et al. 2004). All further discussion of RBP9 in this thesis will refer to the 90kD isoform.

RBP9 is a 729 amino acid-long protein. Its structure is made up of a proline rich domain at its N-terminus, followed by a SPRY (SPIa and the RYanodine receptor) domain, important for protein-protein interactions, LISH (LIssencephaly type-1 like Homology), and CTLH (C-Terminal to LisH) domains, and a C-terminal CRA domain (CT11-RanBP9), which includes a nuclear localization sequence. It is ubiquitously expressed in mammalian cell lines and tissue (Poirer et al. 2006).

Though the possible function(s) of RBP9 are still actively under investigation, it is widely thought to be a scaffolding protein. RBP9 has been shown to interact with the intracellular portions of a number of membrane receptors, including several GPCRs, and diverse modulatory effects on receptor-mediated signaling have been described for RBP9 (Murrin and Talbot 2007).
A recent study has also reported the propensity of RBP9 to interact with the transcription-activating domain of the transcription factor GAL-4 (Tucker et al. 2009). This finding, also partially observed in our laboratory, is extremely pertinent to any interaction data which utilizes the yeast 2-hybrid (Y2H) assay. As will be described in the following chapter, reconstitution of yeast GAL-4, resulting from an interaction between bait and prey proteins, is the basis of determining protein interactions in the Y2H assay. More directly put, because RBP9 is capable of interacting with the required portion of GAL-4, theoretically, it should be pulled out as a supposed interactor in every successful Y2H screen. Though this result is not always observed in our laboratory, the limitation this finding suggests for any interaction data acquired from use of the Y2H assay should be recognized. Though some studies detailed in this section, which report an interaction between RBP9 and a receptor protein initially utilize the Y2H system, all interactions that are discussed here have been confirmed through at least one, and often several, other experimental methods. Most studies also report a functional relationship detected as well.

Table 1.1 outlines interactions that RBP9 has been shown to have with various receptor proteins to date, and the proposed functional significance of their interaction. Included in this list are several GPCRs, namely, the metabotropic glutamate receptors 2 and 8 (Seebahan et al. 2008), the mu opioid receptor (MOR; Talbot et al. 2009), and the D1 dopamine receptor (D1R; Rex et al. 2010). Other, non-GPCR receptors, including thyroid receptors, steroid receptors, and receptor tyrosine kinases, have also been shown to interact with RBP9.
### Table 1.1: RBP9 interacts with a wide range of receptor proteins

<table>
<thead>
<tr>
<th>Receptor Name</th>
<th>Receptor Type</th>
<th>Functional Significance of Interaction</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Dopamine Receptor</td>
<td>GPCR</td>
<td>-Interaction of RanBP9 and 10 with both PKC gamma and delta AND D1 DA receptor.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Both RBP9 and 10 OE decreases D1R-stimulated cAMP activity but only RBP9 OE decreases receptor expression, as indicated by a 50% change in binding of D1R-specific radioligand SCH23390.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-There is a RBP9/10-dependent increase in D1R phosphorylation that was blocked by a PKC inhibitor</td>
<td>Rex et al. 2010</td>
</tr>
<tr>
<td>Mu Opioid Receptor</td>
<td>GPCR</td>
<td>-OE of RBP9 decreased DAMGO-mediated internalization of MOR</td>
<td>Talbot et al. 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-No change in Naloxone binding was observed when RBP9 was overexpressed</td>
<td></td>
</tr>
<tr>
<td>metabolotropic</td>
<td></td>
<td>-Merely states interaction</td>
<td>Seebahan et al. 2008</td>
</tr>
<tr>
<td>Glutamate Receptor 2,8</td>
<td>GPCR</td>
<td>-Merely states interaction</td>
<td></td>
</tr>
<tr>
<td>Thyroid hormone receptor Beta2, alpha 1, Beta 1; oestrogen receptor</td>
<td>Thyroid hormone receptor; steroid receptor</td>
<td>-RBP9 interacts with DNA binding domain of thyroid hormone receptors</td>
<td>Poirier et al. 2006</td>
</tr>
<tr>
<td>Axl and Sky</td>
<td>Receptor protein tyrosine kinases</td>
<td>-Merely states interaction</td>
<td>Hafizi et al. 2005</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Integrin adhesion receptor</td>
<td>-RBP9 interacts with and co-localizes with the beta-2 integrin, LFA-1</td>
<td>Denti et al. 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-RBP9 interacts with the beta-1 integrin cytoplasmic domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Transfection of RBP9 synergizes with LFA-1 signaling in transcriptional activation of an AP-1 dependent promoter</td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>Receptor protein tyrosine kinase</td>
<td>-RBP9 interacts with the tyrosine kinase domain of MET</td>
<td>Wang et al. 2002</td>
</tr>
<tr>
<td>Androgen Receptor, Glucocorticoid</td>
<td>Steroid hormone receptor</td>
<td>-RBP9 increased transcriptional activity of AR and GR in response to R1881</td>
<td>Rao et al. 2002</td>
</tr>
</tbody>
</table>
As shown in Table 1.1, there are a wide range of receptor subtypes shown to interact with RBP9. Diverse functional effects on the receptors have been reported for RBP9, including effects on trafficking (MOR), expression (D1R), receptor phosphorylation (D1R), downstream signaling (D1R and MET) and transcriptional activity (thyroid hormone receptors, LFA-1, androgen and glucocorticoid receptors).

Based on this compilation of data, it appears likely that RBP9 has the capacity to interact with several receptor proteins. It is unlikely that this current list is exhaustive. Furthermore, there is a wide range of functional mechanisms attributed to RBP9, for modulation of receptor signaling. For GPCRs, three different modulatory effects have been shown, including a role for RBP9 in receptor trafficking, expression, and phosphorylation. All of these studies pioneer exploration into a functional relationship between GPCRs and RBP9. Further experimentation must be conducted to fine-tune our current understanding of a functional relationship between RBP9 and G protein-coupled receptor proteins.
1.4 Rationale and Hypothesis

G protein-coupled receptors are responsible for mediating the vast majority of signaling across cell membranes. They are involved in numerous physiological processes, such as sensory perception, regulation of elements of the autonomic nervous system including heart rate and blood pressure, and are involved in mediating reward pathways and determining mood. Research has shown that GPCRs are involved in countless human pathologies, including affective disorders, drug addiction, and neurodegenerative diseases. It is estimated that GPCRs are the targets for up to half of currently marketed pharmaceuticals (Robas et al. 2003, Flower 1999), indicating that they are excellent targets for further drug research, aimed at treating the many pathological conditions with which GPCRs are associated. Having a thorough understanding of GPCR mechanisms of action, means of regulation, and functional attributes is of prime importance.

Investigation of GPCR interacting proteins, and subsequent characterization of functional relationships between GPCRs and their binding partners, has widened our understanding of GPCR signaling and regulation. It is becoming evident that GPCRs function within a signalplex and that GPCR- interacting partners provide additional targets for pharmaceutical research (Milligan 2005; Kabbani and Levenson 2007; Ferre et al. 2008; Bockaert et al. 2010).

Parkinson’s disease is a neurological disorder resulting in decreased dopamine transmission in the human striatum. It is the second most common neurodegenerative disorder, after Alzheimer’s disease. The adenosine A2a receptor, a GPCR, has been implicated in
Parkinson’s disease because caffeine, an A2aR antagonist, has repeatedly been shown to have a protective effect on the development of Parkinson’s disease in humans, as well as in multiple animal models of the disease (Ross et al. 2002; Ascherio et al. 2001; Benedetti et al. 2000; Hernan et al. 2002; Chen et al. 2001; Boehmler et al. 2009; Ossowska et al. 2005; Somayajulu-Nitu et al. 2009; Kachroo et al. 2010; Tan et al. 2008; Ascherio et al. 2006; Morelli and Simola 2010). Gaining an understanding of the regulation of the A2aR through investigation of its larger signaling complex may not only further our understanding of general GPCR function, but also assist in the understanding and treatment of Parkinson’s disease.

The basic hypothesis of this work is that the A2aR interacts with intracellular proteins, and that these interacting proteins modulate the receptor’s signaling, processing, or regulation. Targeting these interacting proteins with pharmaceuticals opens another avenue to modulate the normal, or perhaps more importantly, aberrant functions of GPCRs. Previously, searches for interactors of the D2R and MOR have been conducted in our lab (Kabbani et al. 2002; Justice-Bitner et al. in preparation), resulting in the identification of a number of interacting proteins.

Only a handful of interacting proteins for the A2aR have been identified to date. In this thesis, the existence of additional A2aR interacting proteins will be investigated via the Y2H method. A thorough confirmation of the novel interaction between the A2aR and Ran Binding Protein 9 will be made, using GST-Pulldown (GST-PD), Co-immunoprecipitation (Co-IP), and Co-localization assays. Evidence for an interaction between RBP9 and other GPCRs, namely the dopamine D2R and the classical opioid receptors, will also be presented. Furthermore, evidence of an alteration in expression levels of three GPCRs, the A2aR, D2R, and mu opioid receptor
MOR), will be shown in response to RBP9 siRNA knockdown, indicating a functional relationship between RBP9 and several GPCRs. The central thesis of this dissertation is that RBP9 is a promiscuous interactor of GPCRs, and is capable of affecting the expression of at least three GPCRs, the A2a adenosine receptor, the mu opioid receptor, and the D2 dopamine receptor in mammalian cells.
Chapter Two: Identification of A2a Adenosine Receptor Interacting Proteins via the Yeast Two-Hybrid System

2.1 Introduction

In humans, there are four adenosine receptor subtypes, A1, A2a, A2b, and A3. Activation of A1 and A3 are coupled to Go\textsubscript{i} proteins, and a subsequent inhibition of adenylyl cyclase. Activation of A2a and A2b, conversely, cause an increase in adenylyl cyclase. (For review, see Fredholm et al. 2000) Recent studies of GPCRs have investigated a larger signalplex of receptor interacting proteins, to fully understand intracellular processes resulting from receptor activation (Klinger et al. 2002; Milligan 2005; Kabbani and Levenson 2007; Ferre et al. 2008; Zezula and Freissmuth 2008; Jin et al. 2010).

To more fully understand adenosine receptor signaling, this work explores the possible existence of adenosine receptor interacting proteins. The Y2H system was used in order to discover novel A2aR interacting proteins. In this assay, cytoplasmic segments of the A2a adenosine receptor were used as bait and a human brain cDNA library of constructs were used as prey. The A2a adenosine receptor, like other G protein-coupled receptors, is comprised of seven transmembrane domains, connected intracellularly by three cytoplasmic loops and the carboxyl terminal tail. The cytoplasmic regions are thought to be sites for mediating receptor interactions with intracellular proteins.
2.2 Materials and Methods

The Y2H assay, developed by Fields and Song (1989) as a means for identifying protein interactors, is dependent on the yeast GAL4 transcription factor. By dividing the GAL4 DNA-binding domain from its transcription-activating domain, the protein is rendered unable to facilitate transcription. In this state, each domain is fused to potential interacting proteins; a bait protein is connected to the GAL4 DNA-binding domain and a prey protein, either previously identified or acquired from a cDNA library of potential interactors, is linked to the transcription activating domain. If an interaction takes place, the two proteins will come into close proximity, allowing for the reconstitution of GAL4. As a result, transcription of the yeast lacZ gene is now possible, followed by translation to the beta-galactosidase (β-gal) enzyme. This enzyme is capable of catalyzing a chemical conversion which results in blue pigmentation of yeast colonies. Therefore, at the conclusion of the Y2H and β-gal assays, those yeast colonies containing interacting proteins will be easily observable by their blue pigmentation. In a Y2H screen, used to search a library of unknown proteins, the interacting protein must be further identified by isolation of the DNA sequence from the yeast cell, followed by sequence analysis and use of the Basic Local Alignment Search Tool (BLAST) to identify the clone.

To identify novel A2aR interacting proteins, all four intracellular portions of the A2aR were cloned into the pAS 2-1 vector for use as bait in a Y2H screen. The pAS 2-1 vector (BD Biosciences) enables the fusion of a bait protein to the DNA-binding domain of GAL4. An expressed sequence tag (EST) for the human A2aR in the pCMV-SPORT6 vector was obtained from ATCC (Manassas, VA) and DNA was isolated. Forward and reverse primers were designed
to include EcoRI and BamHI restriction enzymes, respectively, for insertion into the pAS 2-1 vector. All primers were obtained from Integrated DNA Technologies. See Table 2.1 for primer sequences.
Table 2.1: Primer sequences for Yeast two-Hybrid Screens

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2aR ICL1 For</td>
<td>GGA GAA TTC GTG TGG CTC AAC AGC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>A2aR ICL1 Rev</td>
<td>GGA GGA TCC GTA GTT GGT GAC GTT</td>
<td>BamHI</td>
</tr>
<tr>
<td>A2aR ICL2 For</td>
<td>GGA GAA TTC GAC CGC TAC ATT GCC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>A2aR ICL2 Rev</td>
<td>GGA GGA TCC TGC TGC CGG TCA CCA AG</td>
<td>BamHI</td>
</tr>
<tr>
<td>A2aR ICL3 For</td>
<td>GGA GAA TTC GCG GAT CTT CCT GGC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>A2aR ICL3 Rev</td>
<td>GGA GGA TCC TGA CTT GGC AGC ATG</td>
<td>BamHI</td>
</tr>
<tr>
<td>A2aR C-tail For</td>
<td>GGA GAA TTC CGT ATC CGC GAG TTC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>A2aR C-tail Rev</td>
<td>GGA GGA TCC GGA CAC TCC TGC TCC</td>
<td>BamHI</td>
</tr>
</tbody>
</table>
Polymerase Chain Reaction (PCR) was conducted using primers listed in Table 2.1 and the A2aR EST as template DNA. PCR products were run on a DNA gel to determine success of PCR based on correct sizes for A2aR intracellular portions. Following PCR, end products were digested and ligated into pAS 2-1 vector at EcoRI and BamHI restriction sites. Finally, constructs underwent sequence analysis to confirm correct insertion into vector and the absence of nucleotide deletions or alterations. Constructs will be referred to hereafter as ICL1-pAS, ICL2-pAS, ICL3-pAS, or C-tail-pAS.

Each intracellular portion was transformed into the MAV3 yeast strain for a Y2H screen. A human fetal brain MATCHMAKER cDNA library, containing approximately $3.5 \times 10^6$ DNA constructs, was obtained from Clontech Laboratories and used to identify putative interactors. Each DNA sequence in the library had been inserted into the pACT 2 vector, which contains the sequence for the GAL4 transcription activating domain.
2.3 Results

All four intracellular segments (ICL1, ICL2, ICL3, and the C-tail) of the A2aR were assessed for use as bait in a Y2H screen. It was found that ICL1 auto-activates this system, as shown by co-transformation of ICL1-pAS and empty pACT 2 vector resulting in blue pigmentation of yeast in the β-gal assay. Therefore, no further investigation of interactors for ICL1 can be made using the Y2H method. A screen was attempted with each of the three remaining intracellular portions of the A2aR; however, an attempt at use of ICL3 as bait did not produce adequate growth of yeast to efficiently conduct a screen. Successful sequential transformations of the remaining two A2aR intracellular portions were made with the cDNA library. Following the transformation of MAV3 yeast with ICL2 and library clones, many yeast colonies grew on quadruple dropout selection plates lacking leucine, tryptophan, adenine, and uracil (-Leu, -Trp, -Ade, -Ura), indicating the possibility that these yeast colonies contained interacting proteins. However, when assessed in the β-gal assay, no yeast colonies yielded apparent interactors, as indicated by the lack of blue pigmentation. Lastly, a successful complete screen was achieved using the A2aR C-tail as bait. This experiment screened approximately 0.5 – 1 million clones, as based upon assessment of transformation efficiency. One hundred-six colonies grew and were re-streaked for confirmation on quadruple dropout selection plates supplemented with 10mM 3-aminotriazole. In the subsequent β-gal assay, 20 colonies showed blue pigmentation, indicating that they contained interacting proteins. Of those, 8 were successfully sequenced, resulting in 6 distinct putative interacting proteins for the intracellular C-terminal tail of the A2aR (Table 2.2).
Table 2.2

<table>
<thead>
<tr>
<th>Putative interacting proteins for the A2aR C-tail</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHD3</strong></td>
<td>Chromodomains DNA binding domain 3</td>
</tr>
<tr>
<td><strong>Aka.</strong> ATP-dependent helicase CHD3; Mi-2 autoantigen 240kD protein; Mi2-alpha; Zinc finger helicase</td>
<td>Chromatin remodeling; Histone deacetylation; Transcriptional Regulation</td>
</tr>
<tr>
<td><strong>SKIV2L2</strong></td>
<td>Superkiller viralicidic activity 2-like 2</td>
</tr>
<tr>
<td><strong>Aka.</strong> ATP-dependent helicase SKIV2L2; Dob1; KIAA0052; MGC142069; Mtr4; fSAP118</td>
<td>Pre-mRNA splicing</td>
</tr>
<tr>
<td><strong>BZW2</strong></td>
<td>Basic Leucine zipper and W2 domains 2</td>
</tr>
<tr>
<td><strong>Aka.</strong> Basic leucine zipper and W2 domain-containing protein 2, HSPC028, MSTP017</td>
<td>Translation initiation?</td>
</tr>
<tr>
<td><strong>RINT1</strong></td>
<td>RAD50 Interactor 1</td>
</tr>
<tr>
<td><strong>ATP6V1G1</strong></td>
<td>ATPase, H+ transporting lysosomal 13 kD, V1 subunit G1</td>
</tr>
<tr>
<td><strong>RBP9/RBPM</strong></td>
<td>Ran Binding Protein 9</td>
</tr>
<tr>
<td></td>
<td>Scaffolding, mediation of receptor signaling</td>
</tr>
</tbody>
</table>
A brief overview of what is known about the first 5 putative interactors will be given, followed by a more detailed account of Ran Binding Protein 9, as the remainder of this thesis will largely focus on this protein and its interaction with the A2aR.

**Chromodomain helicase DNA binding domain 3**

Chromodomain helicase DNA binding domain 3 (CHD3) was first identified in 1997 (Woodage et al. 1997) as a human homologue of a murine gene CHD1, which contains chromatin organization modifier domains, a Snf2-related helicase/ATPase domain and a DNA-binding domain. Little is known about the function of the CHD3 protein to date, although it has been cited as a member of a large complex responsible for histone deacetylase and chromatin remodeling (Tong et al. 1998; Wade et al. 1998; Schultz 2001). The CHD family of proteins in general is thought to be important for chromatin remodeling and transcriptional regulation. The most highly conserved regions between the murine CHD1 and human CHD3 are in the helicase/ATPase domains (68.8% amino acid similarity), with very limited similarity within the DNA-binding domain (17.9%) (Woodage et al. 1997). CHD3 is the only protein in this family that contains PHD zinc finger domains.

The structure of the CHD-3 protein consists of an N-terminal region, followed by the 2 PHD zinc finger domains, 2 chromodomains, an approximately 500 amino acid long helicase domain, and a DNA binding domain of similar amino acid length, followed by a short C-terminal tail. The region pulled out in the Y2H screen with the A2aR tail corresponded to the last 175 amino acids in the C-terminal region of CHD-3.
**Superkiller viralicidic activity 2-like 2**

First isolated from human myeloid KG-1 cell line, Superkiller viralicidic activity 2-like 2 (SKIV2L2) has an ATP/GTP binding site (Nomura 1994), and is thought to be involved in pre-mRNA splicing, based on SKIV2L2 mutations/depletion studies that altered the proper formation of ribosomal proteins (de la Cruz et al. 1998). It has also been shown that a splice site mutation in SKIV2L2 results in defective cell proliferation in zebrafish melanocytes (Yang et al. 2007). The segment of SKIV2L2 pulled out in the Y2H screen using the A2aR C-tail as bait encompassed the first 287 amino acids in the protein sequence, as well as 12 nucleotides upstream from the translation start site.

**Basic Leucine Zipper and W2 Domains 2**

The portion of Basic Leucine Zipper and W2 Domains 2 (BZW2) sequence pulled out in the screen with the A2aR C-tail encompassed nearly all of the 473 amino acid-long protein, minus the N-terminal 131 amino acids and a short 50 amino acid-long region proximal to the C-terminal. Very little is currently known about this protein’s function. Speculation about its role is taken from the known functions of the leucine zipper domain, which are often present in the DNA binding domain of transcription factors.
**RAD-50 Interactor 1**

The portion of RAD-50 Interactor 1 (RINT1) sequence pulled out in the screen with the A2aR C-tail corresponded to the first 260 amino acids of the protein sequence, as well as 102 base pairs upstream of the ATG translational start site.

It has been shown that RINT1 binds Rad50, which has a role in DNA break repair and cell cycle checkpoints. This interaction is specific to late S and G2/M phases of the cell cycle, and a defective G2/M checkpoint was observed in radiation-treated breast cancer cells expressing a truncated RINT1 (Xiao et al. 2001). Therefore, it is thought that RINT1 may be involved in cell cycle control after DNA damage.

**ATPase, H+ transporting lysosomal 13 kD, V1 subunit G1**

ATPase, H+ transporting lysosomal 13kD, V1 subunit G1 (ATP6V1G1) is a small protein, made up of only 117 amino acids. The DNA sequence pulled out in the Y2H screen with the A2aR tail encompassed the entire translated portion, as well as a short region upstream of the ATG translational start site, and a long sequence, approximately 350 base pairs following the translational stop codon.

ATP6V1G1 is a component of the multi-subunit enzyme, vacuolar ATPase, which regulates acidification of intracellular organelles. This process plays several significant roles in the cell, including receptor-mediated endocytosis and formation of proton gradients in synaptic vescicles (Stevens and Forgac 1997).
**Ran Binding Protein 9**

As detailed in the Literature Review, RBP9 is a protein just over 700 amino acids in length. Its major domains include a proline-rich N-terminus, a SPRYdomain, which is important for protein-protein interactions, LISH and CTLH (LISsencephaly type-1 like Homology and C-Terminal to LiSH) domains, and a C-terminal CRA domain, including a Nuclear Localization Sequence. RBP9 is shown to have subcellular localization to the plasma membrane (Denti et al. 2004), cytoplasmic regions, and nucleus (Rao et al. 2002) and is ubiquitously expressed in tissue and mammalian cell lines.

The DNA sequence that was pulled out in the Y2H screen with the A2aR tail encompassed a middle region, ranging from amino acid 112 to amino acid 425. This region spanned the entire SPRY domain and LiSH domains, but did not include either the N-terminal proline-rich domain or C-terminal regions.

As mentioned previously, RBP9 is thought to be a scaffolding protein, with a number of interactions documented for a variety of receptor proteins. These interactions and relevant functional relationships are cataloged in Table 1.1. GPCRs previously published as interacting with RBP9 include the mu opioid receptor (MOR; Talbot et al. 2009), the D1 dopamine receptor (D1R; Rex et al. 2010) and the metabotropic glutamate receptors 2 and 8 (mGluR2 and mGluR8; Seebahn et al. 2008).

Because there is evidence of RBP9 interacting with a number of GPCRs, in addition to the A2aR C-tail screen conducted in our laboratory, data pertaining to the specific binding site(s) of RBP9 to GPCRs would be valuable.
Data reported concerning the D1 dopamine receptor consisted of co-immunoprecipitation and co-localization experiments using full-length RBP9 and receptor; therefore, no specific information can be ascertained regarding the binding sites required on either protein (Rex et al. 2010). In regards to the mu opioid receptor, a Y2H screen using cDNA encoding Asn$^{332}$-Pro$^{399}$ of the MOR C-terminal tail pulled out a large portion of RBP9, ranging from Asn$^{141}$-His$^{729}$, where the translated portion is terminated, as well as additional 3’ untranslated material (Talbot et al. 2009). For the glutamate receptors, Y2H assays and GST-pulldown experiments utilizing the C-terminal tail of these receptors indicated an interaction with RBP9. Furthermore, the CTLH domain and adjacent portion leading up to, but not including, the C-terminal CRA domain of RBP9 specifically interacted with the C-tail of mGlu2, whereas other portions of RBP9 did not (Seebahn et al. 2008).

Because three of the GPCRs known to interact with RBP9 (A2aR, MOR, and mGluR2) specifically implicate the C-tail regions of the receptor, comparisons of the regions used in these experiments were conducted using an online alignment tool from The European Molecular Biology Open Software Suite, the EMBOSS pairwise alignment algorithm, which can be accessed at http://www.ebi.ac.uk/Tools/emboss/align/index.html.

Results of these alignments showed only limited identity and similarity between receptor tail regions. Specifically, full C-terminal A2aR and MOR sequences reported to interact with RBP9 show only a 14.4% identity and an 18.9% similarity. Regions for the A2aR and mGluR2 showed only a 3.2% identity and 7.1% similarity. The MOR and mGluR2 C-terminal regions showed the least identity (2.7%) and similarity (5.4%).
Figure 2.1 illustrates alignments of the A2aR and MOR C-tails; Figure 2.2 shows alignments of A2aR and mGluR2 tail regions; and Figure 2.3 compares C-tails of mGluR2 and MOR.
**Figure 2.1** - comparison of A2aR (top line) and MOR (bottom line) C-terminal regions reported to interact with RBP9

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**Figure 2.2** - comparison of A2aR (top line) and mGluR2 (bottom line) C-terminal regions reported to interact with RBP9

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**Figure 2.3** - comparison of MOR (top line) and mGluR2 (bottom line) C-terminal regions reported to interact with RBP9

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<tr>
<td>EMBoss_001</td>
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</table>
2.4 Conclusions

In the interest of identifying novel interactors of the adenosine A2a receptor, Y2H screens were attempted for all four intracellular portions of receptor. As the first intracellular loop auto-activated the Y2H assay, no conclusion can be made about potential interactors for that region. The second intracellular loop appeared to produce candidate interactors which grew on quadruple selection plates, however, did not pass the β-gal assay. The third intracellular loop, though able to grow isolated MAV103 yeast colonies, did not produce sufficient growth in media to conduct a thorough screen. However, a screen using the forth intracellular portion, the C-terminal tail, resulted in six proteins that are potentially interactors for this region.

The C-tail of the A2aR is the longest intracellular portion of this receptor, including 122 amino acids. Other intracellular portions used to conduct screens are 13, 20 and 36 amino acids, respectively, for the ICL1, ICL2, and ICL3. One possibility is that smaller portions, in isolation, are unable to assume their final structure, limiting their ability to interact with other proteins.

A well-acknowledged limitation of the Y2H assay is its propensity to produce false positives. Therefore, significant effort must be made to confirm the interactions initially discovered through this method. A number of candidate interacting proteins which emerged from the A2aR C-tail screen have functions related to DNA and RNA processing, in the initial efforts to produce a protein. Because the A2aR is not believed to localize to the nucleus or have any relationship to these processes, those possible interacting proteins were not pursued.
One candidate A2aR interactor, RBP9, appeared of great interest, largely because of other reports which illustrated its interaction with receptor proteins and, in particular, with other GPCR proteins. Though several possible roles for RBP9 and its interaction with receptor proteins have been supported, its cellular function is still in the process of being elucidated. Based on the literature and our observations, it is possible that RBP9 is a more general interactor for GPCRs than has previously been believed, and its potential role in GPCR regulation is of significant interest.

In an initial attempt to identify potential binding regions between GPCRs and RBP9, alignments of three GPCR C-tail regions, all reported to interact with RBP9, were performed. The A2aR C-tail and MOR C-tail showed a higher degree of similarity than either the A2aR and mGluR2, or the MOR and mGluR2; however, no consensus sequence between the three receptors could be identified as a possible binding site for RBP9. Therefore, this leaves the possibilities that quaternary structure is key, or that post-translational modifications, such as phosphorylation of these proteins, are implicated in the interaction.

As mentioned in the previous chapter, RBP9 has the ability to interact with the transcription activating domain of the GAL-4 protein. Therefore, several additional experiments must be conducted to determine the validity of this possible interaction. Because of a number of reported instances where other GPCRs interact with RBP9, it is worthwhile to continue investigation of the interaction between the A2aR and RBP9, despite its limited relevance as a prey protein in the Y2H assay. Several different experiments assessing this possible interaction will be presented in the following chapter.
Chapter Three: Confirmation of the interaction between RBP9 and the A2aR and other GPCRs

3.1 Introduction

The Y2H method was utilized to identify potential interacting proteins for the C-terminal tail of the A2a adenosine receptor, as detailed in the previous chapter. This is an efficient way to screen a large number of clones for putative interactors. However, as the Y2H assay is known to produce a significant number of false positive results, all potential interacting proteins must be subject to several additional experiments to confirm the validity of the interaction.

In order to confirm the initial data produced in the Y2H screen, three distinct experiments were used to assess protein interactions. Glutathione-S-Transferase-pulldowns, Co-immunoprecipitations, and Co-localizations, as used in the following experiments, each have individual strengths and limitations.

Glutathione-S-Transferase-pulldown (GST-PD) experiments investigate a direct interaction between two proteins under in vitro conditions. The advantage of this method is that there are no additional proteins present that could possibly perform a linking function between the two proteins of interest. However, the disadvantage is that the system is artificial; it is not taking place in an actual mammalian cell. Additionally, the data reported using the GST-PD assay, to some degree, localizes the interaction on each protein, because only partial protein segments were used in these experiments.

Co-immunoprecipitation (Co-IP) experiments report an interaction, either direct or indirect, in an in vivo environment. The advantage of this assay is that the context of the
interaction is a mammalian cell, which is of greater physiological relevance than an *in vitro* experiment. In these experiments, full length proteins were used to investigate an interaction. Additionally, it is of value to identify a cell line which endogenously expresses both proteins of interest. Co-IPs presented in this chapter were performed using two cell lines, HEK-293 cells, which endogenously express RBP9 but must be transfected with A2aR, and SHSY5Y neuroblastoma cells, which endogenously express both proteins. The limitation of this assay is that it allows for the possibility that the two proteins of interest do not *directly* interact, but are bound within a complex of proteins.

Co-localization experiments allow a researcher to visualize the location of two or more proteins within a mammalian cell using fluorescent secondary antibodies. The advantage of this experiment is that the location of two or more proteins can be observed within a cell, showing any overlap in localization, as well as the region(s) in which each protein is enriched, for example, in the nucleus, at the membrane, or within intracellular organelles. The limitation to this assay is that it has the capacity only to report a co-localization of two proteins, *not* an interaction. As with Co-IPs, it is of greater physiological relevance to identify a cell line in which both proteins of interest are endogenously expressed within the same cell.

Each of the above-outlined assays are utilized in this chapter to confirm the interaction between the adenosine A2a receptor and RBP9. Additionally, Co-IPs were conducted between RBP9 and other GPCRs, namely, the D2-long dopamine receptor (D2LR), and each of the classical opioid receptors, the mu-opioid receptor (MOR), the kappa-opioid receptor (KOR), and
the delta-opioid receptor (DOR), to assess the possibility that RBP9 not only interacts with the A2aR, but is a more promiscuous interactor of G protein-coupled receptor proteins.
3.2 Materials and Methods

3.2.1 GST Pulldown

3.2.1.1 Primer Design, Construct Formation, and Protein Production

In order to create GST-fusion proteins and S-tagged proteins to be used in a GST-PD experiment, primers were designed to insert the C-tail and ICL2 of the A2aR into the pGEX-4T-1 expression vector (containing a GST-tag; Amersham Biosciences, Piscataway, NJ) and a fragment of RBP9 which was pulled out as prey in the Y2H screen, including the SPRY and LiSH domains, into the Pet30a expression vector (containing an S-tag). See Table 3.1 for primer sequences.
Table 3.1: Primer Sequences for GST-Pulldown experiments

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<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Site</th>
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<td>RBP9 For</td>
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<td>Bam H I</td>
</tr>
<tr>
<td>RBP9 Rev</td>
<td>CTC GAG GGT CTG CCA TTC TCC TTC TCG ATC</td>
<td>Xho I</td>
</tr>
<tr>
<td>A2aR ICL2 For</td>
<td>GGA TCC GAC CGC TAC ATT GCC ATC CGC</td>
<td>BamH I</td>
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<tr>
<td>A2aR ICL2 Rev</td>
<td>CTC GAG AGC CCT CGT GCC GGT CAC CAA</td>
<td>Xho I</td>
</tr>
<tr>
<td>A2aR C-tail For</td>
<td>GTT GGA TCC CGT ATC CGC GAG TTC CGC CAG</td>
<td>BamH I</td>
</tr>
<tr>
<td>A2aR C-tail Rev</td>
<td>GCC CTC GAG TCA TCA GGACAC TCC TGC TCC</td>
<td>Xho I</td>
</tr>
</tbody>
</table>
PCR was conducted using primers listed in Table 3.1 and an A2aR EST or HEK-293 cDNA as template. PCR products were run on a DNA gel to determine success of PCR based on correct sizes for A2aR intracellular portions and RBP9. Following PCR, end products were digested and ligated into the PGEX-4T-1 or Pet30a expression vectors at BamH I and Xho I restriction sites. Finally, constructs underwent sequence analysis to confirm correct insertion into vector and the absence of nucleotide deletions or alterations. Constructs will be referred to hereafter as GST-A2aR C-tail, GST-A2aR ICL2 or RBP9 S-tag.

Constructs were transfected into the *Escherichia coli* strain BL21 (DE3) and proteins induced using the ZYP-5052 auto-induction media, as previously described by Studier (2005; also De Cotiis et al., 2008).

### 3.2.1.2 Experimental Procedures

The GST-PD utilizes Glutathione Sepharose 4B beads (GS4B; GE Healthcare, Piscataway, NJ) to immobilize a GST-fusion protein, in these experiments, either GST-A2aR ICL2 or GST-A2aR C-tail. After incubation of the GST-fused protein with RBP9 S-tag, mixtures are washed several times with 0.1 M phosphate-buffered saline, pH 7.4 (PBS), to remove unbound protein and run on an SDS polyacrylamide gel. Proteins are subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane is then blocked in Tris-buffered saline with Tween-20 (TBST; 20 mM Tris-base, pH 7.4, 275 mM NaCl, 3 mM KCl, 1% Tween-20) containing 10% non-fat milk, for at least one hour at room temperature or overnight at 4°C. Membranes are then probed using horseradish peroxidase (HRP) conjugated S-tag antibody
(1:5000 dilution, Novagen, Madison, WI) in TBST solution containing 5% non-fat milk for one hour, at room temperature. After antibody incubation, the membrane is washed at least three times with TBST to remove excess antibody. Membranes are then treated with Enhanced Chemiluminescence (ECL) Plus reagent (GE Healthcare) and immunoreactive proteins are observed by exposure on autoradiography film (Amersham Hyperfilm ECL; GE Healthcare).

### 3.2.2 Co-Immunoprecipitation

#### 3.2.2.1 A2aR Primer Design, Construct Formation, Cell Culture, and Transfections into HEK-293 cells

In order to conduct Co-Immunoprecipitation experiments, we desired to have a full-length A2aR construct with a histochemical tag. Primers were designed to insert full-length A2aR into the pCMV-3B vector, containing a -myc tag. See Table 3.2 for primer sequences.
Table 3.2: Primer Sequences for Cell Culture Experiments

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<tr>
<th>Primer</th>
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<td>Hind III</td>
</tr>
<tr>
<td>A2aR Full Length Rev</td>
<td>GAA CTC GAG TCA TCA GGA CAC TCC TGC TCC</td>
<td>Xho I</td>
</tr>
</tbody>
</table>
As described above, PCR was conducted using primers listed in Table 3.2 and A2aR EST as template. PCR product was run on a DNA gel to determine success of PCR based on correct size for full length A2aR. Digest and ligation were performed to insert full length A2aR into the pCMV-3B vector using Hind III and Xho I restriction sites. Sequence analysis confirmed correct insertion into vector and the lack of nucleotide deletions or alterations. Construct is hereafter referred to as A2aR-myc.

Human Embryonic Kidney (HEK) 293 cells and SHSY5Y cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified chamber containing 5% CO₂. For Co-IP experiments, A2aR-myc was transiently transfected into Hek-293 cells using Effectene transfection reagent (Qiagen, Valencia, CA) according to manufacturer’s instructions.

3.2.2.2 D2LR, MOR, KOR, DOR- HEK-293 Cell Culture

Several HEK-293 cells lines, stably transfected with Flag-tagged GPCRs were used for Co-IP experiments. HEK-293 cells stably expressing the D2 long dopamine receptor (D2LR-HEK), mu opioid receptor (MOR-HEK), or delta opioid receptor (DOR-HEK) were all generous gifts from Dr. Mark von Zastrow, University of California, San Francisco. HEK-293 cells stably expressing the kappa opioid receptor (KOR-HEK) were a generous gift from Dr. Liu Chen (Temple University, Philadelphia). All cell lines were maintained in DMEM supplemented with 10% FBS and 400 μg/ml geneticin (Invitrogen, Carlsbad, CA). As with other cell lines described above, D2L-Heks,
MOR-HEKs, DOR-HEKs, and KOR-HEKs were incubated at 37°C in a humidified chamber containing 5% CO₂.

3.2.2.3: Experimental Procedure

For Co-IP experiments involving the A2aR, whole cell lysates were harvested from either SHSY5Y cells or A2aR-myc transiently-transfected HEK-293 cells. For those involving either the D2LR or any of the classical opioid receptors, cell lysates were harvested from HEK-293 cells stably expressing the specific receptor. Briefly, cells were resuspended in Tris-based lysis buffer (containing 1% Triton) and sonicated to break up whole cells and membranes. Lysates were solubilized by nutation for 20 minutes and centrifuged for 20 minutes at 12,000xg. Supernatant was collected and protein concentration assessed using Bradford reagent (Bio Rad Laboratories, Hercules, CA).

For Co-IP experiments involving the A2aR, cell lysates (500μg) were incubated with 3μL mouse monoclonal anti-A2aR antibody (Abnova) or 3μL mouse monoclonal anti-myc antibody (Upstate Biotechnology) overnight at 4°C, to immunoprecipitate endogenous or transfected A2aR. GammaBind Protein G Sepharose beads (GE Healthcare) were then incubated with lysates and antibody for 4 hours at 4°C, to immobilize immunocomplexes. Mixtures were washed 4-5 times with cold 0.1 M PBS, pH 7.4, to remove unbound proteins. An identical protocol was followed for Co-IP experiments involving the D2LR, MOR, DOR, and KOR, except that 3μL M2-Flag mouse monoclonal antibody (Sigma) was used to immunoprecipitate the receptor protein from cell lysate, prior to bead incubation.
Western blots were performed by running washed immunocomplexes on an SDS-polyacrylamide gel, combined with 2x SDS-PAGE loading dye (125 mM Tris-HCL, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue, 20% glycerol) and then transferred to PVDF membranes. Membranes were blocked in TBST containing 10% non-fat milk, overnight at 4°C. For antibody incubation, membranes were probed with rabbit polyclonal anti-RBP9 (Santa Cruz) primary antibody at a 1:200 dilution, in TBST containing 5% non-fat milk, for one hour at room temperature. Membranes were thoroughly washed with TBST to remove any unbound primary antibody, before incubating with HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) at a 1:20,000 dilution. Again, to remove excess antibody, blots were washed thoroughly with TBST, and proteins were visualized using ECL Plus reagent and exposure on autoradiography film.

3.2.3 Co-localization

3.2.3.1 Construct and Transfection

As described previously, HEK-293 cells and SHSY5Y cells were maintained in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified chamber containing 5% CO₂. As in Co-IP experiments, described in Section 3.2.2.1, A2aR-myc was transiently transfected into HEK-293 cells using Effectene transfection reagent (Qiagen, Valencia, CA) as instructed by the manufacturer. For fluorescent microscopy, cells were grown on collagen-coated coverslips (BD Biosciences, Bedford MA) for 24 hours after transfection.
3.2.3.2 Experimental Procedure

To visualize A2aR and RBP9 proteins, cells were fixed using 4% paraformaldehyde for 15 minutes, followed by three PBS washes, and blocked for one hour at room temperature in PBS-based block buffer (containing 5% goat serum and 0.1% Triton). Dual-labeling of RBP9 and A2aR was conducted using mouse monoclonal A2aR antibody (Abnova) and rabbit polyclonal RBP9 antibody (Santa Cruz), or no primary antibody control, for one hour at room temperature. Three PBS washes removed any unbound primary antibody. Secondary antibody incubations were conducted at room temperature for 30 minutes, using Cy2-conjugated goat ant-rabbit and rhodamine-conjugated goat anti-mouse (Jackson Immunoresearch, West Grove, PA). Three PBS washes removed unbound secondary antibody. Coverslips were then mounted on microscope slides with ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, Oregon). Fluorescent images were obtained with a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY), and digital images were captured and imported with the LSM 5 image browser (Carl Zeiss Inc.).
3.3 Results

3.3.1 GST Pulldown

In order to confirm the direct interaction between the A2aR C-tail and RBP9, we assessed the ability of a truncated portion of RBP9, including the SPRY and LISH domains, to associate with a GST-A2aR C-tail fusion protein in a GST-PD experiment.

Figure 3.1 shows a Western blot, containing bacterial lysate expressing the RBP9 S-tag truncation protein, which produces an immunoreactive band of ~32 kDa when probed with anti-S-tag antibody (Lane 1). This size band corresponds to the expected size of the truncated portion of RBP9. An identical band is seen when bacterial lysate containing RBP9 S-tag was incubated with GST-A2aR C-tail coated beads (Figure 3.1, Lane 4), but not when RBP9 S-tag lysate was incubated with GST protein coated beads (Figure 3.1, Lane 2) or with beads alone (Figure 3.1, Lane 3). These results indicate a direct interaction between the A2aR C-tail and RBP9 partial proteins in bacterial lysate.
Figure 3.1: GST-A2aR C-tail pulls down RBP9 S-tag from bacterial lysate. A 32kD fragment of RBP9 produced in bacterial lysate is shown in Lane 1. Control experiments using GST protein and beads incubated with RBP9 S-tag do not exhibit an interaction (Lanes 2 and 3, respectively). GST-A2aR C-tail interacts with RBP9 S-tag partial protein (Lane 4).
Additionally, a GST-PD experiment was performed between A2aR ICL2 and the same truncated portion of RBP9 as described for Figure 3.1. As shown again in Figure 3.2, the RBP9 S-tag truncation protein produces an immunoreactive band of ~32 kDa (Lane 1). The same band is seen when bacterial lysate containing RBP9 S-tag was incubated with GST-A2aR ICL2 coated beads (Figure 3.2, Lane 4), but not when RBP9 S-tag lysate was incubated with GST protein coated beads, or with beads alone (Figure 3.2, Lanes 2 and 3, respectively). These results indicate a direct interaction also between the A2aR ICL2 and RBP9 partial proteins.
**Figure 3.2**

GST tagged A2aR-ICL2 pulls down Ran BP9-Stag

**Figure 3.2:** GST-A2aR ICL2 pulls down RBP9 S-tag from bacterial lysate. A 32kD fragment of RBP9 produced in bacterial lysate is shown in Lane 1. Control experiments using GST protein and beads incubated with RBP9 S-tag do not exhibit an interaction (Lanes 2 and 3, respectively). GST-A2aR ICL2 interacts with RBP9 S-tag partial protein (Lane 4).
3.3.2 Co-Immunoprecipitation

In order to assess the interaction of RBP9 and the A2aR full-length proteins *in vivo*, Co-IP experiments were performed in both HEK-293 cells transiently transfected with A2aR-myc, and SHSY5Y cell lines, which express both RBP9 and A2aR endogenously.

Figure 3.3 shows a Western blot containing HEK-293 cell lysate expressing full length RBP9, which produces an immunoreactive band of 90kDa when probed with an antibody specific to RBP9 (Lane 1). This size band corresponds to the expected size of full-length RBP9. An identical band is seen when HEK-293 cell lysate containing RBP9 was incubated with an A2aR-specific antibody (Figure 3.3, Lane 3), but not when HEK-293 cell lysate was incubated with beads alone (Figure 3.3, Lane 2). These results indicate an interaction between full length A2aR and RBP9 proteins in mammalian cells.
Figure 3.3: RBP9 immunoprecipitates with A2aR in A2aR-myc transfected HEK-293 cells using an anti-A2aR antibody. Endogenously expressed, 90kD RBP9 is shown in Lane 1. Control experiment, using no IP antibody does not exhibit an interaction (Lane 2). Anti-A2aR antibody immunoprecipitates RBP9 in HEK-293 cells (Lane 3).
In order to confirm the specificity of the A2aR antibody in the above experiment, a Co-IP was conducted under identical cell conditions as those reported for Figure 3.3, except that an antibody to the -myc histochemical tag on the A2aR construct was used to IP the receptor.

Figure 3.4 demonstrates RBP9 in HEK-293 lysate (Lane 1). That same band is seen when HEK-293 lysate containing transfected A2aR-myc is incubated with anti-myc antibody (Figure 3.4, Lane 3), but not when lysate is incubated with beads alone (Figure 3.4, Lane 2).
**Figure 3.4**

IP: anti-myc
IB: anti-RBP9
A2aR- transfected HEK 293 cells

**Figure 3.4**: RBP9 immunoprecipitates with A2aR in A2aR-myc transfected HEK-293 cells using an anti-myc antibody. Endogenously expressed, 90kD RBP9 is shown in Lane 1. Control experiment, using no IP antibody does not exhibit an interaction (Lane 2). Anti-myc antibody immunoprecipitates RBP9 in HEK-293 cells (Lane 3).
Additionally, a Co-IP experiment was performed between full length A2aR and RBP9 in SHSY5Y neuroblastoma cells endogenously expressing both proteins. As shown in Figure 3.5, a Western blot containing SHSY5Y cell lysate expressing RBP9 produces an immunoreactive band of 90 kDa when probed with an antibody specific to RBP9 (Lane 1). An identical band is seen when SHSY5Y cell lysate containing RBP9 was incubated with an A2aR-specific antibody (Figure 3.5, Lane 4), but not when SHSY5Y lysate was incubated with beads alone (Figure 3.5, Lane 2) or when SHSY5Y lysate was incubated with a control IP antibody, recognizing GFP and raised in the same species as the A2aR IP antibody (Figure 3.5, Lane 3). These results indicate an interaction between full length, endogenously expressed A2aR and RBP9 proteins in neuronal mammalian cells.
**Figure 3.5:** RBP9 immunoprecipitates with endogenous A2aR in SHSY5Y cells using an anti-A2aR antibody. Endogenously expressed, 90kD RBP9 is shown in Lane 1. Control experiments, using no IP antibody (Lane 2) and mouse GFP IP antibody (Lane 3) do not exhibit an interaction. Anti-A2aR antibody immunoprecipitates RBP9 in SHSY5Y cells (Lane 4).
To assess the possible interaction between RBP9 and other G protein-coupled receptor proteins, Co-IP experiments were also conducted using D2LR-HEK, MOR-HEK, DOR-HEK, and KOR-HEK cell lines, which stably express the GPCRs.

Figure 3.6 shows a Western blot containing D2LR-HEK-293 cell lysate expressing full length RBP9, at 90 kDa, when probed with an antibody specific to RBP9 (Lane 1). The same band is seen when D2LR-HEK-293 cell lysate containing RBP9 was incubated with an M2-Flag antibody (Figure 3.6, Lane 3), but not when D2LR-HEK-293 cell lysate was incubated with beads alone (Figure 3.6, Lane 2). These results indicate an interaction between the D2L dopamine receptor and RBP9 in mammalian cells.
Figure 3.6: RBP9 immunoprecipitates with D2LR in D2LR-flag stably transfected HEK-293 cells using an anti-Flag antibody. Endogenously expressed, 90kD RBP9 is shown in Lane 1. Control experiment, using no IP antibody does not exhibit an interaction (Lane 2). Anti-Flag antibody immunoprecipitates RBP9 in D2LR-HEK-293 cells (Lane 3).
Likewise, in Figure 3.7, a Western blot containing MOR-HEK-293 cell lysate shows full length RBP9, at 90 kDa, when probed with a RBP9-specific antibody (Lane 1). As in the previous experiments, the same band is seen when MOR-HEK-293 cell lysate containing RBP9 was incubated with an M2-Flag antibody (Figure 3.7, Lane 3), but not when MOR-HEK-293 cell lysate was incubated with beads alone (Figure 3.7, Lane 2). These results indicate an interaction between the mu opioid receptor and RBP9 in mammalian cells.
Figure 3.7

Figure 3.7: RBP9 immunoprecipitates with MOR in MOR-flag stably transfected HEK-293 cells using an anti-Flag antibody. Endogenously expressed, 90kD RBP9 is shown in Lane 1. Control experiment, using no IP antibody does not exhibit an interaction (Lane 2). Anti-Flag antibody immunoprecipitates RBP9 in MOR-HEK-293 cells (Lane 3).
DOR and KOR- stably expressing HEK-293 cells were also used to investigate a possible interaction between these GPCRs and RBP9. In Figure 3.8, a Western blot containing DOR-HEK-293 cell lysate shows full length RBP9, at 90 kDa, when probed with a RBP9-specific antibody (Lane 1). As in the previous experiments, a similar-sized band is seen when DOR-HEK-293 cell lysate containing RBP9 was incubated with an M2-Flag antibody (Figure 3.8, Lane 3), but not when DOR-HEK-293 cell lysate was incubated with mouse GFP antibody, a negative control IP antibody raised in the same species as the Flag IP ab (Figure 3.8, Lane 2). KOR-HEK-293 cell lysate also shows full length RBP9 at 90 kDa, using a RBP9-specific antibody (Figure 3.8, Lane 4). Again, a 90kD band is seen when KOR-HEK-293 cell lysate containing RBP9 was incubated with an M2-Flag antibody (Figure 3.8, Lane 6), but not when KOR-HEK-293 cell lysate was incubated with the mouse GFP antibody (Figure 3.8, Lane 5). These results indicate an interaction between both the delta and kappa opioid receptors and RBP9 in mammalian cells.
Figure 3.8

Figure 3.8: RBP9 immunoprecipitates with DOR and KOR in stably-transfected HEK-293 cells expressing each receptor, using an anti-Flag antibody. Endogenously expressed, 90kD RBP9 is shown in DOR-HEK and KOR-HEK cell lysates (Lanes 1 and 4, respectively). Anti-Flag antibody immunoprecipitates RBP9 in DOR-HEK-293 cells (Lane 3) and in KOR-HEK-293 cells (Lane 6). Control experiments, using a GFP IP antibody, do not exhibit an interaction (Lanes 2 and 5).
3.3.3 Co-localization

In order to assess localization and possible co-localization of full-length RBP9 and A2aR in vivo, dual-labeling of proteins was conducted in two mammalian cell lines, HEK-293 cells transiently transfected with A2aR-myc, and SHSY5Y cells endogenously expressing both proteins.

Seen in Figure 3.9, SHSY5Y neuroblastoma cells express endogenous A2aR, as observed when probed with a primary antibody specific to the A2aR and a rhodamine fluorescent secondary antibody (red, top left panel). A2aR can be seen perinuclearly with some membrane association in this experiment. This cell line also endogenously expresses RBP9, as can be observed when probed with a primary antibody specific to RBP9 and a Cy2 fluorescent secondary antibody (green, top right panel). RBP9 is shown perinuclearly, as well as in the nucleus. Overlay is shown with and without DAPI nuclear staining (blue, bottom left and right panels, respectively). These results indicate an overlap (yellow) in localization between endogenous A2aR and RBP9 proteins in mammalian cells.
Figure 3.9: Endogenous A2aR and RBP9 co-localize in SHSY5Y neuroblastoma cells. In the top left panel, A2aR is recognized by A2aR-specific primary antibody and rhodamine (red) secondary antibody. In the top right panel, RBP9 is recognized by RBP9-specific primary antibody and Cy2 (green) secondary antibody. Co-localization shown in bottom left and right panels. Blue is DAPI nuclear staining.
Similar to Figure 3.9, Figure 3.10 shows localization and co-localization of A2aR and RBP9. In this experiment, HEK-293 cells transiently transfected with full-length A2aR and endogenously expressing RBP9 were stained with primary antibodies specific to A2aR (red, top left panel) and RBP9 (green, top right panel) using rhodamine and Cy2 fluorescent secondary antibodies, respectively. Bottom left panel shows overlay with DAPI nuclear staining and bottom right panel shows overlay without DAPI nuclear staining. In this cell line, A2aR largely shows membrane association. RBP9 is strongly expressed throughout the cytoplasm, as well as at the membrane. In the overlay, co-localization of A2aR and RBP9 can be seen at the membrane (orange).
Figure 3.10: Transiently transfected A2aR and endogenous RBP9 co-localize in HEK-293 cells. In the top left panel, A2aR is recognized by A2aR-specific primary antibody and rhodamine (red) secondary antibody. In the top right panel, RBP9 is recognized by RBP9-specific primary antibody and Cy2 (green) secondary antibody. Co-localization (orange) is shown in bottom left and right panels. Blue is DAPI nuclear staining.
3.4 Conclusions

In an effort to confirm the interaction between RBP9 and A2aR, as well as other GPCR proteins, GST-PD, Co-IP, and Co-localization experiments were conducted and presented above. The GST-PD experiment assesses a direct interaction between specific regions of the proteins, whereas the Co-IP experiments examine an interaction between full-length proteins in actual mammalian cells. Additionally, Co-localization experiments allow one to visualize the intracellular localization of each protein individually *in vivo*, and show the possible overlap of two or more proteins of interest.

Throughout these experiments, conducted using bacterial lysates or several mammalian cell lines, three different antibodies recognizing full-length or truncated forms of A2aR (an A2aR-specific antibody, a GST-antibody, and a myc-tag antibody) and two different antibodies recognizing full-length or truncated versions of RBP9 (a RBP9-specific antibody and an S-tag antibody) were all used to identify a positive interaction between these two proteins. Controls, to rule out a possible artifactual interaction of RBP9 with Glutathione Sepharose beads, GST-protein, Protein G beads, and a non-related antibody from the same species as other IP antibodies, recognizing GFP, all showed no interaction. Additionally, the A2aR and RBP9 were shown to co-localize in two distinct mammalian cell lines. As a result of this compilation of data, it appears that RBP9 and A2aR are *bone fide* interacting proteins. To my knowledge, this is the first report indicating an interaction between these two proteins.

Additionally, in lieu of several recent reports indicating an interaction between other GPCRs and RBP9, Co-IP experiments were conducted between RBP9 and the D2L dopamine
receptor and the classical opioid receptor proteins. In all cases, RBP9 was immunoprecipitated by a Flag antibody recognizing the receptor proteins in HEK-293 cells. To my knowledge, this is also the first report indicating an interaction between RBP9 and the D2L dopamine receptor, delta opioid receptor, and kappa opioid receptor.

As shown in the GST-PD experiments, RBP9 appears to interact with both the ICL2 and C-tail of the A2aR. This supports the idea presented in Chapter 2, that aspects of GPCR quaternary structure are responsible for binding to RBP9.

In several of the Co-IP experiments, there is an observable increase in RBP9 molecular weight in the IP lane, as compared to that in the cell lysate lane. Because both bands are recognized by an antibody specific to RBP9, and molecular weight is altered only slightly, it is my belief that the slower migration of protein in the IP lane is due to an increase in overall amount of protein present in that lane.

As a result of the data presented here, and previous publications citing an interaction between RBP9 and the D1 dopamine receptor, metabotropic glutamate receptors, and the mu opioid receptor, it appears that RBP9 is a rather promiscuous interactor of several GPCR proteins, and possibly of other receptor proteins which are not G protein-coupled. Furthermore, it would follow that RBP9 may share a common function in its relationship with several or all of these receptor proteins, although further work must be done to confirm this plausible possibility.

In the following chapter, the possibility of a functional relationship between RBP9 and several of the receptor proteins investigated in this chapter will be explored in further detail.
Chapter Four: Knockdown of RBP9 alters the expression of the A2a Adenosine Receptor as well as other GPCRs

4.1 Introduction

To date, RBP9 has been shown to interact with a handful of GPCRs, including the D1 dopamine receptor, the mu opioid receptor, and the metabotropic glutamate receptors 2 and 8. It is possible that a similar functional relationship may exist between RBP9 and more than one, or all, of its receptor binding partners. No functional data was reported for the interaction between RBP9 and the glutamate receptors; however, functional relationships were described involving both the MOR and D1R.

An interaction between RBP9 and the MOR was described by Talbot et al. (2009). Furthermore, these authors showed that over-expression of RBP9 in MOR stably-transfected HEK-293 cells decreased DAMGO-mediated internalization of MOR; however, did not result in an observable change in Naloxone binding (Talbot et al. 2009). Therefore, these authors conclude that RBP9 plays a role in agonist-induced internalization of the MOR.

Rex et al. characterized the interaction between RBP9 and the D1R, as well as showed an interaction between RBP9 and Protein Kinase C (PKC) gamma and delta subtypes in the same study (2010). These authors showed that over-expression of RBP9 decreased D1R expression, as indicated by a 50% change in binding of a D1R-specific radioligand, and also, that RBP9 over-expression decreased D1R-stimulated cAMP activity. Furthermore, they reported a RBP9-dependent increase in D1R phosphorylation that could be blocked by a PKC inhibitor (Rex et al.
Therefore, these authors suggest that RBP9 plays a functional role in D1R expression, downstream signaling, and PKC-mediated D1R phosphorylation.

RBP9 is ubiquitously expressed in cell types and tissues, showing particularly high expression in HEK-293 cells, testis and oesophagus, and was also present throughout the CNS (Poirier et al. 2006). In an effort to assess the functional relationship between RBP9 and the A2aR, and possibly other GPCRs, this work employs the small interfering RNA (siRNA) knockdown technique to remove the abundant expression of RBP9 in cell culture. Possible changes involving GPCR proteins were then examined.

In this chapter, it is shown that knocking down RBP9 using siRNA alters the expression of three of the GPCRs for which we have previously observed an interaction in stably-transfected HEK-293 cells. This data supports the hypothesis that RBP9 may have a similar functional role in its relationship with several GPCRs.
4.2 Materials and Methods

4.2.1 Cell Culture, siRNA, and Transfections

SHSY5Y cells were maintained in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified chamber containing 5% CO₂. HEK-293 cells stably transfected with flag-tagged A2a adenosine receptor (A2aR-HEK) were a generous gift from Dr. Jurgen Zezula, Medical University Vienna/Center for Biomolecular Medicine and Pharmacology, Vienna, Austria. HEK-293 cells stably transfected with flag-tagged D2 long dopamine receptor (D2LR-HEK) or flag-tagged mu opioid receptor (MOR-HEK) were both a generous gift from Dr. Mark von Zastrow, University of California, San Francisco. All three cell lines were maintained in DMEM supplemented with 10% FBS and 400 μg/ml geneticin (Invitrogen, Carlsbad, CA), and were incubated at 37°C in a humidified chamber containing 5% CO₂.

Small interfering RNA (siRNA) to RBP9 (Product Identification: SASI_Hs01_00036366), as well as Universal Negative Control siRNA (NT-siRNA) (Product Identification: SIC001) were acquired from Sigma-Aldrich, Inc., Saint Louis, MO. Transfections of RBP9 siRNA, NT-siRNA, or H₂O Controls were carried out using Lipofectamine 2000 Reagent (Invitrogen Corp., Carlsbad, CA), according to manufacturer’s instructions. Cells were harvested approximately 48 hours after performing transfections.
4.2.2 Experimental Procedures and Statistical Analysis

Whole cell lysates were harvested by resuspending cells in Tris-based lysis buffer (containing 1% Triton) and sonicating. Lysates were solubilized by nutation for 20 minutes and centrifuged for 20 minutes at 12,000xg. Supernatant was collected and total protein concentration assessed using Bradford reagent (Bio Rad Laboratories, Hercules, CA).

Western blots were performed by running processed lysates on an SDS-polyacrylamide gel, combined with 2x SDS-PAGE loading dye (125 mM Trisma-HCL, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, 20% glycerol) and then transferred to PVDF membranes. Membranes were blocked in TBST containing 10% non-fat milk, overnight at 4°C.

All cell lines described in the previous section were investigated for effective knockdown of RBP9 using RBP9 siRNA, prior to assessment of GPCR expression changes. Briefly, cell lysates, transfected with RBP9 siRNA or controls, were harvested, run on a gel, transferred, and blocked as described above. An antibody directed at RBP9 (Santa Cruz) was then used to probe for changes in expression level of that protein.

After confirmation of effective RBP9 knockdown, changes in GPCR expression were assessed. When HEK-293 cell lines stably-transfected with Flag-tagged receptors were used, membranes were probed with M2 Flag antibody (Sigma-Aldrich, Inc., Saint Louis, MO, Product Identification: F-2165) at a 1:5000 dilution. Alternatively, if SHSY5Y cells were harvested, rabbit D2DR antibody (Santa Cruz Biotechnology, Product Identification: sc-9113) was used.

All primary antibody incubations took place in TBST containing 5% non-fat milk, for one hour at room temperature. Membranes were thoroughly washed with TBST to remove any
unbound primary antibody, before incubating with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Jackson ImmunoResearch) at a 1: 20,000 dilution. Again, to remove excess antibody, blots were washed thoroughly with TBST, and proteins were visualized using ECL Plus reagent and exposure on autoradiography film.

Although protein concentrations were assessed, and equal protein loaded between lanes of SDS-polyacrylamide gels, a loading control antibody was utilized as a secondary control for loading variations between lanes. Antibodies to GPCR proteins were removed by incubating membranes with strip buffer (6.25mM Tris-HCL, 2% SDS and 100mM beta-mercaptoethanol) for 15-20 minutes in 50°C H₂O bath. Membranes were washed in TBST, blocked overnight as described above, and re-probed with beta-Actin (β-actin) primary antibody (Sigma) at a 1:5000 dilution, for one hour at room temperature. Membranes were washed thoroughly with TBST to remove excess antibody and incubated with HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) at a 1: 20,000 dilution. Following further washes, proteins were visualized using ECL Plus reagent and exposure on autoradiography film (GE Healthcare).

Autoradiography films of GPCR proteins and β-actin were scanned, and protein bands were quantified using the Image J software package (NIH). GPCR band intensity was normalized to β-actin, to account for possible variations in loading. Data were subject to a two-tailed Student's t-test for assessment of a statistically significant difference in receptor expression between RBP9 siRNA knockdown and control conditions.
4.3 Results

4.3.1 siRNA knockdown of RBP9

In order to investigate a functional role of RBP9 in GPCR regulation, RBP9 siRNA knockdown experiments were performed. To first assess the effectiveness of RBP9 siRNA, several siRNAs were acquired and tested at various concentrations to compare for greatest alteration in RBP9 protein expression (data not shown). Figure 4.1 shows optimal conditions chosen for knockdown of RBP9 in three different HEK-293 cell lines, stably expressing A2aR, MOR, or D2LR and transiently transfected with 600 pmoles siRNA to RBP9 or equal volume H$_2$O control per plate. Figure 4.1, Panel A, shows a Western blot on which RBP9 protein expression is observed in these three cell lines. Lanes 1, 3, and 5 represent siRNA knockdown conditions and Lanes 2, 4, and 6 are corresponding controls. More intense bands can be observed in all control conditions, indicating a successful knockdown of RBP9 under siRNA-transfected conditions. Change in band intensity is quantified for A2aR-HEKs (Panel B), MOR-HEKs (Panel C), and D2LR-HEKs (Panel D), showing an overall 40-70% knockdown of RBP9 after siRNA transfection, as compared to control transfection.
Figure 4.1: RBP9 siRNA successfully knocks down RBP9 protein in HEK-293 cells stably transfected with A2aR, MOR, or D2LR. A. Western blot showing lysate from A2aR-HEK-293 cells, transfected with 600pmol siRNA to RBP9 or equal volume H₂O control (Lanes 1 and 2, respectively), MOR-HEK-293 cells, transfected with 600pmol siRNA to RBP9 or equal volume H₂O control (Lanes 3 and 4, respectively), D2LR-HEK-293 cells, transfected with 600pmol siRNA to RBP9 or equal volume H₂O control (Lanes 5 and 6, respectively). B. Quantification of change in RBP9 protein expression in A2aR-HEK-293 cells, as assessed by RBP9-specific antibody shows an approximate 40% knockdown of RBP9 as compared to control. C. Quantification of change in RBP9 protein expression in MOR-HEK-293 cells, as assessed by RBP9-specific antibody shows an approximate 70% knockdown of RBP9 as compared to control. D. Quantification of change in RBP9 protein expression in D2LR-HEK-293 cells, as assessed by RBP9-specific antibody shows an approximate 70% knockdown of RBP9 as compared to control.
4.3.1.1 siRNA knockdown of RBP9 results in a decrease in expression of the A2aR

Because the primary focus of this thesis is the relationship between the A2a adenosine receptor and novel interacting protein RBP9, the initial functional experiments focus on knocking down RBP9 in cell culture and observing any effect on the overall expression of A2aR.

A2aR-flag protein expression was assessed, in A2aR-HEK cells, when 600 picomoles siRNA to RBP9 were transfected vs. identical transfections using equal volume H₂O. Figure 4.2, Panel A, shows a representative Western blot demonstrating A2aR-HEK cell lysate transfected with siRNA (Lane 1) or control (Lane 2) and probed using an anti-Flag antibody (row 1) or an anti-β-actin antibody (row 2). Figure 4.2, Panel B, shows the quantification of A2aR-Flag band intensity for five separate experiments, after normalizing each to β-actin. All quantifications were done using Image J software. Figure 4.2, Panel C, shows a graphic representation of the summation of data presented in Panel B, showing an average decrease in A2aR-Flag protein expression of 30.0% under RBP9 siRNA-transfected conditions, as compared to control. Data was analyzed using a two-tailed Student’s t-test, using mean ± SEM, and showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value< 0.003.
Figure 4.2

A. Representative western blot showing A2aR-Flag and β-actin expression levels, under RBP9 siRNA-transfected or control-transfected conditions.

B. Quantification of A2aR-Flag protein expression for 5 separate experiments in RBP9 siRNA or control-transfected conditions, after normalization to β-actin.

C. Graphic representation of reduction in A2aR-Flag protein expression under RBP9 siRNA-transfected conditions, as compared to control. Data presented is the average for 5 experiments, and normalized to β-actin. Student’s T-test, using mean ± SEM, showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value < 0.003.
In order to control for the possibility that any, non-specific siRNA knockdown in this cell line may result in a similar change in A2aR expression, a non-targeting, control siRNA (NT-siRNA) was acquired from Sigma-Aldrich and used in an identical experiment as described above. Briefly, 600 picomoles RBP9 siRNA or NT-siRNA were transfected into A2aR-HEK cells. As before, any alteration in expression of the A2aR was observed between conditions, by Western blot and quantification of band intensity. Figure 4.3, Panel A, shows a representative Western blot demonstrating A2aR-HEK cell lysate transfected with RBP9 siRNA (Lane 1) or NT-siRNA (Lane 2) and probed using an anti-Flag antibody (row 1) or an anti-β-actin antibody (row 2). Figure 4.3, Panel B, shows the quantification of A2aR-Flag band intensity for five separate experiments, after normalizing each to β-actin. All quantifications were done using Image J software. Figure 4.3, Panel C, shows a graphic representation of the summation of data presented in Panel B, showing an average decrease in A2aR-Flag protein expression of 32.9% under RBP9 siRNA-transfected conditions, as compared to NT-siRNA-transfected control. Data was analyzed using a two-tailed Student’s t-test, using mean ± SEM, and showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value< 0.0005.
Figure 4.3: SiRNA knockdown of RBP9 results in a 32.9% decrease in overall A2aR expression in A2aR-stably transfected HEK-293 cells. A. Representative western blot showing A2aR-Flag and β-actin expression levels, under RBP9 siRNA-transfected or NT-siRNA-transfected conditions. B. Quantification of A2aR-Flag protein expression for 5 separate experiments in RBP9 siRNA or control conditions, after normalization to β-actin. C. Graphic representation of reduction in A2aR-Flag protein expression under RBP9 siRNA-transfected conditions, as compared to NT-siRNA control. Data presented is the average for 5 experiments, and normalized to β-actin. Student’s T-test, using mean ± SEM, showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and NT-siRNA control conditions, with a p-value<0.0005.
It can be observed through the data presented in Figures 4.2 and 4.3 that knockdown of RBP9 results in a decrease in expression of the A2a Adenosine Receptor. Virtually identical results were observed when either NT-siRNA or H2O were used as the control, showing a 32.9% or a 30.0% decrease in A2aR expression in those cells transfected with RBP9 siRNA.

### 4.3.1.2 siRNA knockdown of RBP9 results in an increase in expression of the MOR

To assess whether or not knockdown of RBP9 has similar effects on multiple GPCRs, MOR-Flag protein expression was also assessed, under similar conditions as described above. As with the initial experiment detailed in section 4.3.1.1, 600 picomoles siRNA to RBP9 or equal volume H2O were transfected, this time into MOR-HEK cells. Figure 4.4, Panel A, shows a representative Western blot demonstrating MOR-HEK cell lysate transfected with siRNA (Lane 1) or Control (Lane 2) and probed using an anti-Flag antibody (row 1) or an anti-β-actin antibody (row 2). Figure 4.4, Panel B, shows the quantification of MOR-Flag band intensity for five separate experiments, after normalizing each to β-actin. All quantifications were done using Image J software. Figure 4.4, Panel C, shows a graphic representation of the summation of data presented in Panel B, showing an average increase in MOR-Flag protein expression of 42% under RBP9 siRNA-transfected conditions, as compared to control. Data was analyzed using a two-tailed Student’s t-test, using mean ± SEM, and showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value<0.007.
Figure 4.4: SiRNA knockdown of RBP9 results in a 42% increase in overall MOR expression in MOR-stably transfected HEK-293 cells. A. Representative Western blot showing MOR-Flag and β-actin expression levels, under RBP9 siRNA-transfected or control-transfected conditions. B. Quantification of MOR-Flag protein expression for 5 separate experiments in RBP9 siRNA or control-transfected conditions, after normalization to β-actin. C. Graphic representation of the increase in MOR-Flag protein expression under RBP9 siRNA-transfected conditions, as compared to control. Data presented is the average for 5 experiments, and normalized to β-actin. Student’s T-test, using mean ± SEM, showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value< 0.007.
4.3.1.3 siRNA knockdown of RBP9 results in an increase in expression of the D2LR/D2R

A potential change in expression of the D2LR in response to siRNA knockdown of RBP9 was also investigated. Similar to experiments detailed in sections 4.3.1.1 and 4.3.1.2, 600 picomoles siRNA to RBP9 or equal volume H₂O were transfected into D2LR-HEK cells. Figure 4.5, Panel A, shows a representative Western blot demonstrating D2LR-HEK cell lysate transfected with siRNA (Lane 1) or Control (Lane 2) and probed using an anti-Flag antibody (row 1) or an anti-β-actin antibody (row 2). Figure 4.5, Panel B, shows the quantification of D2LR-Flag band intensity for five separate experiments, after normalizing each to β-actin. All quantifications were done using Image J software. Figure 4.5, Panel C, shows a graphic representation of the summation of data presented in Panel B, showing an average increase in D2LR-Flag protein expression of 43% under RBP9 siRNA-transfected conditions, as compared to control. Data was analyzed using a two-tailed Student’s t-test, using mean ± SEM, and showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value< 0.011.
Figure 4.5

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Figure 4.5: SiRNA knockdown of RBP9 results in a 43% increase in overall D2LR expression in D2LR-stably transfected HEK-293 cells. **A.** Representative Western blot showing D2LR-Flag and β-actin expression levels, under RBP9 siRNA-transfected or control-transfected conditions. **B.** Quantification of D2LR-Flag protein expression for 5 separate experiments in RBP9 siRNA or control-transfected conditions, after normalization to β-actin. **C.** Graphic representation of the increase in D2LR-Flag protein expression under RBP9 siRNA-transfected conditions, as compared to control. Data presented is the average for 5 experiments, and normalized to β-actin. Student’s T-test, using mean ± SEM, showed a statistically significant change in receptor expression between RBP9 siRNA knockdown and control conditions, with a p-value < 0.011.
In order to assess the universality of receptor expression alteration in multiple cell lines, identical experiments to those described previously were conducted in SHSY5Y cells, which endogenously express the GPCR proteins. Because of limitations surrounding antibodies which directly recognize GPCR proteins, investigation was limited to D2 receptor expression in response to RBP9 siRNA knockdown.

As in previous experiments, 600 picomoles siRNA to RBP9 were transfected, this time into SHSY5Y cells. NT-siRNA or equal volume H₂O was used as control. Figure 4.6, Panel A, shows a representative Western blot demonstrating SHSY5Y cell lysate transfected with siRNA (Lane 1) or Control (Lane 2) and probed using an anti-D2R antibody (row 1) or an anti-β-actin antibody (row 2). Figure 4.6, Panel B, shows the quantification of endogenously expressed D2R Western blot band intensity for ten separate experiments, after normalizing each to β-actin. All quantifications were done using Image J software. Figure 4.5, Panel C, shows a graphic representation of the summation of data presented in Panel B, showing an average increase in D2R protein expression of 44% under RBP9 siRNA-transfected conditions, as compared to control. Data was analyzed using a two-tailed Student’s t-test, using mean ± SEM, and showed a change in receptor expression between RBP9 siRNA knockdown and control conditions, with a significant p-value< 0.024.
Figure 4.6

A.

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<td>*9</td>
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B.

C. p-value < 0.024
Figure 4.6: SiRNA knockdown of RBP9 results in a 44% increase in overall D2R expression in SHSY5Y cells. A. Representative Western blot showing D2R and β-actin expression levels, under RBP9 siRNA-transfected or control-transfected conditions. B. Quantification of endogenously expressed D2R protein expression for 10 separate experiments in RBP9 siRNA or control-transfected conditions, after normalization to β-actin. Starred experiments are those using NT-siRNA as control. All others are H2O-transfected controls. C. Graphic representation of the increase in D2R protein expression under RBP9 siRNA-transfected conditions, as compared to control. Data presented is the average for 10 experiments, and normalized to β-actin. Student’s T-test, using mean ± SEM, showed a change in receptor expression between RBP9 siRNA knockdown and control conditions, though with a significant p-value< 0.024.
4.4 Conclusions

In an effort to discover a functional relationship between RBP9 and the A2aR, as well as other GPCRs, production of the RBP9 protein was dampened using the siRNA knockdown technique, in HEK-293 cells stably expressing the A2aR, MOR, or D2LR and SHSY5Y cells. GPCR expression levels were then assessed by Western Blot.

Interestingly, in stably transfected HEK-293 cells, an alteration in all three receptor proteins was observed with RBP9 knockdown as compared to control, and these results were shown to be highly statistically significant. Perhaps even more interesting, however, is that the observed effect on GPCR expression was receptor-specific. Overall expression of the A2aR decreased with a knockdown of RBP9, whereas overall expression of both the MOR and D2LR increased with RBP9 knockdown.

Because of limitations in antibodies which directly recognize GPCRs, the experiments conducted in SHSY5Y cells, which endogenously express the GPCRs, were confined to investigation of the D2R expression. A similar result to that observed for the D2LR in HEK-293 cells was seen in SHSY5Y cells, in that there was a statistically significant increase in D2R expression in response to RBP9 siRNA knockdown. Furthermore, quantifications from both cell lines resulted in a quite similar percentage of increased D2R expression. Specifically, in HEK-293 cells, the D2LR expression increased by an average of 43%, and in SHSY5Y cells, the D2R expression increased by an average of 44%, under RBP9 siRNA knockdown conditions, as compared to controls. Replication of the effect seen in HEK-293 cells, in a cell line which
endogenously expresses both RBP9 and the D2R receptor, supports the idea that RBP9 may have a regulatory effect on GPCR expression in several different cell types.

To my knowledge, this is the first report showing a RBP9-dependent alteration in the expression of any of these three receptor proteins. However, the idea of RBP9 having an effect on GPCR expression is not completely without precedent. Rex et al. (2010) showed that an overexpression of RBP9 resulted in a decrease in the D1 dopamine receptor protein, in HEK-293T cells, as assessed by a binding assay using a D1R-specific radioligand.

It would be interesting to investigate whether the effect RBP9 has on GPCR expression is family-specific. In other words, if the adenosine A1, A2b, and A3 receptors, which are all also GPCR family members, also showed a decrease in expression in response to RBP9 knockdown, that would support this possibility.

Because we observed a dopamine D2LR increase in response to a knockdown of RBP9, it is also tempting to associate the dopamine D1R decrease in response to an overexpression of RBP9 (Rex et al. 2010), in support of a family-specific effect of RBP9. However, several points must be clarified prior to making this claim. First, the assumption that an overexpression vs. a knockdown of RBP9 would have opposing effects on receptor expression requires confirmation. Furthermore, the other 3 dopamine receptor GPCR family members, D3, D4, and D5, as well as other GPCR families would warrant investigation.

The observation that RBP9 knockdown has an effect on the expression levels of three separate GPCR proteins is a novel finding. Implications of this finding are as yet undetermined,
and there are several possible mechanisms by which this effect could be achieved. These possibilities will be discussed further in the following chapter.
Chapter Five: Discussion

5.1 Review

The initial overall goal of this work was to investigate the possibility that there are adenosine A2a receptor interacting proteins capable of binding and regulating the function of the receptor. The concept that GPCR functional properties are regulated by a number of binding proteins has received increasing support in recent years (Milligan 2005; Kabbani and Levenson 2007; Ferre et al. 2008; Zezula and Freissmuth 2008, Jin et al. 2010). However, prior to this study, only seven proteins showing a direct interaction with the A2aR had been identified. Four known interactors for the A2aR are fellow GPCRs, and only three are intracellular regulatory proteins, as compared to over fifteen known regulatory interactors for the mu opioid receptor.

In this thesis, a number of candidate interactors have been identified for the C-terminal tail of the A2aR. Among them is Ran Binding Protein 9, a 90kD protein widely expressed in various tissues and cell types. In addition to showing support for an interaction between RBP9 and the A2aR in GST-pulldown, co-immunoprecipitation, and co-localization experiments, Co-IP experiments provide evidence that RBP9 also interacts with the D2LR, MOR, KOR and DOR.

The functional role of RBP9 is not well understood. This thesis, as well as three other studies published in the last 2 years, demonstrate RBP9 interacting with GPCR proteins (Seebahn et al. 2008, Talbot et al. 2009, Rex et al. 2010). It has been speculated that RBP9 performs a scaffolding role (Murrin and Talbot 2007), and has been shown to affect receptor internalization (Talbot et al. 2009), GPCR phosphorylation, and expression (Rex et al. 2010).
However, several other roles have been proposed for RBP9’s function, including acting as a transcriptional co-activator (Poirier et al. 2006, Denti et al. 2004, Rao et al. 2002), and mediating aspects of downstream signaling (Wang et al. 2002).

In this thesis work, a highly replicable and highly significant alteration of A2aR, MOR, and D2LR expression in stably transfected HEK-293 cells has been shown, when RBP9 expression was knocked down using siRNA. The effect seen on D2 receptor expression was confirmed in SHSY5Y cells, which endogenously express both RBP9 and the receptor protein. To my knowledge, this is the first instance where a functional role for RBP9 has been investigated using the siRNA knockdown method. Interestingly, however, dampening the expression of RBP9 did not have the same effect on the expression of all three receptors. Whereas the A2aR protein showed decreased presence with RBP9 knockdown, both the D2R and MOR had an opposite result, showing increased expression with RBP9 knockdown.

One other publication recently showed an effect of RBP9 on GPCR expression. Rex et al. observed a decrease in the expression of the D1 dopamine receptor in response to over-expression of RBP9 (2010). However, no proposal for the means by which RBP9 may be regulating receptor expression has, as yet, been suggested or tested.
5.2 Possible models for a functional role of RBP9

There are three general mechanisms of protein regulation, in which RBP9’s involvement would allow it to have the observed regulatory effect on GPCR expression. These possibilities include the idea that RBP9 may be involved in the general protein production process, a degradation pathway, or may be a part of a stabilizing/destabilizing effect on receptor proteins already present and functioning at the membrane. Each of these possibilities has some degree of support which can be found in the literature; however, none have yet been evaluated in the context of GPCR expression. In the subsequent paragraphs, each of these possibilities will be evaluated in greater detail.

First, RBP9 may have a role in the protein production process. This involvement could take place at the level of gene transcription, translation, or post-translational modifications. The widespread intracellular localization of RBP9 does not eliminate its involvement in any of these significantly well-understood processes. More to the point, RBP9 has been shown to have nuclear (Rao et al. 2002), cytosolic, as well as membrane (Denti et al. 2004) associations. Localization experiments presented in this thesis have also shown endogenous RBP9 expression in all of these subcellular regions.

Several findings allow for the possibility that RBP9 has some involvement in gene transcription. Lakshmana et al. show a nuclear localization sequence within the C-terminal region of RBP9 (2009). This work and other publications have shown RBP9 expression in the nucleus (Rao et al. 2002). Several lines of research show RBP9 interacting with transcription factors. Specifically, Tucker et al. point out that RBP9 interacts with the DNA binding domain of
the yeast GAL4 transcription factor (2009). Additionally, RBP9 has been shown to interact with the androgen and glucocorticoid receptors, which are steroid receptors capable of regulating transcription (Rao et al. 2002). These authors furthermore demonstrate that RBP9 is capable of increasing ligand-mediated transcriptional activity of both receptor proteins, as indicated by reporter plasmids (Rao et al. 2002). Again, Poirier and colleagues show that the C-terminal region of RBP9 interacts with the DNA binding domain of thyroid hormone nuclear receptors; and moreover, over-expression of RBP9 increased activation of two thyroid hormone receptor response elements (2006). Therefore, it certainly appears possible that RBP9 is a transcriptional co-factor, and is thereby exerting an effect on the expression of many proteins, including the change we observed on G protein-coupled receptor expression.

Because RBP9 has also been shown at the plasma membrane (Denti et al. 2004), and this work, in conjunction with other publications, support the idea that RBP9 is a promiscuous GPCR interactor, it seems likely that RBP9 is transiently present at the intracellular surface of the plasma membrane, where it has the ability to interact with many receptor proteins and receive information pertaining to receptor activation or desensitization. It may then subsequently translocate to the nucleus to play a role in regulation of receptor transcription (Figure 5.1, panel A).

Investigation of this possible mechanism of action would be aided by utilizing the qRT-PCR technique under cellular conditions where RBP9 is expressed normally as compared to knocked down. By using this technique, transcription of GPCRs could be directly monitored, and the possible role of RBP9 at this stage of protein production assessed. In addition, it may be
worthwhile to investigate RBP9 localization changes in response to manipulation of the receptor signaling pathway using known agonists or antagonists for the receptor. By doing so, any possible translocation of RBP9 between the plasma membrane and nucleus, in response to receptor stimulation, would be observable.

Protein production is not the only process in which RBP9 may be exerting the observed effect on GPCR expression. A second possibility is that RBP9 is involved in a degradation pathway. As mentioned, the investigation of this protein’s functional role is still ongoing; however, there is minimal evidence in the literature to support this theory at present. One publication is worthy of note in discussion of this option. Ideguchi and colleagues report an interaction between RBP9 and USP-11, a de-ubiquitinating enzyme (2002). These authors demonstrate that RBP9 appears to be de-ubiquitinated by USP-11 in a dose-dependent manner (Ideguchi et al. 2002). The publication focuses on RBP9 merely as a substrate for USP-11, and does not discuss a possible role for RBP9 in the ubiquitination/de-ubiquitination process. However, this thesis shows that RBP9 has some involvement in protein expression, and an interaction between RBP9 and a member of the protein-degradation pathway has been demonstrated (Ideguchi et al. 2002). Therefore, the possibility remains, though not directly supported, that RBP9 may be exerting its effects on protein expression in some manner connected with ubiquitin-dependent protein degradation (Figure 5.1, Panel B).

A third possibility is that RBP9 binds GPCRs and has a stabilizing/destabilizing effect on a functioning receptor protein. Denti et al. demonstrated a plasma-membrane localization of endogenous RBP9 (2004). These authors also reported that RBP9 is phosphorylated on serine
residues, and that it is this phosphorylated version of RBP9 that is enriched at the plasma membrane (Denti et al. 2004). Rex et al. showed not only an interaction between RBP9 and the D1 dopamine receptor, but also between RBP9 and Protein Kinase C (2010). Furthermore, they showed that there is a RBP9-dependent increase in D1R phosphorylation that is blocked by a PKC inhibitor, as well as decreased receptor expression in response to RBP9 overexpression (Rex et al. 2010). These links between RBP9 and phosphorylation are of interest because phosphorylation is one of the key components in desensitization, internalization, and down-regulation of GPCRs (reviewed by Freedman and Lefkowitz 1996; Gurevich and Gurevich 2006). Therefore, though much more exploration into this possibility is necessary, it remains a possibility that RBP9’s involvement in GPCR expression may be a latter effect of a participatory role for RBP9 in receptor phosphorylation (Figure 5.1, Panel C).

To investigate this possible mechanism of action, it would be worthwhile to stimulate PKC and observe any changes in receptor expression under conditions where RBP9 is normally expressed or knocked down. Synthetic PKC activators, such as phorbol 12-myristate 13-acetate, or inhibitors, including Gö6983 and Gö6976, have been shown useful for evaluation of the role of PKC in GPCR phosphorylation (Palmer and Stiles 1999 and Rex et al. 2010, respectively).
Figure 5.1: Potential mechanisms of action by which RBP9 is regulating GPCR expression. Panel A, RBP9 is transiently present at the plasma membrane, where it receives information regarding receptor activation or desensitization, then translocates to the nucleus to participate in transcription regulation. Panel B, via its known interaction with de-ubiquitinating enzyme USP-11, RBP9 plays a role in blocking or assisting receptor down-regulation. Panel C, RBP9 regulates receptor phosphorylation, and thereby, stability at the membrane, through its known interaction with PKC.
5.3 Clinical Significance

The adenosine A2a receptor is associated with a variety of disease states, as are many other GPCRs. Particularly of interest to our laboratory are GPCRs which are implicated in neurological disorders, including Parkinson’s disease, schizophrenia, psychiatric disorders, and others. In several disease states, alterations in receptor expression levels could prove useful, in compensating for other losses, such as increased or decreased neurotransmission. For example, in Parkinson’s disease, where there is decreased dopaminergic signaling in the nigrostriatal pathway, maximizing post-synaptic dopamine receptor expression could provide an overall increase in neurotransmitter signaling, thereby assisting in the alleviation of motor dysfunction associated with the disease.

In addition to mechanisms directly involving dopamine and its receptors, the A2aR has also been a target for PD research, largely because it has been shown that caffeine, an A2aR antagonist, is protective against the development of Parkinson’s disease in humans (Ross et al. 2002; Tan et al. 2008; Hernan et al. 2002, and others), and that A2aR knockout animals show decreased dopaminergic toxicity in the MPTP model of Parkinson’s disease (Chen et al. 2001).

As demonstrated in this work, RBP9 is a novel A2aR and D2R interacting protein. Furthermore, siRNA knockdown of RBP9 decreases the expression of the A2aR in A2aR stably-transfected HEK-293 cells and increases the expression of the D2R in SHSY5Y cells and in HEK-293 cells, stably expressing the D2LR. Increase in the expression of the MOR, in response to RBP9 siRNA, has also been demonstrated. The mechanism by which RBP9 exerts this effect on the expression of the A2aR and other GPCRs remains, as yet, undetermined. However,
manipulation of GPCR expression may be a valuable tool for the development of new drug treatments in a number of disease states, including Parkinson’s disease.

Interestingly, in a microarray analysis of brain tissue from sporadic PD patients and controls, RBP9 was identified as one of 892 highly dysregulated genes in the substantia nigra of PD patients (Moran and Graeber 2008). However, no proposition as to its involvement in a PD pathway has been established. To my knowledge, this thesis presents the first link between RBP9 and other proteins believed to be involved in PD pathology.

As described above, it is possible that RBP9, through its interaction with the A2aR, has a stabilizing/destabilizing effect on the receptor. In this paradigm, reducing the potential for interaction between RBP9 and the A2aR is the key factor responsible for the effect seen on A2aR with RBP9 siRNA knockdown, i.e., decreased expression of the A2aR. As shown by Chen and colleagues, A2aR knockout animals showed reduced dopaminergic toxicity in the MPTP model of Parkinson’s disease (2001). Therefore, in this paradigm, disruption of the A2aR-RBP9 interaction may also reduce toxicity of dopaminergic cells, presenting a new target for treatment of the motor symptoms associated with PD.

As previously mentioned, there are a number of non-motor symptoms associated with PD, in addition to the classically recognized resting tremor, rigidity, and postural imbalance. It is possible that disruption of the A2aR-RBP9 interaction, by allowing increased dopaminergic signaling, may improve some of these non-motor symptoms, as well. Those symptoms which are responsive to L-dopa treatment could also be aided by disruption of the A2aR-RBP9 interaction. Fatigue, which has been cited as the most severe PD symptom in up to a third of PD
patients (Friedman and Friedman 1991), has been shown to progress less in PD patients receiving L-dopa instead of placebo (Schifitto et al. 2008), and could therefore also be improved by manipulation of A2aR expression. Additionally, cognitive deficits associated with PD may be related to loss of DA in the striatum, through basal ganglia-thalamocortical connections (Borek et al. 2006). It is possible that these deficits could also be improved through manipulation of A2aR expression.

There are a number of non-motor symptoms which appear to be hindered by L-dopa treatment, which could also be hindered by any treatment which creates a similar increase in dopaminergic signaling. Sleep disturbances, though present even in PD patients not taking medication, fall into this group. Arousal and inhibited rapid-eye-movement sleep are associated with stimulation of dopaminergic neurons (Ongini et al. 1985; Trampus et al. 1991) and L-dopa or dopamine agonist treatment was shown to be a strong predictor of sleep disturbance (van Hilten et al. 1994). However, in the studies associating dopaminergic signaling with sleep and arousal, it is the D1 dopamine receptor, and not striatal D2Rs, which are thought to be involved in these disturbances. Therefore, the proposed increase in dopaminergic signaling associated with breaking the interaction between RBP9 and the A2aR may not promote further sleep disturbances associated with commonly-used PD treatments.

In addition to PD, there are several other diseases that involve aberrant GPCR signaling, for which manipulation of GPCR expression levels may prove helpful. For example, overactivity in central brain DA pathways (Nikolaus et al. 2009 and others), as well as insufficient signaling of cortical DA (Masana et al. 2010 and others), have been proposed to result in different
symptoms associated with schizophrenia. Also, some mood disorders, including anxiety and depression, are thought to be due, at least in part, to a deficiency of serotonin (Wong et al. 2005 and others). Several GPCRs for serotonin may be involved in these pathologies; however, a down regulation of the serotonin 1A autoreceptor expression has recently been shown to facilitate antidepressant actions (Abert and Le Francois 2010). A third example is GPCRs for somatostatin and acetylcholine, which have been implicated in Alzheimer’s disease. Specifically, inefficient somatostatin receptor function may lead to the development of β-amyloid plaques (Saito et al. 2005), characteristically seen in brains of Alzheimer’s disease patients. The muscarinic receptor (mAChR) M1 mediates learning and memory through acetylcholine signaling, and decreased concentration of this receptor and dysfunction of the receptor system has been implicated in Alzheimer’s disease (Joseph et al. 1993).

Although the possible regulation of each of these receptors would require additional investigation, the benefit of regulating GPCR expression for treatment of disease is not limited to Parkinson’s disease. Regulation of receptor expression, in lieu of increased neurotransmitter signaling or receptor blockade, may prove to be another means of improving symptoms associated with many diseases, including the examples listed above. As RBP9 appears to interact with and regulate expression of multiple GPCRs, development of an agent to specifically disrupt a RBP9-GPCR interaction is a challenging, yet potentially quite useful tool, for the treatment of a number of pathologies.
5.4 Remaining Questions and Future Studies

This work suggests several novel concepts relevant to G protein-coupled receptor interactions and regulation. However, several questions remain unresolved.

One question raised by this work is the degree of promiscuity RBP9 has for interacting with GPCRs, and possibly other receptor proteins. This dissertation shows an interaction between RBP9 and the A2aR, D2R, MOR, KOR, and DOR. It would be worthwhile to investigate what other receptor proteins RBP9 interacts with, or perhaps more interestingly, which RBP9 does not interact with. This is a possible area for future investigation. Other adenosine receptor and dopamine receptor family members would be of interest, as well as non-related GPCRs, and non-GPCR receptor proteins. From the work presented in this thesis, it is evident that RBP9 has a very high propensity for interaction with GPCRs. It is therefore a possibility that RBP9 performs a related function in its interaction with these similar binding partners.

As illustrated in this work, RBP9 siRNA knockdown alters the expression of several GPCR proteins stably-transfected into the same cell line. However, in experiments carried out under identical conditions, particular receptors demonstrated an increase in expression, and others demonstrated a highly replicable decrease. Because HEK-293 cells were the basis for observing expression changes for all receptors, the increase or decrease cannot be attributed to alterations in cell type, and rather, appears to be dependent on the particular receptor. The fact that D2R expression increased in both HEK-293 and SHSY5Y cells, in response to RBP9 knockdown, supports the concept that RBP9 modulates expression in a receptor-specific
manner; however, comparison of other receptors in several cell lines is required to confirm this conclusion.

Particular characteristic(s) or condition(s) pertaining to a specific protein, which designate the RBP9-regulated increase vs. decrease in expression, are still unclear. It is possible that protein-specific promoter elements, or modifications in receptor state, such as phosphorylation or glycosylation, determine whether knockdown of RBP9 results in a receptor increase or decrease in expression. Because it is known that the A2aR and D2R form antagonistic functional heteromers (Hillion et al. 2002; Canals et al. 2003; Kamiya et al. 2003), and this thesis shows that RBP9 interacts with and exerts opposing effects on the expression of these two receptors, the possibility exists that RBP9 is involved in regulation of the A2aR-D2R heteromer. RBP9 may affect the formation or regulation of this heteromer by influencing the ability of the two receptors to interact, or by affecting expression levels of the receptors, either individually or jointly. To more concretely address this issue, it may first be helpful to determine the general mechanism- protein production, degradation, or stabilization (described in Section 5.2), in which RBP9 plays a role.

Functional experiments described in this thesis were conducted using a knockdown of RBP9. As shown in Figure 4.1, RBP9 expression was reduced approximately 40-70% in various experiments, 24 hours after siRNA transfection. The half-life of RBP9 has not been established to date. To determine the optimal amount of time to allow for siRNA transfections, prior to harvesting cells, it would be valuable to first assess the rate of RBP9 turnover by conducting either a pulse chase analysis or cyclohexamide blocking experiment.
Additionally, it would be worthwhile to evaluate whether an overexpression of RBP9 would also show an alteration in GPCR expression. One hypothesis is that RBP9 overexpression would carry with it the opposite directional alteration in expression, to that observed in the RBP9 knockdown experiments. However, RBP9 is already very highly expressed in many cell types, and particularly in HEK-293 cells. Indeed, Poirier et al. compared mRNA levels of RBP9 to GAPDH in five cell lines, and though RBP9 was expressed well in all cell lines, its highest relative expression was in HEK-293 cells (2006). Therefore, it is my supposition that an overexpression of RBP9 would not be as effective as a knockdown of this protein, in assessing a functional role. Use of another cell line could be beneficial, in assessing any functional changes related to a knockdown and particularly, an overexpression, of RBP9.

Perhaps the most interesting question presented as a result of this work is the mechanism by which RBP9 is regulating the expression of GPCRs. Those mechanisms that seem most likely are elaborated on in Section 5.2, including a role for RBP9 in protein production, degradation, and/or stabilization. In each of these proposed scenarios, the interaction between RBP9 and the GPCR is vital for alteration in receptor expression. To confirm the importance of RBP9-GPCR binding on alteration of receptor expression, a competing peptide experiment could be performed. Specifically, design of a peptide, which is capable of binding the receptor in place of RBP9, would inhibit the RBP9-GPCR interaction in a dominant negative fashion, and allow for observation of changes to receptor expression which could be attributed to the RBP9-receptor interaction.
However, one challenge in designing this experiment is to disrupt only the GPCR-RBP9 interaction, without inhibiting any interactions that may be occurring in a similar binding region on the GPCR. Particularly, since the C-tail of the A2aR has been implicated in all of its known interactions with intracellular proteins, care should be taken to localize the specific binding site(s) for RBP9, and design of the competing peptide should target only the region required for RBP9 binding, in order to minimize potentially confounding disruption of other interactions.

Lastly, the cellular conditions under which RBP9 interacts with the GPCRs could be better understood. Treatment of cells with receptor agonists or antagonists may assist or inhibit the RBP9- GPCR interaction. It is possible that receptor activation or antagonism would influence recruitment of RBP9 to the membrane, thereby affecting the ability of these two proteins to interact. Preliminary investigations conducted in conjunction with this thesis work have not shown an effect of the A2aR agonist CGS-21680, or caffeine, (both used at 1uM concentration, for 24 hours, in SHSY5Y cells and HEK-293 cells stably expressing the A2aR) on overall expression of either the A2aR or RBP9 (data not shown); however, time course for treatment, ideal amount of agonist or antagonist used, as well as possible alterations in the interaction between the receptor and RBP9 are conditions yet to be investigated.
5.5 Conclusion

Ran Binding Protein 9 has been identified as a novel interacting protein for the A2a adenosine receptor, as well as for the D2LR, MOR, KOR, and DOR. It has been shown to play a role in expression of three GPCRs, the A2aR, D2LR, and MOR in stably-transfected HEK-293 cells, and in SHSY5Y neuroblastoma cells for the D2R.

The means by which RBP9 is exerting a regulatory role on receptor expression is still unknown. Increased understanding of the mechanism by which RBP9 is affecting receptor expression could be beneficial, not only for understanding general GPCR function, but also as a novel target for treatment of various neuropathologies, particularly those associated with aberrant signaling through GPCRs.
References


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