RGS2 MODULATION OF DOPAMINE D2 RECEPTOR TRAFFICKING IN NEUROBLASTOMA CELLS

By

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A thesis submitted to the graduate faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ART AND SCIENCES

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Biomedical Science

August 2015

Winston-Salem, North Carolina

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DEDICATIONS AND ACKNOWLEDGEMENTS:

I am exceptionally grateful for Dr. Rong Chen for allowing me to join her lab as an undergraduate student and serving as my mentor for the past 2 years. Dr. Chen has continually provided me with the knowledge, skillsets and support that allowed me to develop into a successful student, both in my undergraduate and graduate studies. She continued to set an exemplary model of the hard work, passion and motivation that is required to become a successful scientist. I am thankful for her honest advice and faith in my potential as a student and researcher.

I would also like to thank Dr. Allyn Howlett and Dr. Glen Marrs for being a part of my committee and providing valuable insight on my research projects to help me develop into a better scientist. I would like to acknowledge Dr. Howlett for all of her sage advice and support with regards to my career development and helping forge the path to continuing my research and education. I would also like to thank Dr. Marrs for not only mentoring me in various confocal microscopy techniques, but also for supporting and encouraging my passion for microscopy in the biological sciences.

I am endlessly grateful for the love and support that family has provided me that have allowed me to continue through my education and be where I am today. Additionally, I would like to express my gratitude to my incredibly supportive fellow students and friends, Molly McGinnis, Sarah Ewin, Jamie Rose, and Zachary Zabarsky, for encouraging me through many obstacles, teaching,

sharing advice and proof reading endless drafts of papers. Finally, I cannot fully express my appreciation for my boyfriend, Tadas, for providing me with constant support, strength and humor (and sometimes lab materials) through an incredibly hectic and, at times, overwhelming year. Thank you for always believing in me, even when I did not, and providing constant encouragement to keep going.

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LIST OF ABBREVIATIONS

ADP, adenosine diphosphate

ANOVA, analysis of variance

CHO, Chinese hamster ovary cells

CNS, central nervous system

D2R, dopamine D2 receptor

DA, dopamine

DMEM, Dulbecco's Modified Eagle Medium

ERK, extracellular signal-regulated kinases

GAIP, G-alpha interacting protein

GPCR, G protein coupled receptor

GRK, G protein receptor kinases

GTP, guanosine triphosphate

HEK,human embryonic kidney cells

N2A, neuro-2A cells

PBS, phosphate buffered saline

PKC, protein kinase C

PMA, phorbol 12-myristate 13-acetat

PTX, pertussis toxin

NA, numerical aperture

RGS, regulator of G protein signaling

TM, transmembrane

ABSTRACT

Dysregulated D2R expression and function is implicated in the pathophysiology of many diseases including Parkinson's disease, schizophrenia, and drug addiction. D2R trafficking is vital to proper D2R function. While the expression and activity of D2Rs are altered in the pathology of these diseases, the underlying mechanisms of these changes remain unclear. As D2 receptors have a critical role in the pathophysiology of disease and serve as potential as a therapeutic target, the current study aimed to characterize the trafficking of D2R in a neuronal cell culture model. D2R internalization and recycling are critical for D2R function, including receptor desensitization and resensitization. Specifically, this study focused on the modulation of D2R trafficking by the regulator of G protein signaling, RGS2. Though RGS2 is most commonly associated with termination of GPCR signaling through accelerated GTPase activity, this study reveals a novel role of RGS2 knockdown in the modulation of quinpirole, a D2/D3 agonist, stimulated D2R internalization, recycling and β-arrestin translocation and distribution. This study provides evidence that RGS2 plays a vital role in modulation of D2R trafficking and could potentially serve as a target for manipulation of D2R activity *in vivo*.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 DOPAMINE AND D2 RECEPTORS

Dopamine (DA) is a catecholaminergic neurotransmitter that is critically involved in the functions of many central nervous system including reward, working memory, sleep, feeding, learning and attention. Dopamine also plays an important role in the periphery by contributing to hormonal regulation, sympathetic system regulation, cardiovascular function, and renal function (Lokhandwala and Barrett, 1982; Mannelli *et al.*, 1999; Hussain and Lokhandwala, 2003). Dopamine innervations are numerous in the brain and dopamine neurons in the midbrain serve as the main source of dopamine for the mammalian central nervous system (Chinta and Andersen, 2005). Due to its vital regulation of neuronal processes, dopamine is of particular interest in the etiology of many diseases, including mood disorders, movement disorders and drug addiction (Davis *et al.*, 1991; Koob and Simon, 2009). For instance, loss of dopaminergic neurons is most commonly associated with Parkinson's disease, resulting in less brain dopamine content and subsequent dysfunctional movement. Additionally, alterations in dopaminergic transmission are a key focus in the study of drug addiction. Chronic use of psychostimulants produces a wide range of changes in both midbrain dopamine neurons and their forebrain projections (Nestler, 2004). Additionally, repeated exposure to psychostimulants, like cocaine, is associated with behavioral desensitization and decreased extracellular dopamine responses in the accumbens and caudate (Segal and Kuczenski, 1992).

Dopamine Receptors:

The dopaminergic system consists of 5 subtypes of dopamine G-protein coupled receptors (GPCRs) that are prominent in the central nervous system (CNS). Dopamine receptors are classified into two major groups: D_1-like (D_1 and D_5) and D_2-like receptors (D_2, D_3 and D_4) (Missale *et al.*, 1998). The members of each subfamily are highly homologous in their transmembrane domains and display unique pharmacological properties. The two major groups of dopamine receptors are differentiated by their ability to positively or negatively modulate adenylyl cyclase activity and cAMP production. Dopamine receptors also activate specific subtypes of G proteins, which then confer different neuronal responses to neurotransmitter signals through a direct regulation of ion channel function or through regulation of second messengers. The D_1-like receptors are located postsynaptically and are known to activate Gs-coupled GPCRs, resulting in downstream activation of adenylyl cyclase and production of cAMP. The D_2-like receptors can be located both presynaptically and postsynaptically on dopaminergic neurons and stimulate Gαi/o GPCRs, resulting in an inhibition of adenylyl cyclase and a decrease in cAMP production (Sibley and Monsma, 1992; Rankin *et al.*, 2009; Beaulieu and Gainetdinov, 2011).

D2-like Receptors: D2R

D2 receptors (D2R) serve as key modulators of dopaminergic neuron activity through the negative-feedback mechanism. D2Rs are Gαi/o coupled and

function to inhibit DA synthesis, neuronal firing and DA release (Brown *et al.*, 1985; Wang *et al.*, 2012). The structure of D2Rs consists of seven transmembrane (TM) domains with an extracellular N-terminus and an intracellular C-terminus. Two distinct protein isoforms of the receptors exist: the short-form D2S and the long-form D2L, which differ in length by 29 amino acids. The D2S isoform is reported to be more abundantly expressed presynaptically while the D2L isoform is more abundantly expressed postsynaptically (Usiello *et al.*, 2000). This varied neuronal distribution of isoforms could contribute to the varying roles of presynaptic and postsynaptic D2 receptors (Usiello *et al.*, 2000). D2Rs are most abundantly expressed in the striatum, the nucleus accumbens, as well as the olfactory tubercle. D2Rs are also expressed at notable levels in the ventral tegmental area, substantia nigra, hypothalamus, amygdala, hippocampus, and cortical areas (Seeman and Van Tol, 1994; Missale *et al.*, 1998; Gerfen, 2000). Due to the critical role of dopamine transmission in numerous physiological processes, the functional characteristics of dopamine receptors, in particular D2R, have been studied extensively. In particular, locomotor activity has been shown to be controlled by D1, D2, and D3 receptors (Sibley, 1999). Many other important functions rely upon activation of D2 receptors in the brain. D2R are implicated as critically important in reinforcement and reward, and therefore are of particular interest in the study of drug addiction and learning (Hyman *et al.*, 2006; Di Chiara and Bassareo, 2007; Koob and Volkow, 2010).

Gαi/o specificity and function:

D2R couples to PTX-sensitive G proteins, specifically Gαi/o, to initiate many signaling pathways. Gαi/o proteins inhibit adenylyl cyclase and are inactivated by pertussis toxin (PTX) (Civelli *et al.*, 1991). PTX uncouples Gαi/o proteins from the receptor by ADP ribosylation of the subunits at a conserved carboxyl-terminal domain cysteine (Cys) residue (Jennings and Linder, 2010). Four main subtypes of Gαi/o subunits exist: Gαi1, Gαi2, Gαi3 and Gαo. Each of these subunits is known to be involved in different signaling events as well as coupling to different GPCRs. D2Rs have been shown to couple to and activate all subtypes of Gαi/o proteins though with varying efficiency based on cell type (Ghahremani *et al.*, 1999; Obadiah *et al.*, 1999; Nickolls and Strange, 2004). To better understand D2R specificity of binding to Gαi/o subunits, PTX-insensitive mutants were developed, containing a carboxyl-terminus cysteine residue mutated to a serine or isoleucine residue, that prevents ADP-ribosylation (Clark and Traynor, 2006). Utilizing PTX-insensitive Gαi/o subunit mutants, previous studies have reported that D2R-mediated inhibition of adenylyl cyclase and forskolin-stimulated cAMP accumulation is highly dependent upon Gαi2 proteins in particular. In contrast, Gαi/o subunits play differential roles in D2R-mediated calcium mobilization as this signaling event is not Gαi/o dependent, but rather Gβγ subunit-dependent (Ghahremani *et al.*, 1999). In addition to inhibition of adenylyl cyclase and mobilization of calcium, various other vital signaling pathways are mediated by D2R-like extracellular signal-regulated kinase (ERK) activation. Other evidence indicates that Gαi2, in particular, is involved in D2R-

mediated ERK activation (Quan *et al.*, 2008). Taken together, these studies generally characterize the specificity and variability of Gαi/o subunits involved in D2R-mediated signaling events.

D2R implications in disease:

D2 receptors are common targets for the treatment of various diseases including movement, endocrine and mental health disorders caused by dysfunctional dopaminergic transmission (Thomas *et al.*, 2008; DI Cho *et al.*, 2010). Ample evidence demonstrates altered D2R function in many disease models. For instance, in a rodent model of schizophrenia, enhanced effects of the D2/D3 agonist, quinpirole, on dopamine neuron activity and altered locomotor response to D2R activation are observed. These findings further support the existing hypothesis that postsynaptic D2R function is altered in schizophrenic patients (Perez and Lodge, 2012). Additionally, altered D2R expression is observed in drug addiction, particularly after psychostimulant abuse. Reduced availability of striatal D2Rs is consistently observed after chronic exposure to cocaine and amphetamine (Chen *et al.*, 1998; Wang *et al.*, 2012). Though aberrant D2R function and expression are observed in various disease models including schizophrenia and drug addiction, the underlying molecular mechanisms of these alterations are not fully understood. Further investigation of alterations in D2R trafficking and signaling are critical for understanding the relationship between D2R function and dopaminergic transmission implicated in

various disease states.

1.2. D2R TRAFFICKING: INTERNALIZATION AND RECYCLING

D2R Internalization

Following agonist stimulation, GPCRs internalize through a number of distinct, highly regulated mechanisms. Internalization of D2Rs plays a critical role in controlling D2R desensitization and subsequent dopamine transmission (Kabbani *et al.*, 2004). In particular, previous literature has characterized D2R internalization in response to agonist stimulation. For instance, D2R internalization was increased after stimulation with DA or the D2 agonist, bromocriptine (10 μM) for 30 minutes in CHO cells. Additionally, internalization was not seen after pre-treatment with the D1 agonist, SKF38393, or D2 antagonist sulpiride, thus verifying D2R-specific effects in CHO cells (Goggi *et al.*, 2007).

D2Rs internalize in a dynamin-dependent and G protein receptor kinase (GRK) dependent manner, involving critical scaffolding proteins like β-arrestin. Phosphorylation of the receptor is a key step for its internalization and desensitization. In particular, GRKs have been shown to phosphorylate D2Rs. GRKs are a family of six serine/threonine kinases that negatively regulate the activity of GPCRs by phosphorylation of the activated receptors (Pitcher *et al.*, 1998). GRK structure and activation are unique from many other kinases. GRK activation does not require phosphorylation of the activation loop but rather

activation occurs upon binding to an active GPCR (Gurevich *et al.*, 2012). Phosphorylation of GPCRs results in rapid desensitization thus impairment of signaling. These phosphorylated residues also act as binding sites for β-arrestin. (Namkung and Sibley, 2004; D Cho *et al.*, 2010).

β-arrestin serves as a vital intermediate in agonist-dependent desensitization of GPCRs including D2Rs. There are two main subtypes of non-visual arrestins: β-arrestin 2 and β-arrestin 3 (Reiter *et al.*, 2012). The intracellular trafficking pattern of GPCRs with β-arrestin delineates into two main groups: class A and class B receptors. Class A receptors are characterized by binding to β-arrestin with a low affinity while class B receptors bind to β-arrestin with a high affinity (Quan *et al.*, 2008). Class A receptors are dissociate from β-arrestin once the receptors are integrated into the clathrin coated pits, while Class B receptors remain associated with β-arrestin on the surface of endocytotic vesicles. In particular, it has been shown that β-arrestin 2 is necessary for D2R internalization (Celver *et al.*, 2013). β-arrestin facilitates this effect by uncoupling the G protein and preferentially binding to the receptor which is phosphorylated upon prolonged stimulation by GRK2 (Attramadal *et al.*, 1992; Ferguson *et al.*, 1996; Lohse *et al.*, 2014). In addition to facilitating desensitization, β-arrestin also promotes receptor clathrin-mediated endocytosis by acting as an adapter protein to link the receptor to the endocytotic machinery, which occurs through a direct interaction with clathrin (Goodman *et al.*, 1996).

After β-arrestin binds the endocytotic machinery such as AP-2 and clathrin, clathrin-mediated endocytosis of D2Rs is mediated by dynamin. Dynamin comes into play by pinching off the coated vesicles from the plasma membrane of the cell, allowing for internalization of the receptor (Danino and Hinshaw, 2001). Dynamin completes this function through the use of GTPase activity. Dynamin is found in three main isoforms; dynamin-1, dynamin-2, and dynamin-3; all of which are implicated in synaptic function (Noda *et al.*, 1993; Okamoto *et al.*, 2001). The importance of dynamin in GPCR endocytosis has been further supported by studies expressing dominant-negative constructs of dynamin mutants (K44A or K44E), which results in blockade of clathrin-mediated endocytosis (Herskovits *et al.*, 1993; Damke *et al.*, 1994). Dynamin is therefore implicated in a number of GPCR trafficking events, including receptor-mediated endocytosis and the internalization of caveolae (Henley *et al.*, 1998).

To summarize, D2R internalization is a highly regulated process that requires the proper activation and function of numerous proteins like GRK, dynamin and β-arrestin. Due to the critical role of D2R internalization for desensitization and D2R function, it is imperative to improve our understanding of the molecular mechanisms regulating this process.

D2R Recycling

After D2Rs undergo internalization and are desensitized, there are two main post-endocytotic fates: recycling back to the plasma membrane or being targeted for degradation (Iwata *et al.*, 1999). When D2R degradation is prevented, an increase in D2R recycling and D2R response to quinpirole (3 μM) is observed in HEK 293 cells (Bartlett *et al.*, 2005). Further, D2R recycling has been shown to play a critical role in resensitization and maintaining surface availability (Kim *et al.*, 2004; Bartlett *et al.*, 2005; Thompson *et al.*, 2010). The balance between D2R internalization and recycling dictates the availability of D2R surface and therefore plays a key role in various aspects of D2R function such as signaling and resensitization. Internalized surface D2Rs appear to redistribute from endocytic vesicles to the cell surface within 30 minutes in HEK293 cells using immunocytochemical staining (Vickery and Von Zastrow, 1999). Though the specific mechanism of D2R recycling remains unclear, various accessory proteins have been implicated in D2R recycling, including G protein coupled receptor associated sorting protein-1 (GASP-1), GAIP (RGS19) interacting protein C-terminus (GIPC), β-arrestin 2, and Ras-associated binding (Rab) proteins; specifically Rab4 and Rab11 (Macey *et al.*, 2004; Beaulieu *et al.*, 2005; Thompson *et al.*, 2010; Li *et al.*, 2012; Varsano *et al.*, 2012). These proteins are commonly associated with the sorting for the receptors, thus promoting D2R recycling.

D2R trafficking has been shown to be critical to D2R function. Though some details are known about the mechanisms of D2R internalization and recycling, modulation of these processes still remains unclear. Due to the importance of D2R desensitization and resensitization in proper signaling and function, it is imperative to further investigate the underlying molecular mechanisms regulating D2R internalization and recycling, specifically potential protein targets that may modulate these events.

1.3: REGULATOR OF G-PROTEIN SIGNALING-2 (RGS2)

Many studies suggest potential roles of RGS proteins in the central dopaminergic system, involving both acute and long-term adaption to pharmacological treatments (Burchett *et al.*, 1999; Robinet *et al.*, 2001), as well as an involvement in the development of neurodegenerative diseases (Tekumalla *et al.*, 2001). Due to this potential role of RGS proteins in the dopaminergic system and pathophysiology of disease, it is imperative to further investigate the underlying molecular mechanisms of RGS regulation of D2R trafficking and function.

RGS protein structure and function

Regulators of G-protein Signaling (RGS) proteins are a diverse family of proteins that play a critical role in termination of GPCR signaling. All RGS proteins contain a conserved 120 amino acid sequence which constitutes the RGS domain. The RGS protein subtypes can vary in their overall structure. For instance, RGS2 and RGS7 contain only the RGS domain with an N-terminal and C-terminal extension while RGS4 or RGS10 have a more complex structure containing multiple other domains (Kehrl and Sinnarajah, 2002). The RGS domain is composed of nine α-helices folded into two small subdomains. Additionally, the RGS domain consists of three highly conserved regions where the RGS protein can bind to G-protein switch sites, allowing for an inhibition of G protein-dependent signaling (Tesmer *et al.*, 1997; Wieland *et al.*, 2007). Studies

have shown that mutations of these three regions of the RGS domain dramatically reduces RGS protein GTPase activating protein (GAP) activity (Kimple *et al.*, 2011). RGS proteins are distributed throughout various tissues, including brain, heart, kidney and liver. In the brain, there are over 20 subtypes of mammalian RGS proteins expressed which are distributed in a brain region-dependent manner (Gold *et al.*, 1997).

RGS2 Function

RGS proteins act as GAPs to facilitate the termination of GPCR signaling through both GTPase-dependent and GTPase-independent mechanisms. RGS proteins increase GTP hydrolysis by accelerating the re-association of activated GTP-bound Gα proteins to its basal GDP-bound state and assist in termination of effector activation by both Gα and Gβγ subunits, therefore regulating the kinetics and amplitude of signaling. Additionally, RGS proteins generate GDP bound Gα from GTP bound Gα, resulting in the re-formation of the GPCR heterotrimers, thus termination of βγ signaling (Willars, 2006). RGS proteins can inhibit GPCR-mediated signaling in a GTPase-independent manner by binding to the Gα subunit and antagonizing sites on the Gα subunit necessary for activation of downstream effectors. The GTPase-independent mechanisms of G protein activity inhibition have been demonstrated through the ability of RGS proteins to inhibit activity of G proteins bound to hydrolysis-resistant GTPγs (Hepler *et al.*, 1997). RGS proteins demonstrate GAP activity against particularly Gαi/o family

receptors, while some show effectiveness against Gαq family members (Kimple *et al.*, 2011).

Though RGS proteins are more classically associated with modulation of GPCR signaling through potentiation of GTPase activity, recent literature suggest that RGS proteins may also play a modulatory role in GPCR trafficking. Previous literature has alluded to other RGS proteins playing a similar role in D2Rs or GPCR surface expression and trafficking. For instance, overexpression of RGS9 in HEK-293 cells blocked the decrease in D2R surface expression after the treatment with dopamine (Celver *et al.*, 2010). Additionally, overexpression of RGS4 in HEK293 cells resulted in the attenuation of agonist-induced reduction in surface delta-opioid receptors (Leontiadis *et al.*, 2009). Moreover, RGS proteins are versatile proteins with a broad range of functional implications, many of which are not fully understood.

RGS2 structure, localization, and function:

RGS2 is a small RGS protein, containing an RGS domain, an N-terminal and C-terminal. The N-terminal domain of RGS2 is of particular interest as it is necessary and sufficient for plasma membrane targeting of RGS2 (Kehrl and Sinnarajah, 2002). Additionally, the N-terminus has been implicated in regulating the degradation of RGS2 proteins (Bodenstein *et al.*, 2007). RGS2 localizes robustly in various regions of the brain, many of which also have a high

expression of D2Rs. Using in situ hybridization in rat brains, RGS2 mRNA expression localized in highest concentration in the striatum, cerebral cortex, hippocampus, thalamic nuclei and hindbrain regions like the dorsal raphe nuclei (Grafstein-Dunn *et al.*, 2001; Taymans *et al.*, 2002). The subcellular localization of RGS proteins varies between subtypes but appears to play a role in the specificity of action. RGS2 and RGS4 are distributed in the nucleus and cytosol in HEK293 cells, but when exposed to specific Gα subunits or GPCRs, there is apparent redistribution to the plasma membrane (Leontiadis *et al.*, 2009). G-protein activation has been shown to recruit RGS proteins to the plasma membrane (Druey *et al.*, 1998; Dulin *et al.*, 1999).

1.4: AIMS AND OBJECTIVES

The primary goal of this study is to characterize the modulatory role of RGS2 proteins on D2R function specifically trafficking, in a neuronal N2A cell culture model. Numerous studies have shown an association between altered D2R expression/function and the pathophysiology of various diseases such as Parkinson's disease, schizophrenia and drug addiction (Beaulieu *et al.*, 2015). Due to its vital role in dopaminergic transmission and dysregulation in many diseases, it is vital to better understand the molecular mechanisms underlying D2R expression and function. RGS proteins have been implicated in the modulation of D2R trafficking, as overexpression of RGS9 in medium spiny striatal neurons inhibited dopamine-induced D2R internalization (Celver *et al.*, 2010). Additionally, RGS proteins are altered in the pathophysiology of many dopamine-related diseases. RGS2 levels are reduced in the striatum of human Parkinson's disease patients (Dusonchet *et al.*, 2014). Alternatively, treatments with amphetamine, cocaine or methamphetamine resulted in an increased RGS2 mRNA expression in the striatum of rats (Burchett *et al.*, 1999). Despite evidence of RGS protein modulation of D2R function, as well as their implication in many of the same diseases, the underlying molecular mechanisms of RGS2, regulation of D2R trafficking remain elusive.

Our study focuses on RGS2 in particular in the modulation of D2R expression based on the anatomic and functional evidence. Firstly, high RGS2

expression levels have been reported in the cell bodies of rat midbrain dopaminergic neurons where dopamine D2 autoreceptors are also expressed (Labouèbe *et al.*, 2007; Calipari *et al.*, 2014). Secondly, RGS2 expression is modulated by D2R cAMP-dependent activity, as the D2R agonist, quinpirole, downregulates and the D2R antagonist, raclopride, upregulates RGS2 mRNA levels in rat striatum (Burchett *et al.*, 1999; Taymans *et al.*, 2003). These findings suggest a physiologically relevant and functional interaction between RGS2 and D2R.

In this study, the regulatory role of RGS2 on D2R trafficking was first evaluated by characterizing quinpirole-induced D2R surface expression in a neuronal N2A cell culture model. Previous literature has demonstrated that decreased D2R surface expression is associated with prolonged receptor stimulation (Vickery and Von Zastrow, 1999). By depleting RGS2 though siRNA knockdown, we were able to evaluate how RGS2 affects stimulated D2R surface expression. Because both D2R internalization and recycling contribute to the surface levels of D2R, the second aim of our study was to evaluate the effect of RGS2 knockdown on D2R internalization. Agonist-induced D2R internalization is both dynamin and β-arrestin-dependent. β-arrestin has been shown to be necessary for D2R internalization, as D2R internalization is abolished when β-arrestin mutants are present (Kim *et al.*, 2004). Due to the critical role of β-arrestin in D2R internalization, this study aimed to characterize the translocation of β-arrestin in both control and RGS2 knockdown cells. In addition to D2R

internalization, D2R recycling is critical to maintaining steady state surface expression of receptors and plays a role in resensitization. Internalized D2Rs have also been shown to recycle back to the plasma membrane, both constitutively and under agonist-stimulated conditions, in HEK293 cells (Vickery and Von Zastrow, 1999). Though previous literature has characterized D2R internalization and recycling independently, the third aim of our study is to concurrently evaluate constitutive and quinpirole-stimulated D2R internalization and recycling.

The main objective of these studies was to further improve our knowledge of the molecular mechanisms governing D2R trafficking, including internalization and recycling, given the importance of D2R function in the pathophysiology of many neurological and psychological disorders. Specifically, this study elucidates the role of RGS2 in the modulation of D2R trafficking.

This chapter is from a manuscript that is prepared for the submission to Molecular Pharmacology

CHAPTER 2

RGS2 MODULATION OF DOPAMINE D2 RECEPTOR TRAFFICKING

ABSTRACT

Dysregulated expression and function of dopamine D2 receptors (D2Rs) are implicated in drug addiction, Parkinson's disease and schizophrenia. The internalization and recycling of D2Rs tightly regulates D2R activity such as desensitization and resensitization. Thus, it is important to understand the molecular and cellular mechanisms governing D2R trafficking. A potential modulator of D2R trafficking is the family of regulator of G-protein signaling (RGS) proteins. This study examined a novel role of RGS2 subtype in modulation of D2R trafficking in neuro2A neuroblastoma cells by knocking down RGS2. Using confocal microscopy, we demonstrated that RGS2 knockdown via siRNA had no effect on constitutive D2R trafficking; however, it altered agonist quinpirole-stimulated D2R trafficking. Quinpirole downregulated surface D2R expression by concurrently accelerating dynamin-dependent D2R internalization and attenuating D2R recycling in cells treated with scrambled siRNA. In contrast, quinpirole increased surface D2R expression by preventing D2R internalization and promoting D2R recycling in RGS2 knockdown cells. β-arrestin was

recruited to the plasma membrane following 5 minutes of quinpirole treatment in both control and RGS2 knockdown cells. However, β-arrestin was dissociated from the membrane after 30 minutes of quinpirole treatment in control cells whereas it remained persistently adjacent to the membrane in RGS2 knockdown cells. This altered β-arrestin translocation may contribute to aberrant D2R trafficking when RGS2 was depleted. These results indicate that RGS2 may be an important modulator of D2R internalization and recycling and a potential target for manipulation of D2R trafficking and activity *in vivo*.

Introduction

Dysregulated function and expression of dopamine D2 receptors (D2Rs) are known to be implicated in the pathology of human neurological diseases including drug addiction, Parkinson's disease and schizophrenia (Scherfler *et al.*, 2006; Volkow *et al.*, 2007; Perez and Lodge, 2012; Wang *et al.*, 2012). For example, chronic exposure to cocaine, methamphetamine, heroin and alcohol in human has been associated with a reduced level of surface D2Rs in the striatum (Volkow *et al.*, 2002, 2007), which is also hypothesized to contribute to drug relapse (Wang *et al.*, 2012). Moreover, enhanced D2R function has been reported in post-mortem patients with schizophrenia (Hietala *et al.*, 1994; Knable *et al.*, 1994; Zakzanis and Hansen, 1998). In contrast, patients with Parkinson's disease have been reported to show a significant decrease in striatal D2R activity (Scherfler *et al.*, 2006). Thus, it is important to understand the molecular machinery that governs D2R trafficking, which is associated with receptor activity such as desensitization and resensitization.

D2Rs belong to the large superfamily of G protein-coupled receptors (GPCRs) and are coupled to Gαi/o for signal transduction. One deciding factor for the strength and duration of D2R function is the surface D2R expression which is determined by the rate of D2R internalization and recycling. D2Rs have been shown to undergo constitutive and agonist-stimulated internalization in HEK293 cells (Vickery and Von Zastrow, 1999). D2R internalization is dependent on β-arrestin and dynamin. For example, β-arrestin knockdown in

HEK293 cells abolished dopamine-stimulated D2R internalization (Cho *et al.*, 2011). Overexpression of a dominant negative dynamin construct (K44A) in HEK293 cells inhibited β-arrestin-dependent internalization of D2Rs (Kim *et al.*, 2001). In contrast to agonist-induced D2R internalization that has been well characterized, there is limited knowledge on D2R recycling which is important for functional resensitization of D2Rs. It was shown previously that internalized D2Rs were able to recycle back to the plasma membrane constitutively and under agonist-stimulated conditions in HEK293 cells (Vickery and Von Zastrow, 1999). A recent study also demonstrated vesicular insertion events of D2Rs in somatic and dendritic surfaces of cultured mouse striatal medium spiny neurons (Li *et al.*, 2012), further confirming that D2R recycling may occur under physiological conditions. Although it is known that agonist treatments cause downregulation of surface D2R expression, it has not been investigated whether both D2R internalization and recycling are altered by D2 agonists. Thus, the first goal of the present study was to concurrently measure the effect of D2R stimulation by a D2/D3 receptor agonist quinpirole on D2R internalization and recycling in neuro2A neuroblastoma (N2A) cells.

A key modulator of D2R signaling is the family of regulators of G-protein signaling (RGS) proteins, which terminate G protein signaling by accelerating GTP hydrolysis (Willars, 2006). There are at least 30 identified RGS subtypes in mammals. In addition to its GTPase activity, emerging evidence indicates that RGS proteins are also capable of modulating GPCR internalization. For

example, overexpression of RGS9, which is co-localized with D2Rs in striatal medium spiny neurons, inhibits dopamine-induced D2R internalization in HEK293 cells (Celver *et al.*, 2010). In the present study, we investigated the modulatory role of RGS2 on D2R trafficking for two reasons. First, RGS2 is abundantly expressed in the cell bodies of rat midbrain dopaminergic neurons where dopamine D2 autoreceptors are also expressed (Labouèbe *et al.*, 2007; Calipari *et al.*, 2014). Dopamine D2 autoreceptors are unlikely to be modulated by RGS9 because RGS9 is absent from midbrain dopaminergic neurons (Mancuso *et al.*, 2010). Second, RGS2 mRNA level is modulated by D2Rs in a cAMP-dependent manner. For example, D2R agonist quinpirole downregulates while D2R antagonist raclopride upregulates the RGS2 mRNA level in the rat striatum (Burchett *et al.*, 1999; Taymans *et al.*, 2003). These data suggest a physiologically relevant interaction between RGS2 and D2Rs *in vivo*. Recent data indicate a novel role of RGS proteins in regulation of trafficking of various GPCRs in addition to their ability to inhibit GTPase activity of G proteins (Celver *et al.*, 2010; Min *et al.*, 2012a). Thus, the second goal of the present study was to examine the role of RGS2 in modulation of D2R internalization and recycling by knocking down RGS2 via siRNA in N2A cells.

Here we reported that RGS2 knockdown had no effect on constitutive D2R internalization and recycling in N2A cells stably expressing D2Rs. Quinpirole treatment reduced surface D2R level by enhancing D2R internalization and attenuating D2R recycling in control cells treated with scrambled siRNA.

However, quinpirole treatment increased surface D2R level by preventing D2R internalization and accelerating D2R recycling in cells treated with RGS2 siRNA. Moreover, quinpirole treatment caused a persistent localization of β-arrestin near the membrane in RGS2 knockdown cells in the time frame when it moved away from the membrane in the control cells. These observations suggest that RGS2 may critically modulate D2R trafficking in N2A cells.

Materials and Methods

Generation of N2A Cells Stably Expressing D2Rs and RGS2 siRNA Knockdown.

The N2A cells obtained from the American Type Culture Collection (Manassas VA) were cultured in Opti-MEM media supplemented with 10% fetal growth serum and 1% penicillin/streptomycin. Cells stably expressing D2Rs (N2A-D2R) were generated by transfecting N2A cells with the short form of human D2Rs (Missouri S&T cDNA Resource Center) and selected by geneticin (400 μg/ml, Life Technologies). The expression of D2Rs was confirmed by the mRNA level and the D2/D3 antagonist [^{3}H]raclopride binding. To knock down the endogenous RGS2 expression, N2A-D2R cells were transiently transfected with mouse RGS2 siRNA (Sigma Aldrich) using Lipofectamine 2000 (Invitrogen). The control cells were transfected with a corresponding scrambled siRNA (Sigma Aldrich). The internalization and recycling of D2Rs in the control and RGS2 knockdown cells were examined 48 hours after the transfection.

RGS2 knockdown was confirmed via Western blot analysis. Briefly, cells were transfected with scrambled or RGS2 siRNA and lysed in solubilization buffer containing 50 mM Tris-HCl (pH7.4), 150 mM NaCl and 1% Triton X-100. Total protein concentrations were determined using BCA Protein Assay (Pierce). Proteins (20 μg) were loaded on 10% Tris-Glycine gels for SDS-polyacrylamide gel electrophoresis and then were transferred to PVDF (polyvinylidene difluoride) membranes. RGS2 levels were detected using mouse anti-RGS antibody (Sigma) and HRP-conjugated rabbit anti-mouse IgG (Santa Cruz). Pierce ECL

Western Blotting substrate was utilized for imaging. Bands were quantified via densitometric analysis using ImageJ (NIH) software. The level of RGS2 was normalized to β-actin relative to control.

Basal and Quinpirole-stimulated Surface D2R Expression. The surface level of D2Rs was examined as described previously (Chen *et al.*, 2013). Briefly, cells were plated on coverslips coated with 0.5 mg/ml poly-D-lysine (Sigma Aldrich) inside wells of 6-well plates. Cells were treated with vehicle (phosphate-buffered saline, PBS) or the D2R/D3R agonist quinpirole (1 μM) for 5, 15 or 30 min at 37°C in the culture medium. The reaction was terminated by replacing the medium with cold PBS. After cells were blocked with 4% normal goat serum for 30 min on ice, surface D2Rs were labeled at 4°C with a mouse anti-D2R antibody (targeting the extracellular N terminus of D2Rs, Santa Cruz Biotechnology) followed by incubation with the secondary goat anti-mouse Alexa Fluor 594 (Invitrogen). Cells were then fixed with 4% paraformaldehyde in PBS followed by permeabilization (0.1% Triton X-100 in PBS) for 10 min. Cells were quenched with 50 mM NH_4Cl in PBS for 20 min to minimize autofluorescence. Intracellular D2Rs were labeled with a rabbit anti-D2R antibody (targeting N terminus of D2Rs, Santa Cruz) followed by incubation with the secondary goat anti-rabbit Alexa Fluor 488 (Invitrogen). Cells were mounted with ProLong Gold anti-fade reagent (Life Technology) for imaging. The D2R surface level was calculated as a ratio of fluorescent intensity of surface (Alexa 594) to intracellular (Alexa 488) D2R labeling for each cell.

Confocal Microscopy. Fluorescent images were acquired with a Zeiss LSM 710 laser scanning confocal microscope. Confocal image planes were acquired with a 63X/NA 1.4 PlanNeoFluor oil-immersion objective. Alexa 405, 488 and 595 were excited at 405 nm, 408 nm and 594 nm with a diode, Argon and HeNe laser, respectively. Alexa 633 and 647 signals were also excited at 633 nm with a HeNe laser. Fluorescent channels were acquired sequentially to prevent cross-excitation of laser signal and bleed-through. All images were acquired with identical settings for excitation intensity, detector sensitivity and pinhole to allow intensity comparison between samples. Images for analysis were obtained by taking a Z-axis stack of image planes (1024 X1024 pixels) with 0.2 µm steps encompassing the entire cell structure and combining image planes into a maximum intensity projection stack. Fluorescent intensity was quantitated for each channel using ImageJ software (NIH). All images were adjusted with a 0.3 level Gaussian blur for presentation purposes only. Alexa 405 emission was pseudo colored as green upon acquisition for optimal presentation appearance.

Quinpirole-stimulated β-ArrestinTranslocation. The N2A-D2R cells were treated with vehicle or quinpirole (1 µM) for 30 min. Surface D2Rs were labeled with a mouse anti-D2R antibody (Santa Cruz) at 4°C followed by secondary goat anti-mouse Alexa Fluor 594 (Invitrogen). Then cells were fixed and permeabilized. Cytoplasmic β-arrestin was subsequently labeled with rabbit anti-β-arrestin (Santa Cruz) followed by secondary goat anti-rabbit Alexa Fluor 405

(Invitrogen). The translocation of β-arrestin towards and away from the membrane was determined by quantitation of gross fluorescence in the outer (adjacent to the membrane) and inner (intracellular) sector that were manually defined using a modified segmentation technique described previously (Goldys, 2009; Torrano *et al.*, 2013; Ujihara *et al.*, 2013). Specifically, the outer boundary of the cell was manually outlined based on the localization of surface D2R staining. An inner boundary was defined as a standardized 5 micron distance from the outer boundary. The outer cell sector is defined as the area between the outer and inner boundaries. The inner sector was defined as the remaining cellular area towards the cell center beginning at the defined inner boundary. Maximum intensity projection images in the outer and inner sections were quantitated using ImageJ software. β-arrestin translocation was determined as a ratio of the Alexa 405 fluorescent intensity of the outer versus the inner sector. Data were expressed as relative to the control.

To assess the redistribution of β-arrestin into vesicles, punctate staining intensity of β-arrestin was quantitated. Aggregation results in a greater intensity of pixels in contrast to diffuse staining therefore β-arrestin punctate quantification was performed as described previously (Matheson *et al.*, 2007; Erickson *et al.*, 2013). Briefly, manual segmentation was used to define each cell and punctate were identified and quantitated using threshold filters to only display pixels that are greater than or equal to 25% of full intensity (64 intensity units), 38% of full intensity (96 intensity units), and 75% of full intensity (128 intensity units). The

following formula was used to integrate the intensity of punctate for each cell: ((intensity at 64 intensity units*64/255)+(intensity at 96 intensity units*96/255)+(intensity at 128 intensity units*128/255)/total β-arrestin fluorescence intensity). β-arrestin punctate redistribution was determined as a ratio of the Alexa 405 fluorescent intensity of relative punctate versus total fluorescence. Data were expressed relative to control.

Constitutive and Quinpirole-stimulated D2R Internalization. D2R internalization was examined using two distinct approaches. The first approach used a modified antibody feeding technique to directly measure internalized D2Rs as described previously (Bartlett *et al.*, 2005; Xiao *et al.*, 2009). Briefly, cells were blocked with 4% normal goat serum (Cell Signaling) in PBS for 30 min and the basal surface D2Rs were labeled with mouse anti-D2R (Santa Cruz) for 45 min at 4°C. Cells were then warmed up and treated with vehicle or quinpirole (1 μM) for 30 min at 37°C to allow internalization of surface D2Rs. The reaction was terminated by replacing the media with cold PBS. The remaining primary antibody-bound surface D2Rs were subsequently incubated with the secondary antibody anti-mouse Alexa 647 (Invitrogen) for quantitation of labeled surface D2Rs that were not internalized. After fixation and permeabilization, internalized surface D2Rs bound with primary D2R antibody were labeled with the secondary goat anti-mouse Alexa 405 (Invitrogen) for quantitation of internalized D2Rs. D2R internalization was calculated as a ratio of fluorescent intensity for labeled surface D2Rs that were internalized (Alexa 405) versus the labeled total surface

D2Rs (Alexa 405+Alexa 647) for each cell. Data were represented as relative to the control.

To confirm that D2R internalization was dynamin-dependent, D2R internalization was assessed in the presence of dynasore, an inhibitor of dynamin-dependent endocytosis. Dynasore blocks endocytosis by inhibiting the GTPase activity of dynamin (Kirchhausen *et al.*, 2008) and has previously been used to inhibit dopamine D1 receptor internalization (Kotowski *et al.*, 2011; Gorshkov and Zhang, 2014). Briefly, cells were incubated with or without dynasore (20 μM) (Cayman Chemical) for 30 minutes at 37°C prior to treatment with either vehicle or quinpirole (1 μM) for 30 minutes. Direct labeling of internalized D2Rs was performed as detailed above. D2R internalization was calculated as a ratio of fluorescent intensity for labeled surface D2Rs that were internalized (Alexa 405) versus the labeled total surface D2Rs (Alexa 405+Alexa 647) for each cell. Data were represented as relative to the control.

The second approach was to measure D2R internalization by blocking D2R recycling at 18°C as described previously (Loder and Melikian, 2003). Briefly, cells were treated with vehicle or quinpirole (1 μM) for 30 min in the culture medium at 18°C under which condition receptor traffic between the early and the recycling endosome is blocked. Cells were then fixed, permeabilized and followed by the surface and intracellular labeling of D2Rs as described above. D2R internalization was calculated as a ratio of fluorescent intensity for the

intracellular Alexa 405 vs. the total D2Rs (Alexa 405+Alexa 647). Since there was no D2R recycling, quinpirole-induced accumulation of intracellular D2Rs resulted from the internalization of surface D2Rs. Data were represented as relative to the control.

Constitutive and Quinpirole-stimulated D2R Recycling. D2R recycling was also determined by modified antibody feeding technique to measure recycled D2Rs that were internalized as described previously (Loskutov *et al.*, 2014). Briefly, basal surface D2Rs were incubated with the primary antibody rabbit anti-D2R at 4°C. Cells were then warmed up to 37°C and treated with vehicle or quinpirole (1 μM) for 30 min to allow internalization of surface D2Rs. After termination of the reaction with cold PBS, the remaining primary antibody-bound, surface D2Rs were labeled with secondary goat anti-rabbit Alexa 405 (total surface D2R). Then cells were warmed up to 37°C again and treated with vehicle or quinpirole for 30 min to allow internalized, primary antibody-bound D2Rs to recycle to the surface, which were labeled with secondary goat anti-rabbit Alexa 555. D2R recycling was calculated as a ratio of the intensity of Alexa 555 (recycled D2Rs that were internalized) vs.405 (total surface D2Rs before internalization and recycling). Data were expressed as relative to the control.

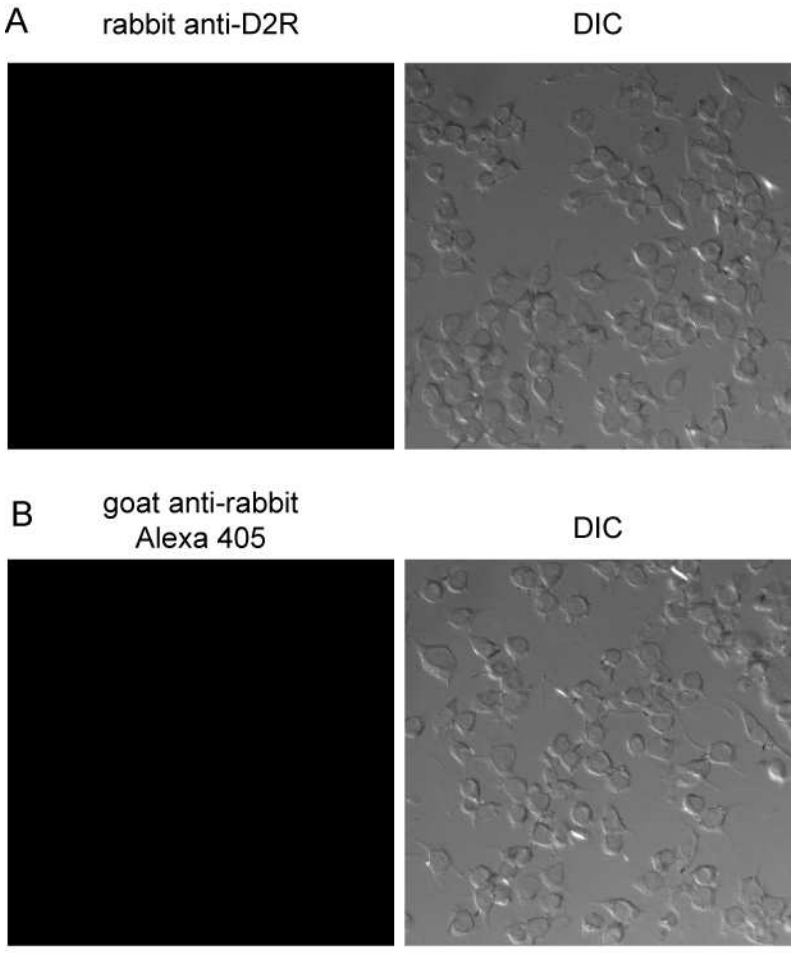
Statistics. Graph Pad Prism 5 (La Jolla, CA, USA) was used for statistical analysis. All data are presented as means±S.E.M. A Student's t-test was used to

compare the difference in RGS2 protein levels between the control and RGS2 knockdown cells. A two-way ANOVA with Bonferroni *post hoc* analysis was used to determine the statistical significance in all other experiments. Statistical significance was set at $p<0.01$.

Results

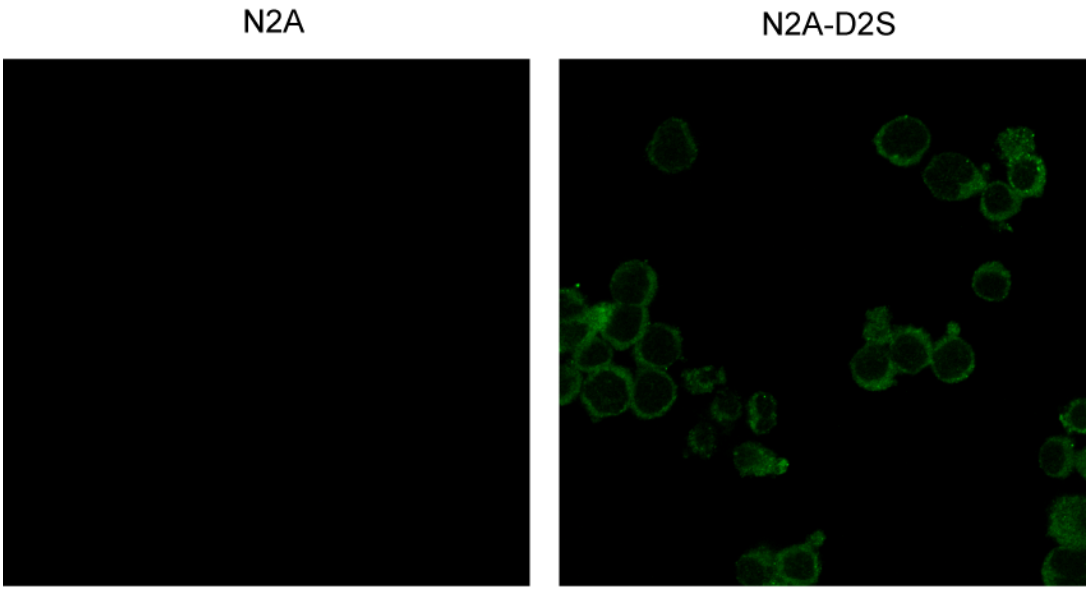
RGS2 Knockdown Diminished Quinpirole-induced Decrease in Surface D2Rs. To determine the knockdown efficiency by RGS2 siRNA, the level of RGS2 was measured in N2A-D2R cells transfected with RGS2 siRNA and scrambled siRNA. RGS2 siRNA knockdown reduced the RGS2 protein level by approximately 62± 7.9% compared to the scrambled siRNA control (n=3). The effect of RGS2 depletion on D2R surface expression was measured in cells treated with vehicle or quinpirole and assessed by confocal microscopy. A negative control to determine the fluorescent background was included in the absence of the primary D2R antibody for each experiment (Fig. S1). The specificity and working condition of the primary D2R antibodies were validated in non-transfected N2A cells that do not express D2Rs (Fig. S2). The surface D2R level was determined by the ratio of surface to intracellular D2R fluorescent intensity (Figs. 1B and 1C; n = 60-80 cells/group). In both control and RGS2 knockdown cells there was no significant difference in D2R surface expression after 5 minutes of quinpirole stimulation. In control cells, quinpirole caused an approximate 21± 2.5% decrease in D2R surface level after 15 minutes of quinpirole treatment and 35± 3.1% decrease after 30 minutes of treatment when compared to the vehicle treatment. However, RGS2 knockdown resulted in 21± 3.6% and 41± 3.8% increase in surface D2R levels after 15 and 30 minutes of quinpirole treatment, respectively, in comparison to the vehicle condition. Basal surface levels of D2Rs did not differ significantly between the control and RGS2 knockdown cells.

S1. Negative control experiments for immunofluorescence. (A) Corresponding differential interference contrast (DIC) and merged fluorescence images of N2A-D2R cells immunostained with primary antibody rabbit anti-D2R. (B) DIC and merged fluorescence images of N2A-D2R cells immunostained with secondary antibody goat anti-rabbit Alexa 405 only. Both negative control experiments for primary or secondary antibodies alone did not display any non-specific fluorescence.



Supplementary Figure 1

S2. Verification of D2R antibody specificity. D2R antibody specificity was verified by labeling N2A and N2A-D2S cells with rabbit anti-D2R antibody (1:100) followed by goat anti-rabbit Alexa 555. Confocal images displayed that no significant immunoreactivity was detected in N2A cells (left) compared to N2A-D2S cells (right).



Supplementary Figure 2

Figure 1. The effect of quinpirole treatment on D2R surface expression. N2A cells stably expressing D2Rs were transiently transfected with either scrambled or RGS2 siRNA. Cells were then treated with vehicle (Veh) (upper row) or quinpirole (1 μM) for 5 minutes (Q5), 15 minutes (Q15) or 30 minutes (Q30). Surface D2Rs were fluorescently labeled (red) followed by fixation, permeabilization and labeling of intracellular D2Rs (green). (A-B) Representative confocal images of surface and intracellular D2Rs in scrambled siRNA (A) and RGS2 siRNA (B) transfected cells. All scale bars, 10 μm. (C) Quantification of surface D2R expression as a ratio of surface to intracellular D2R fluorescent intensity. Quinpirole treatment resulted in a decrease and an increase in D2R surface expression in scrambled and RGS2 siRNA transfected cells, respectively (n=60-80 cells/group, *P<0.01 vs. control, a two-way ANOVA with Bonferroni posthoc analysis). Experiments were replicated four times. Data are expressed as relative to the control.

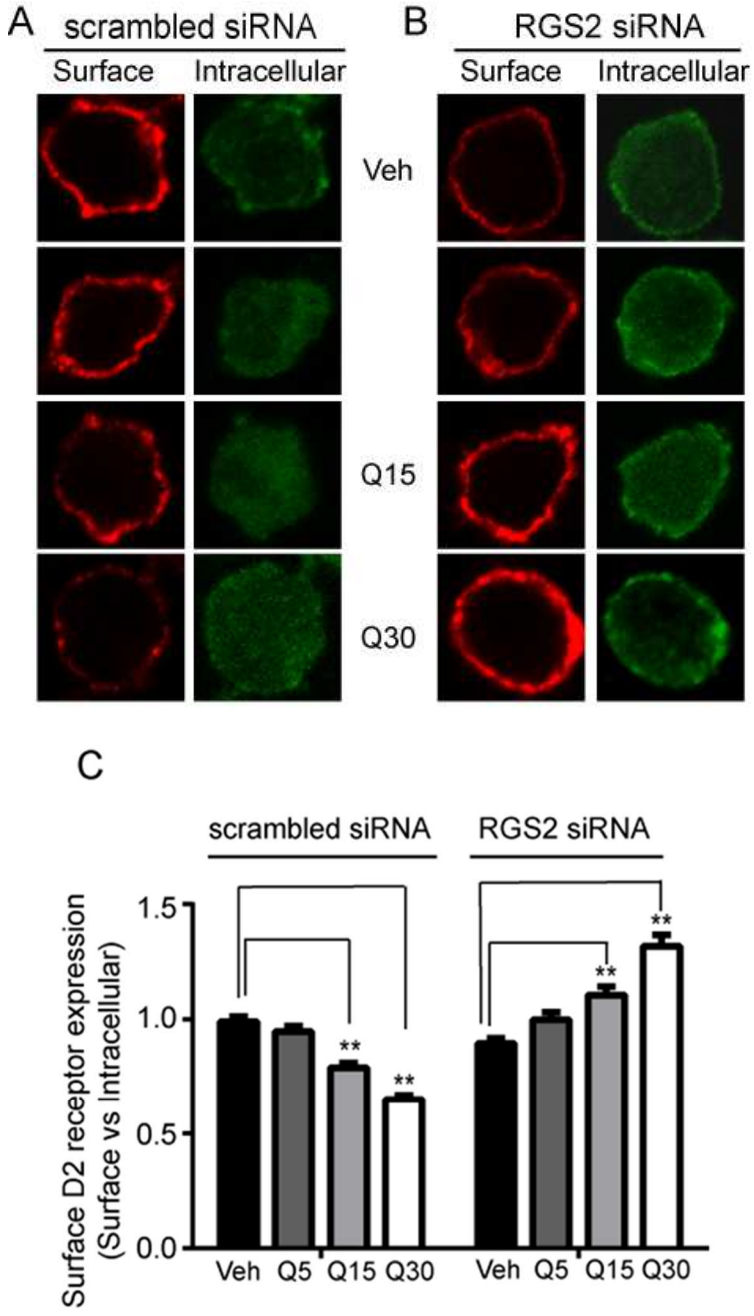

A
scrambled siRNA
Surface
Intracellular
B
RGS2 siRNA
Surface
Intracellular
Veh
Q15
Q30
C
scrambled siRNA
RGS2 siRNA
Surface D2 receptor expression
(Surface vs Intracellular)
1.5
1.0
0.5
0.0
**
**
**
**
Veh
Q5
Q15
Q30
Veh
Q5
Q15
Q30

Figure 1

The Effect of RGS2 Knockdown on Quinpirole-stimulated β-Arrestin Translocation. To determine quinpirole-stimulated β-arrestin translocation towards and away from the membrane, the outer (membrane) and inner (intracellular) sectors were defined as shown in Fig. 2A. The localization of β-arrestin was determined by a ratio of the fluorescent intensity in the outer vs. the inner sector for selected regions in each cell (Figs. 2B and 2C; n =45-56 cells/group). β-arrestin was distributed evenly throughout the cytoplasm of N2A cells at the basal condition. There was 47± 2.7% increase in β-arrestin translocation to the membrane after 5 minutes of quinpirole exposure compared to the vehicle treatment in control cells. Similarly to the control cells, 5 minutes of quinpirole treatment in RGS2 knockdown cells, β-arrestin translocation to the membrane increased by 41± 2.8% compared to the vehicle treatment. Furthermore, extended exposure (30 minutes) of quinpirole resulted in 37± 2.7% decrease of β-arrestin from the outer sector in control cells. However, β-arrestin persistently remained at the outer sector in RGS2 knockdown cells following 30 minutes of quinpirole treatment as only 4± 1.2% was removed from the outer sector.

The redistribution and aggregation of β-arrestin was determined by a ratio of the relative punctate vs. total β-arrestin fluorescence intensity for each cell (Figs. 2E; n =45-56 cells/group). β-arrestin was diffuse throughout the cytoplasm at the basal condition for both control and RGS2 knockdown cells. After 5 minutes of quinpirole stimulation there was 25±1.4% and 26±1.6% increase in relative

punctate intensity in control and RGS2 knockdown cells, respectively. There was 6±1.3% decrease in punctate intensity and punctate were localized away from the plasma membrane after 30 minutes in control cells. In contrast, there was no significant change in punctate intensity in RGS2 knockdown cells and punctate remained localized on or adjacent to the plasma membrane.

Figure 2. Quinpirole-induced β-arrestin translocation. N2A cells were labeled for surface D2Rs (red) followed by cytoplasmic labeling of β-arrestin (green). (A) A schematic illustration of manual segmentation for quantitation of β-arrestin translocation towards and away from the membrane. The outer boundary of the cell was manually outlined based on the localization of surface D2R staining (left, red). An inner boundary was drawn as a standardized 5 micron distance from the outer boundary. The outer sector was defined as the area between the outer and inner boundary while the inner sector was defined as the remaining cellular area towards the cell center beginning at the indicated inner boundary (right). β-arrestin translocation from the membrane to the cytoplasm was determined by the ratio of the outer to the inner sector intensity. (B-C) Representative confocal images of surface D2Rs and β-arrestin in scrambled siRNA (B) and RGS2 siRNA (C) cells after treated with vehicle (Veh) or quinpirole (1μM) for 5 minutes (Q5) or 30 minutes (Q30). Scale bars, 10 μm. (D) Quantification of β-arrestin translocation which was calculated as a ratio of the outer sector versus the inner sector. β-arrestin translocated to the outer sector after 5 minutes of quinpirole treatment in both scrambled siRNA and RGS2 siRNA cells. Extended quinpirole treatment (30 minutes) resulted in the removal of β-arrestin from the outer sector in scrambled siRNA cells whereas it was persistently remained at the outer sector in RGS2 knockdown cells (n=45-56 cells/group, **P<0.01 vs. control, two-way ANOVA with Bonferroni posthoc analysis). Experiments were replicated three times. Data are expressed as relative to the control. (E) Quantification of β-arrestin distribution which was calculated as a ratio of relative punctate versus

total β-arrestin fluorescent intensity (see Materials and Methods) and expressed relative to control. The intensity of β-arrestin punctate structure fluorescence increased significantly in both scrambled and RGS2 siRNA cells after 5 minutes of quinpirole stimulation. The intensity of punctate decreased slightly after 30 minutes of stimulation in scrambled siRNA cells while it remained elevated in RGS2 siRNA cells (n=45-56 cells/group, **P<.01 vs. control, a two-way ANOVA with Bonferroni posthoc analysis).

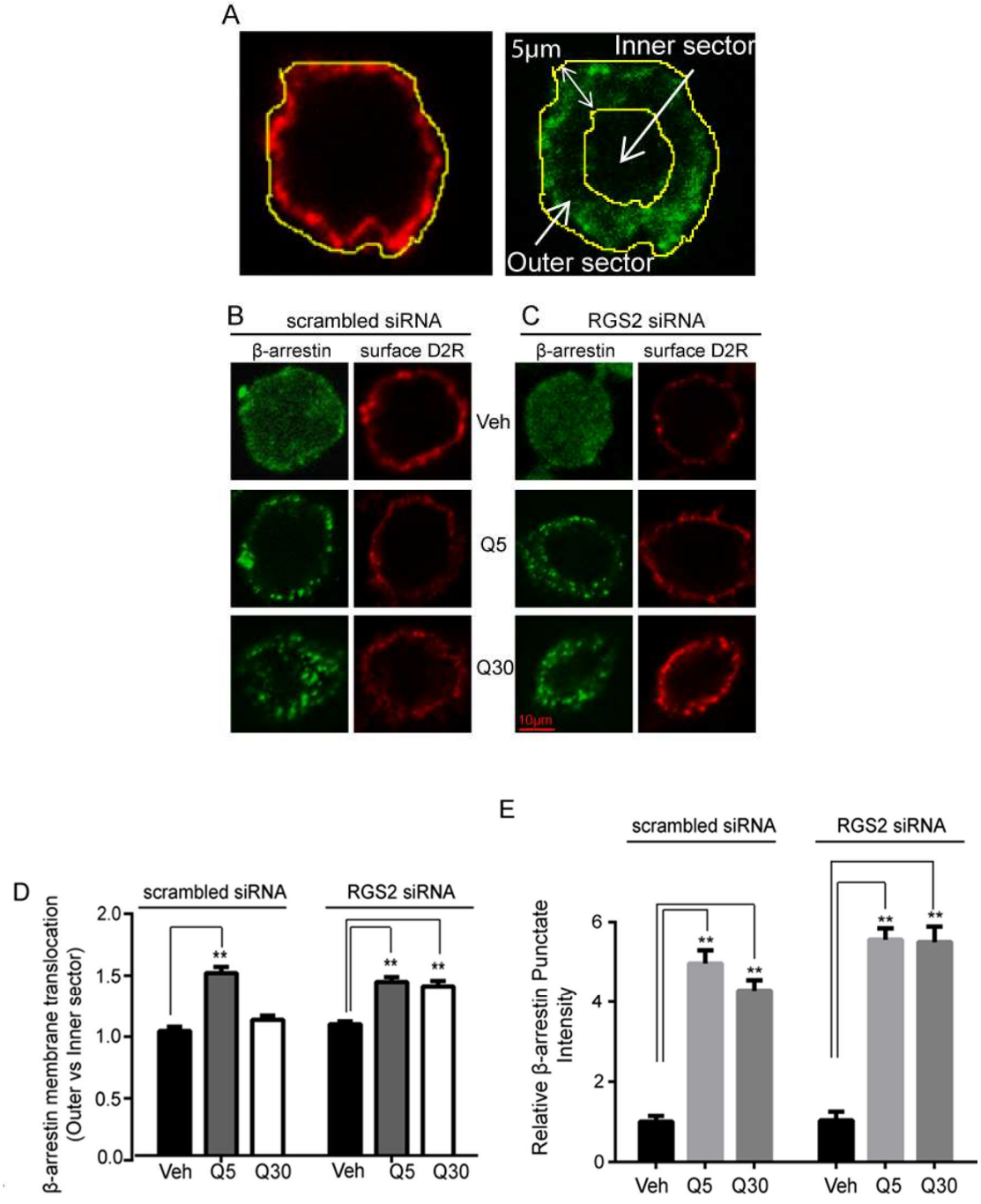

A
5μm
Inner sector
Outer sector
B
scrambled siRNA
β-arrestin
surface D2R
C
RGS2 siRNA
β-arrestin
surface D2R
Veh
Q5
Q30
10μm
D
scrambled siRNA
RGS2 siRNA
β-arrestin membrane translocation
(Outer vs Inner sector)
2.0
1.5
1.0
0.5
0.0
**
Veh
Q5
Q30
E
scrambled siRNA
RGS2 siRNA
Relative β-arrestin Punctate Intensity
6
4
2
0
**

Figure 2

RGS2 Knockdown Prevented Quinpirole-induced D2R Internalization. To determine the role of RGS2 knockdown in D2R internalization, constitutive and quinpirole-stimulated D2R internalization was examined using two distinct approaches. First, we used a modified antibody feeding technique to directly label internalized surface D2Rs as schematically illustrated in Fig. 3A. Data were expressed as a ratio of internalized surface D2Rs vs. the total surface D2Rs (Figs. 3B and 3C; n=55-70 cells/group). D2R internalization did not differ between the control and RGS2 knockdown cells at the basal level; however, quinpirole had differential effects on stimulated D2R internalization in these two types of cells. Treatment with quinpirole for 30 minutes induced 67± 1.8% increase in D2R internalization in control cells when compared to the vehicle treatment. In contrast, RGS2 knockdown abolished quinpirole-induced D2R internalization. Additionally, pre-treatment with the dynamin inhibitor, dynasore, blocked quinpirole-stimulated D2R internalization, demonstrating that D2R internalization is dynamin-dependent (Fig. S3).

D2R internalization was also determined by blocking D2R recycling. Cells were incubated at 18°C to prevent D2R recycling because temperatures under 20°C inhibit receptor traffic between early and recycling endosomes (Loder and Melikian, 2003). The experimental procedure was illustrated in Fig. 4A. Data were expressed as a ratio of intracellular vs. total D2R fluorescent intensity (Figs. 4B and 4C; n=55-70 cells/group). There was no difference in the basal D2R internalization between the control and RGS2 knockdown cells. Furthermore, we

observed 31± 1.5% increase in D2R internalization after 30 min exposure of quinpirole in control cells in comparison to the vehicle treatment; however, D2R internalization did not occur in RGS2 knockdown cells (Fig. 4C). Together, these data demonstrated that RGS2 knockdown significantly attenuated quinpirole-stimulated D2R internalization without compromising constitutive D2R internalization.

Figure 3. The effect of RGS2 knockdown on basal and stimulated D2R internalization determined by direct labeling of internalized D2Rs. (A) A schematic diagram of surface and intracellular D2R labeling for measurement of internalized D2Rs. Surface D2Rs were incubated with mouse anti-D2R followed by stimulation with vehicle (Veh) or quinpirole (1μM) for 30 minutes (Q30). Quinpirole treatment resulted in some labeled surface D2Rs to internalize while some remained on the surface. Remaining surface D2Rs were labeled with Alexa 647 (red). D2Rs that were internalized were identified by labeling with secondary antibody Alexa 405 (green). (B-C) Representative confocal images of surface and internalized D2Rs in scrambled siRNA (B) and RGS2 siRNA (C) transfected cells. Scale bar, 10 μm. (D) Quantification of internalized D2Rs which was represented as a ration of internalized vs. total D2Rs. Quinpirole-induced D2R internalization was observed in scrambled siRNA cells whereas internalization was abolished in RGS2 siRNA treated cells (n=55-70 cells/group, **P<0.01 vs. control, two-way ANOVA with Bonferroni posthoc analysis). No significant difference in constitutive D2R internalization was observed between scrambled and RGS2 knockdown cells. Experiments were replicated three times. Data are expressed as relative to the control.

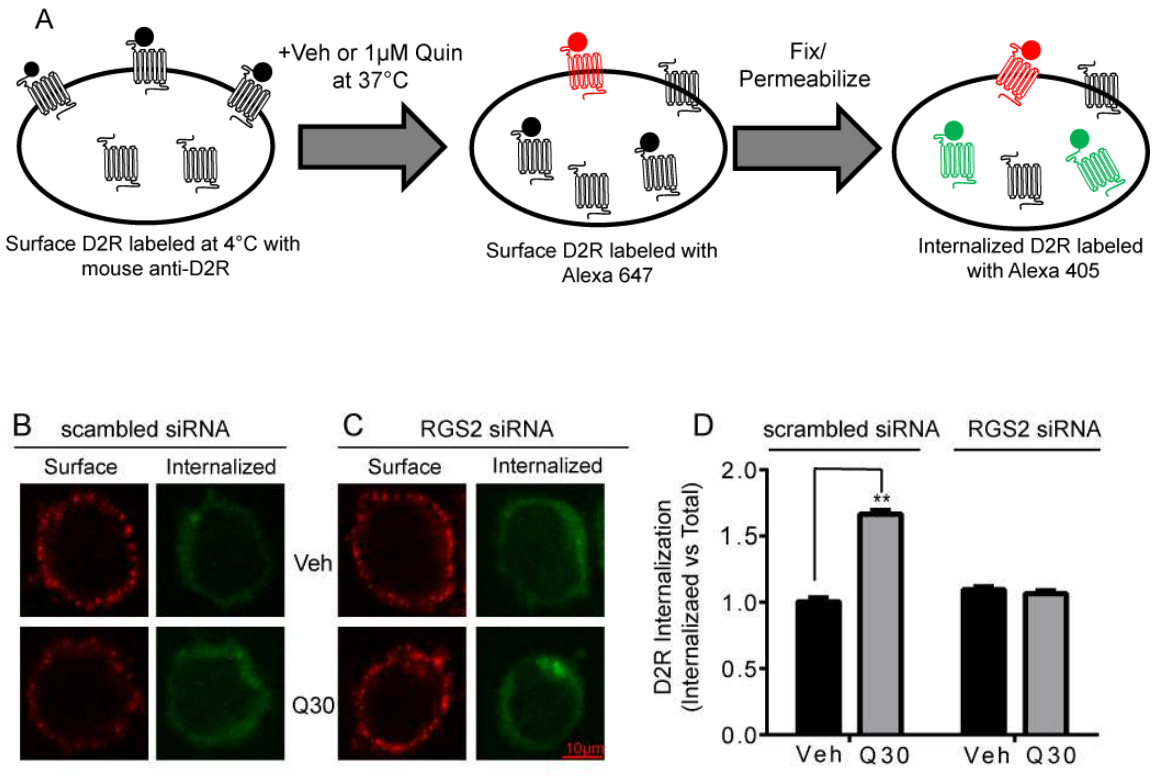
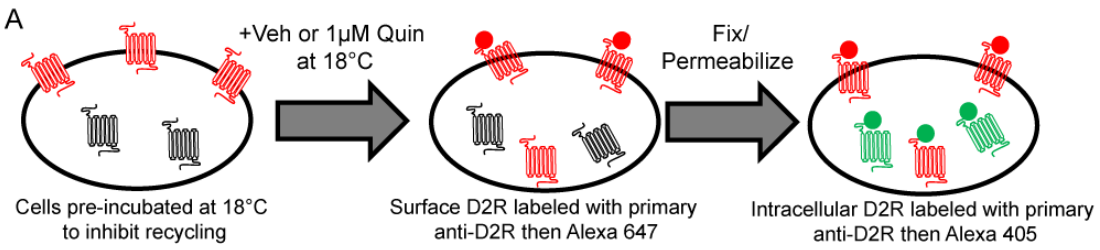
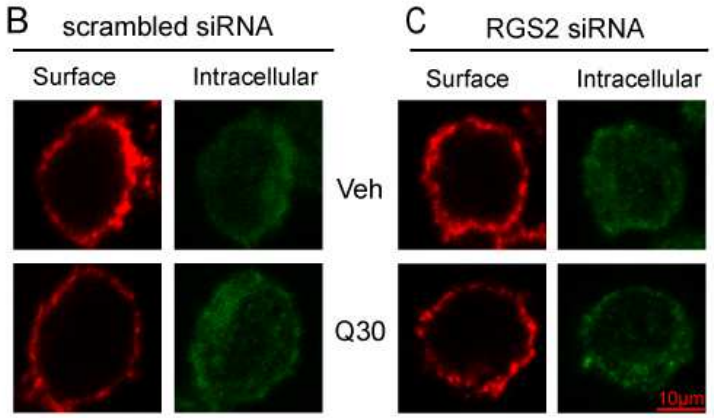

A
+Veh or 1μM Quin
at 37°C
Fix/
Permeabilize
Surface D2R labeled at 4°C with
mouse anti-D2R
Surface D2R labeled with
Alexa 647
Internalized D2R labeled
with Alexa 405
B scambled siRNA
Surface
Internalized
C RGS2 siRNA
Surface
Internalized
Veh
Q30
10μm
D
scrambled siRNA
RGS2 siRNA
D2R Internalization
(Internalizaed vs Total)
2.0
1.5
1.0
0.5
0.0
**
Veh
Q30
Veh
Q30

Figure 3

Figure 4. The effect of RGS2 knockdown on basal and stimulated D2R internalization determined by blocking D2R recycling. (A) A schematic diagram of measurement of D2R internalization by blocking D2R recycling at 18°C. N2A cells were treated with vehicle (Veh) or quinpirole (1μM) for 30 minutes (Q30) at 18°C to block receptor recycling. Cells were then labeled for surface (red) and intracellular D2Rs (green). (B-C) Representative confocal images of surface and intracellular D2Rs in scrambled siRNA (B) and RGS2 siRNA (C) cells. All scale bars 10 μm. (D) Quantification of internalization of D2Rs as a ratio of intracellular vs. total D2R fluorescent intensity. A significant increase in D2R internalization was observed after the quinpirole treatment in scrambled siRNA cells. However, RGS2 knockdown abolished quinpirole-induced D2R internalization (n=55-70 cells/group, **P<0.01vs. control, a two-way ANOVA with Bonferroni posthoc analysis). Experiments were replicated three times. Data are expressed as relative to the control.


A
+Veh or 1μM Quin
at 18°C
Fix/
Permeabilize
Cells pre-incubated at 18°C
to inhibit recycling
Surface D2R labeled with primary
anti-D2R then Alexa 647
Intracellular D2R labeled with primary
anti-D2R then Alexa 405


B
scrambled siRNA
C
RGS2 siRNA
Surface
Intracellular
Surface
Intracellular
Veh
Q30
10μm

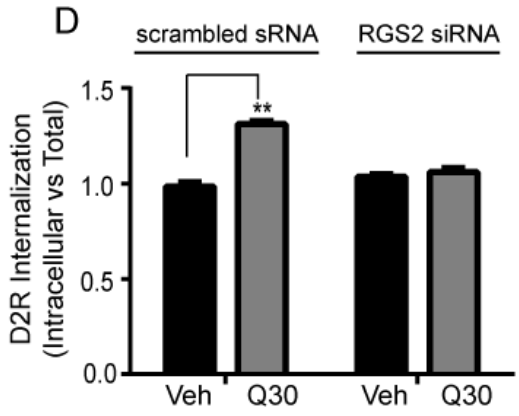
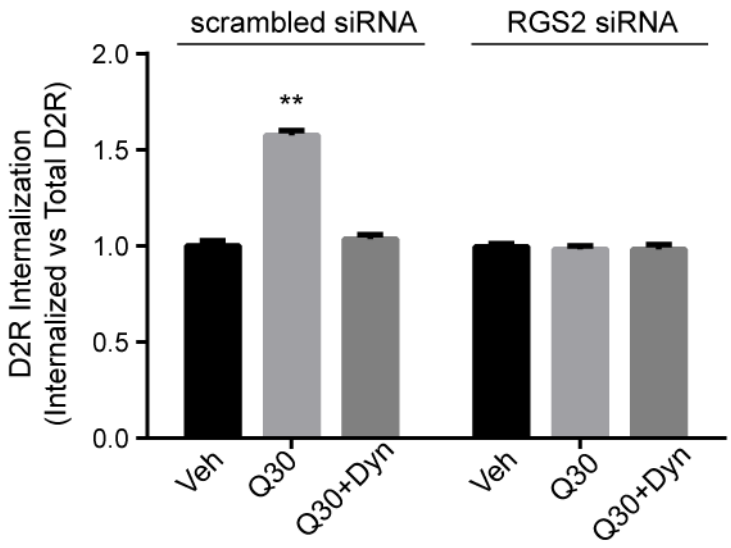

D
scrambled sRNA
RGS2 siRNA
D2R Internalization
(Intracellular vs Total)
1.5
1.0
0.5
0.0
**
Veh
Q30
Veh
Q30

Figure 4

S3. The effect of dynamin inhibitor dynasore on quinpirole-stimulated D2R internalization. Surface D2Rs were incubated with mouse anti-D2R followed by stimulation with vehicle (Veh), quinpirole (1μM) for 30 min (Q30) or quinpirole (1μM, 30 min) in the presence of 20 μM dynasore (Q30+Dyn). Remaining surface D2Rs were labeled with Alexa 647. Internalized D2Rs were identified by labeling with secondary antibody Alexa 405. Confocal images (not shown) of surface and internalized D2Rs in scrambled siRNA and RGS2 siRNA transfected cells were quantitated as a ratio of internalized vs. total D2Rs. Quinpirole-induced D2R internalization was observed in scrambled siRNA cells whereas quinpirole-stimulated internalization was abolished in dynasore pretreated treated cells (n=50-65 cells/group, **P<0.01 vs. control, two-way ANOVA with Bonferroni posthoc analysis). Experiments were replicated three times. Data are expressed as relative to the control.



Supplementary Figure 3

RGS2 Knockdown Accelerated Quinpirole-induced D2R Recycling. The effect of RGS2 knockdown on D2R recycling was examined using a modified antibody feeding technique to directly measure the recycling of internalized D2Rs after quinpirole treatment as illustrated in Fig. 5A. A ratio of fluorescent intensity for recycled D2Rs to the total surface D2Rs was determined (Figs. 5B and 5C; n=55-70 cells/group). Quinpirole treatment reduced D2R recycling by 17± 4.1% compared to the vehicle treatment in the control cells; however, quinpirole caused 48± 3.6% increase in D2R recycling compared to the vehicle treatment in RGS2 knockdown cells.

Figure 5. The effect of RGS2 knockdown on basal and stimulated D2R recycling. (A) A schematic diagram of labeling of recycled D2Rs in N2A cells. Surface D2Rs were incubated with rabbit anti-D2R at 4°C. Then cells were treated with vehicle (Veh) or quinpirole (1μM) for 30 minutes (Q30) at 37°C to allow surface D2Rs to internalize. The remaining primary antibody-bound, surface D2Rs were labeled with secondary goat anti-rabbit Alexa 405 (total surface D2R). Then cells were warmed up to 37°C again and treated with vehicle or quinpirole for 30 minutes to allow internalized, primary antibody-bound D2Rs to recycle to the surface, which were labeled with secondary goat anti-rabbit Alexa 555. (B-C) Representative confocal images of basal surface D2Rs and recycled D2Rs in scrambled siRNA (B) and RGS2 siRNA (C) cells. All scale bars, 10 μm. (D) Quantification of D2R recycling as a ratio of the intensity of Alexa 555 (recycled D2Rs that were internalized) vs. 405 (total surface D2Rs). Quinpirole decreased D2R recycling in control cells whereas quinpirole increased D2R recycling in RGS2 knockdown cells (n=55-70 cells/treatment group, **P<0.01 vs. control, two-way ANOVA with Bonferroni posthoc analysis). Experiments were replicated three times. Data are expressed as relative to the control.

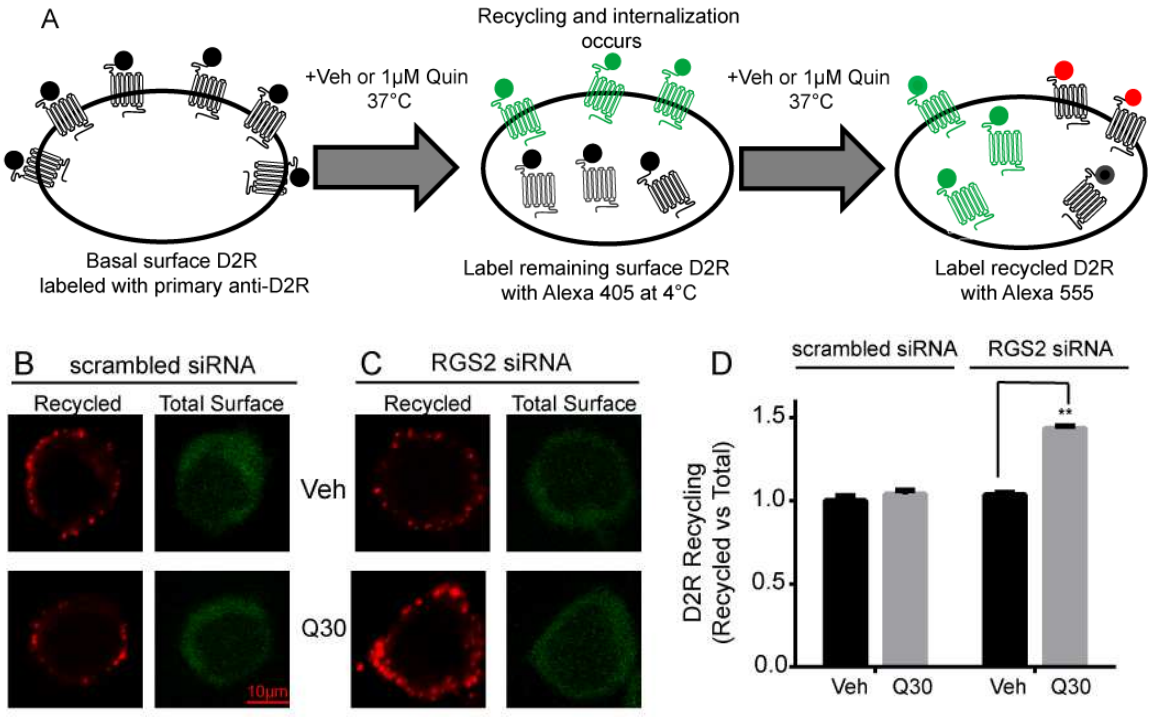

A
Recycling and internalization occurs
+Veh or 1μM Quin 37°C
+Veh or 1μM Quin 37°C
Basal surface D2R labeled with primary anti-D2R
Label remaining surface D2R with Alexa 405 at 4°C
Label recycled D2R with Alexa 555
B scrambled siRNA
C RGS2 siRNA
Recycled
Total Surface
Veh
Q30
10μm
D
scrambled siRNA
RGS2 siRNA
D2R Recycling (Recycled vs Total)
1.5
1.0
0.5
0.0
**
Veh
Q30
Veh
Q30

Figure 5

Discussion

The present study demonstrated for the first time that D2R simulation caused concurrent changes in D2R internalization and recycling in N2A cells. Moreover, we reported a new role of RGS2 in modulation of stimulated D2R trafficking. Quinpirole treatment induced a dose-dependent increase in D2R surface expression in RGS2 knockdown cells, which is in contrast to the dose-dependent decrease in quinpirole-induced D2R surface expression observed in control cells. Agonist-induced decrease in surface D2R expression has been shown in several non-neuronal cell lines. For instance, exposure to dopamine (10 μM) for 15 or 30 min resulted in a reduction in surface and an increase in intracellular D2R level in Chinese hamster ovary (CHO) cells (Goggi *et al.*, 2007). Surface D2R expression was also reduced in HEK293 cells following 60 min of dopamine (10 μM) treatment (Bartlett *et al.*, 2005). This is the first report that RGS2 knockdown reversed the effect of quinpirole treatment on D2R surface expression. Although RGS proteins are known to modulate GPCR signaling through acceleration of GTPase activity, emerging data also indicate a potential role of RGS proteins in regulation of trafficking of various GPCRs. For example, RGS9 overexpression in HEK293 cells abolished dopamine-induced reduction in surface D2R expression (Celver *et al.*, 2010). Overexpression of RGS4 in HEK293 cells attenuated agonist-induced reduction in surface delta-opioid receptors (Leontiadis *et al.*, 2009). Moreover, RGS14 knockdown prevented DAMGO-induced reduction in membrane mu-opioid receptors in the mouse brain

(Rodríguez-Muñoz, de la Torre-Madrid, Sánchez-Blázquez, *et al.*, 2007). These data suggest that the modulatory role of RGS proteins in receptor trafficking is RGS- and cell-type dependent. Here, we reported that RGS2 knockdown attenuated the effect of quinpirole on surface D2R level without altering constitutive D2R trafficking in a neuronal cell line. In contrast to our data, there was a report showing that RGS2 overexpression in HEK293 cells had no effect on D2R internalization (Min *et al.*, 2012b). Although differences in trafficking-related molecules between neuronal and non-neuronal cell lines may contribute to the discrepancy, the major difference in experimental approaches (knockdown vs. overexpression) may be the explanation. When endogenous RGS2 proteins are abundantly expressed in the cells, overexpression may not have an effect due to a potential "ceiling" effect. In agreement with this hypothesis, overexpression of RGS2 in N2A cells did not alter D2R trafficking in our hand (data not shown).

We further investigated whether altered D2R internalization and/or recycling contributed to the increased surface D2R level in RGS2 knockdown cells. Although agonist-induced reduction of surface D2R level has been consistently reported, the effects of D2R agonists on D2R internalization and recycling have not been concurrently characterized. Our data showed that quinpirole treatment significantly accelerated D2R internalization and attenuated D2R recycling when compared to vehicle treatment in control cells, which contributed to the overall reduction of surface D2R expression. In contrast, quinpirole treatment increased

D2R surface expression in RGS2 knockdown cells by increasing D2R recycling and preventing D2R internalization. It is worth noting that the amplitudes of changes in D2R internalization induced by quinpirole were notably variable between the methods used, which likely arose from the methodology used to assess these processes and how data were normalized. For example, there was an approximate 67% increase in D2R internalization following quinpirole treatment when internalized surface D2R was directly measured (Fig. 3B); however, there was only 31% increase in D2R internalization in the presence of quinpirole when D2R recycling was blocked at 18°C (Fig.4B). One potential confounding factor contributing to this discrepancy is that approximate 10% D2Rs can still undergo internalization although 90% D2R internalization was blocked (Koenig *et al.*, 1998).

β-arrestin plays an important role in internalization of GPCRs including D2Rs. Knockdown of β-arrestins in NS20Y neuronal cells abolished agonist-induced D2R internalization (Macey *et al.*, 2004). It has been shown that endocytosis of β2-adrenergic receptors is dependent on arrestin recruitment followed by subsequent interactions with clathrin and the endocytic adaptor AP2 (Claing *et al.*, 2002; Luttrell and Lefkowitz, 2002). Activation of D2Rs by dopamine was shown to induce membrane translocation of β-arrestins within 5 min in HEK293 cells (Kim *et al.*, 2001), which is consistent with our observation. To determine whether the difference in quinpirole-stimulated D2R trafficking between RGS2 knockdown and control cells resulted from impaired β-arrestin translocation, we

examined the trafficking pattern of β-arrestins. Consistent with the literature, β-arrestin membrane translocation occurred after 5 min exposure to quinpirole and was dissociated from the membrane after 30 min in control cells, which paralleled the time course of D2R internalization in the control cells. Moreover, it has been reported that recruited β-arrestin remains on the membrane for up to an hour following stimulation of β2 adrenergic receptors or D2Rs in HEK293 cells (Kim *et al.*, 2001; Oakley *et al.*, 2001). The discrepancy in the duration of β-arrestin membrane localization following D2R stimulation between our study and the literature could have resulted from the differences in cell types and isoforms of D2Rs. The short form of D2Rs (D2S) was expressed in N2A cells in our study whereas the long form (D2L) was transfected in HEK293 cells reported by Kim (2001). D2S has been shown to be more susceptible than D2L to dopamine-induced internalization in HEK293 cells (Kim *et al.*, 2004). Additionally, internalization of D2S, but not D2L, was dynamin-dependent. Thus, these variables likely contributed to the observed difference in β-arrestin trafficking pattern in cells expressing the different isoform of D2Rs. In contrast to the control cells, β-arrestin remained persistently localized on or adjacent to the membrane after 30 min exposure of quinpirole in RGS2 knockdown cells. Because RGS2 knockdown prevented quinpirole-induced D2R internalization, it is possible that knockdown disrupted quinpirole-induced receptor endocytosis by preventing the association of β-arrestin with the endocytic proteins such as clathrin or AP2. Another possibility is that RGS2 knockdown may compromise D2R phosphorylation by GRK2 and/or PKC. It has been shown that D2R can be

phosphorylated by GRK2 and PKC and phosphorylated D2Rs undergo β-arrestin and dynamin-dependent internalization (Kim *et al.*, 2001; Claing *et al.*, 2002; Namkung and Sibley, 2004). Although the role of RGS proteins in modulation of kinase-mediated receptor phosphorylation is largely unknown, RGS14 knockdown was shown to increase morphine-induced phosphorylation of serine 375 in the C terminus of mu-opioid receptors, which causes internalization of the receptors (Rodríguez-Muñoz, de la Torre-Madrid, Gaitán, *et al.*, 2007). Thus, RGS14 may attenuate GRK or PKC from phosphorylating residues of mu-opioid receptors that are required for β-arrestin mediated endocytosis. Accordingly, it is possible that RGS2 knockdown may decrease GRK2 or PKC activity, resulting in attenuated phosphorylation of D2Rs and subsequent inhibition of formation and internalization of the D2R-β-arrestin complex.

D2Rs can undergo constitutive recycling. Internalized surface D2Rs have been shown to redistribute from endocytotic vesicles to the cell surface within 30 min in HEK293 and N2A cells (Vickery and Von Zastrow, 1999). Here we demonstrated for the first time that quinpirole not only increased D2R internalization but also decreased D2R recycling in control cells, which contributed to the reduced surface D2R expression following quinpirole treatment. Although RGS2 knockdown did not affect constitutive D2R recycling, it accelerated D2R recycling following quinpirole treatment. A potential modulator of D2R recycling is the family of Rab proteins. Rab proteins have been identified as a critical modulator of endosomal recycling of many GPCRs

and transporters (Olkkonen and Stenmark, 1997; Stenmark, 2009) . A recent study has shown that Rab4 is associated with constitutive D2R recycling while Rab11 is associated with agonist-stimulated D2R recycling in HEK293 cells (Li *et al.*, 2012). Specifically, Rab4 dominant negative (DN) and Rab11DN mutant constructs abolished basal and dopamine-stimulated endosomal recycling of D2Rs, respectively. Thus, it is possible that RGS2 knockdown may increase D2R recycling by enhancing Rab11activity in the presence of quinpirole. It has been reported that Rab11DN decreases RGS4 function and its translocation to the plasma membrane in HEK293 cells (Bastin and Heximer, 2013). Therefore, the potential reciprocal relationship between RGS2 and Rab proteins is worth further investigation. Finally, we have also observed an enhanced cycling of intracellular D2R to the membrane by quinpirole treatment in RGS2 knockdown cells when compared to the control cells.

Taken together, our study demonstrated that RGS2 is an important modulator of D2R internalization and recycling. Future studies investigating the mechanisms of RGS2 function could provide a more detailed depiction of the physiological role of RGS2 and its regulatory role in D2R trafficking. Understanding the molecular mechanisms underlying D2R trafficking may open new avenues for identifying novel proteins involved in D2R signaling.

Acknowledgments

This work was supported by NIH grants (DA0006634, DA03690) and a pilot grant from the Center for Molecular Communication and Signaling at Wake Forest University. The authors would like to thank for the Wake Forest Microscopic Imaging Core Facility at the Department of Biology

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Wrote or contributed to the writing of the manuscript:

Chen, Luessen, Marrs, Howlett

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CHAPTER 3: OVERALL DISCUSSION

The primary aim of these studies was to elucidate the molecular mechanisms underlying D2R trafficking, including constitutive and agonist-induced internalization and recycling. The results from this study demonstrated a modulatory role of RGS2 on D2R trafficking, as well as providing a more detailed outlook on D2R regulation in a neuronal cell culture model. D2R surface availability is vital for proper dopaminergic transmission and deregulated D2R surface availability is observed in numerous diseases, including schizophrenia and drug addiction (Davis *et al.*, 1991; Koob and Volkow, 2010). Our study confirmed that quinpirole stimulation resulted in a reduction of D2R surface expression in a dose-dependent manner in a neuronal N2A cell line. A key finding of our study was that RGS2 knockdown resulted in a dose-dependent increase in D2R surface expression after quinpirole stimulation, in contrast to a decrease seen in control cells. This novel finding demonstrates that RGS2 proteins play a critical role in controlling D2R surface expression and agonist-induced receptor trafficking patterns.

The balance between D2R internalization and recycling dictates D2R surface availability and therefore plays a key role in various aspects of D2R function, such as signaling and resensitization. Our study evaluated D2R internalization and recycling, concurrently. By doing so, we hoped to further delineate whether the increase d D2R surface expression in RGS2 knockdown

cells was attributed to modification of D2R recycling or internalization. Our study provided novel insight to the modulatory role of RGS2 knockdown on D2R internalization as demonstrated by blunted quinpirole-induced internalization. A better understanding D2R internalization is vital for characterizing the pharmacological effects D2R targeting biochemicals, like PET radiotracers, as D2R internalization has been shown to impact factors like radiotracer binding potentials and binding affinity (Goggi *et al.*, 2007; Guo *et al.*, 2010).

Though the critical role of β-arrestin in D2R internalization has been shown in previous studies, the molecular events underlying arrestin activation and translocation remain unclear. Our study sought to delineate how RGS knockdown may alter β-arrestin translocation, potentially providing a rationale for the observed blunted D2R internalization. We were able to demonstrate that β-arrestin membrane translocation towards the membrane occurred after 5 minutes of quinpirole treatment followed by β-arrestin translocation away from the membrane region after 30 minutes in control cells, which paralleled the time course of D2R internalization, as seen previously. Conversely, we observed that β-arrestin remained persistently localized near the membrane after 30 minutes of quinpirole treatment in RGS2 knockdown cells. Because RGS2 knockdown prevented quinpirole-induced D2R internalization, even after 30 minutes of quinpirole stimulation, these findings together lead us to hypothesize that the decrease in D2R internalization in RGS2 knockdown cells could be associated with altered β-arrestin function mediated by GRK2 or PKC associated D2R

phosphorylation. More specifically, it has been demonstrated that D2R can be phosphorylated directly by GRK2 or PKC. These phosphorylated D2Rs then undergo β-arrestin and dynamin-dependent internalization (Kim et al., 2001; Claing et al., 2002; Namkung and Sibley, 2004). Specific serine and threonine residues within D2Rs are known to be phosphorylation sites for GRK2 and PKC. The phosphorylation of D2R and internalization has been further studied by pharmacological manipulation of these kinases. For instance, the level of phosphorylation of these residues has been shown to be enhanced by PKC activators, like phorbol esters, and increase D2R internalization, even without agonist-stimulation (Namkung and Sibley, 2004; Thibault *et al.*, 2011). The mechanisms by which these kinases phosphorylate D2Rs prior to internalization have also been shown to be highly regulated and condition specific. It has been demonstrated in HEK293 cells that the phosphorylation sites for basal internalization vary from the phosphorylation sites for agonist-induced D2R internalization (Celver *et al.*, 2013). Despite ample knowledge of how GRK2 and PKC influence D2R internalization, limited studies detail how RGS2 or other RGS proteins may affect the function of these kinases.

Another interesting finding of our study was that RGS2 knockdown results in significantly potentiated quinpirole-induced D2R recycling in addition to attenuated D2R internalization. D2R recycling is vital to resensitization but the underlying mechanisms of how D2R recycling is regulated remain unclear. In HEK293 cells, it has been demonstrated that previously internalized D2Rs can

recycle back to the plasma membrane both constitutively and after agonist stimulation (Vickery and Von Zastrow, 1999). Literature also suggests that D2R recycling may be GRK2-dependent, as co-expression of the mutant GRK2 K220R in HEK293 cells resulted in attenuated receptor recycling after stimulation with 10 μM DA for 1 hour (Cho *et al.*, 2013). Additionally, internalized D2Rs must be dephosphorylated and β-arrestin must dissociate prior to recycling back to the plasma membrane (Cho *et al.*, 2010). Despite some mechanistic evidence of the regulation of D2R recycling, the role of RGS proteins on D2R recycling has not been studied. Our finding of potentiated D2R recycling after quinpirole stimulation in RGS2 knockdown cells provides novel evidence that RGS2 may be a potential molecular regulator of D2R recycling *in vitro*.

Previous studies have delved into potential factors that may facilitate agonist-stimulated and constitutive recycling of GPCRs. In particular, the Rab family of proteins has been implicated as critical modulators of endosomal recycling (Olkkonen and Stenmark, 1997). More specifically, Rab4 has been associated with constitutive D2R recycling and Rab11 with agonist stimulated D2R recycling in HEK293 cells (Li et al., 2012). Rab proteins also play a critical role in mediating D2R internalization. In particular, Rab5 is implicated in GPCR internalization and commonly used as a protein marker for internalization into the early endosomes. Previous literature has shown that co-expression of the constitutively active or dominant negative forms of Rab5 in COS-7 cells results in facilitation or inhibition of D2R internalization, respectively (Iwata *et al.*, 1999).

Due to their established role in D2R recycling and internalization, Rab4, Rab5 and Rab11 are key proteins that may be altered by RGS2 knockdown.

To summarize, we hypothesized a model of D2R internalization (left panel) and recycling (right panel), as depicted in Figure 6. We postulate that GRK phosphorylation of D2R upon quinpirole stimulation allows for recruitment of β-arrestin and subsequent binding to the endocytotic machinery AP-2 and clathrin. D2Rs are then able to undergo clathrin-mediated endocytosis facilitated by Rab5. Upon internalization, D2R can then be directed to two fates: degradation or recycling back to the plasma membrane. We hypothesize that D2R recycling is facilitated by Rab11. Finally, D2Rs recycled back to the plasma membrane are then available for agonist binding and resensitization.

Figure 6. Illustrated mechanism of agonist-stimulated D2R internalization and recycling. 1) Upon prolonged stimulation of D2R by quinpirole, a D2 agonist, the activated GTP-bound α subunit undergoes GTP hydrolysis facilitated by RGS2 resulting in exchange of GTP to GDP and termination of signaling. GRK forms a complex with β and γ subunits of the G protein and phosphorylates specific serine and threonine residues located within the 3rd intracellular loop of D2R. 2) Phosphorylation of D2R by GRK results in β-arrestin recruitment to the receptor and subsequent binding of endocytotic machinery, AP-2 and clathrin. 3) AP-2 and clathrin facilitate removal of D2R from the plasma membrane in a clathrin-coated pit. The G protein, GRK and RGS2 dissociate from D2R and Rab5

facilitates endocytosis of the D2R-clathrin complex into the early endosome resulting in desensitization. 4a) Internalized D2Rs can then be targeted for transport into the late endosome and degradation. 4b) Alternatively, internalized D2Rs can be recycled back to the plasma membrane. After D2R is dephosphorylated and β-arrestin dissociates, Rab11 can then facilitate recycling. 5) Upon D2R recycling to the plasma membrane, the receptor is available for agonist binding resulting in recruitment and activation of the G protein subunits thus allowing for resensitization.

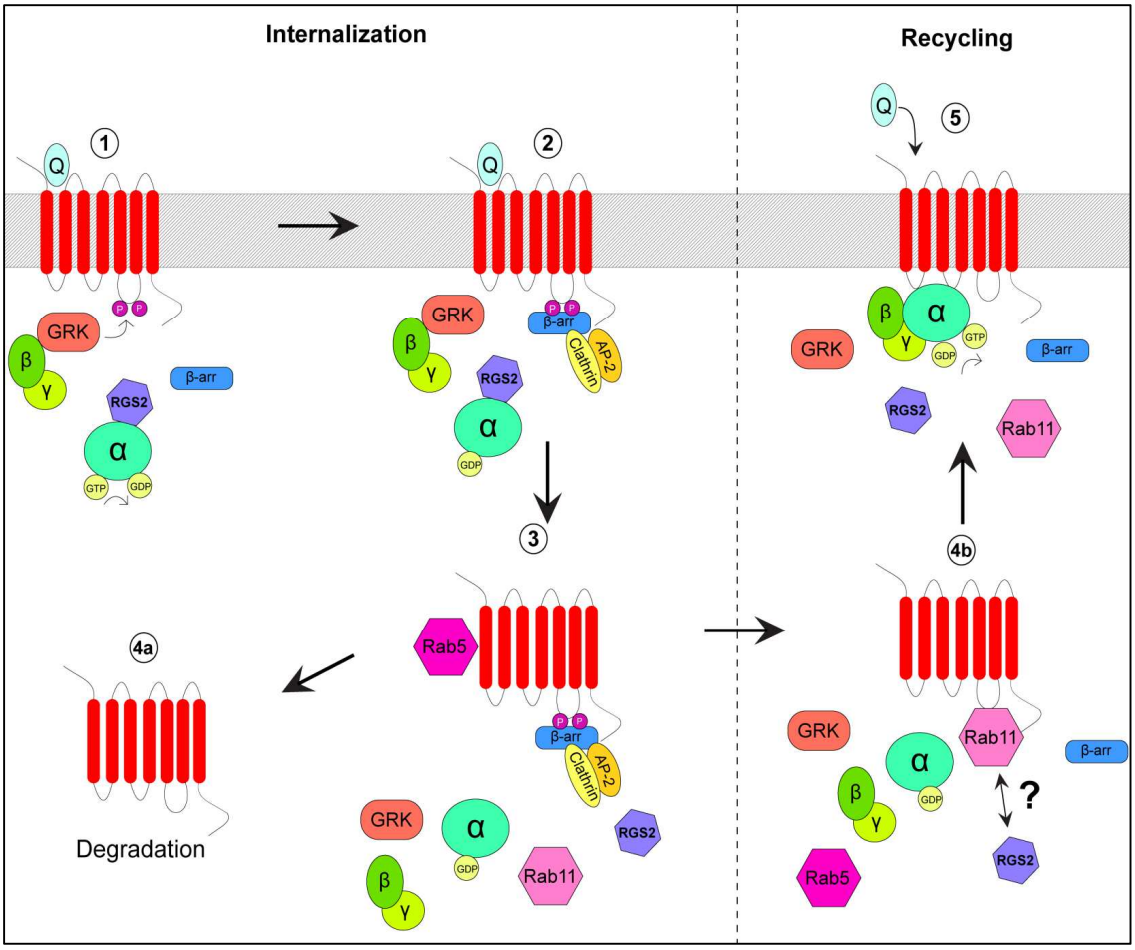
Internalization
Recycling
1
2
3
4a
4b
5
Q
GRK
β
γ
α
β-arr
RGS2
GTP
GDP
P
Clathrin
AP-2
Rab5
Rab11
Degradation
?

Figure 6

Future Directions

Future studies could elucidate the interaction between RGS2 and GRK2 or PKC, providing a more detailed explanation for altered D2R internalization. To do so, inhibition of GRK2 and PKC through specific inhibitors, like βARKct or PMA, respectively, could be used to parse out their contribution to the internalization effects seen in this study. Firstly, to examine the role of PKC in D2R internalization, N2A-D2R cells would be transiently transfected with scramble siRNA or RGS2 siRNA and pre-treated with the PKC inhibitor, PMA, followed by vehicle or quinpirole stimulation. Additionally, pre-treatment of the cells with the recycling inhibitor monensin could be performed to ensure that only internalization is observed. Under these drug conditions we would use the immunocytochemistry methods previously utilized in Chapter 2 to directly label internalized D2Rs thus allowing evaluation of PKC inhibition on D2R internalization. If PKC phosphorylation is altered by RGS2 knockdown, thus inhibiting D2R internalization, we hypothesize that inhibition of PKC will result in a gain of internalization in RGS2 knockdown cells after quinpirole treatment.

These experiments can also be conducted with the GRK2 peptide inhibitor βARKct, which inhibits GRK2 activation by competing for binding with Gβγ (Reinkober *et al.*, 2012). Using immunocytochemistry to directly label internalized D2Rs, we would be able to evaluate the role of GRK2 on D2R internalization in both control and RGS2 knockdown cells. Similarly, we hypothesize that if altered GRK2 function is involved in the inhibition of RGS2

knockdown-associated D2R internalization, then inhibition of GRK2 would result in recovered D2R internalization in RGS2 knockdown cells. The combination of these two sets of experiments would allow characterization of the interactions between RGS2 and GRK2 or PKC with regard to their respective roles in D2R internalization. Moreover, these findings could provide a more detailed mechanistic framework of functional interactions between RGS2 and specific kinases that control D2R internalization.

Though the interactions between RGS2 and these Rab proteins have not been directly studied, previous literature has eluded to functional interactions between Rab proteins and other RGS subtypes. In HEK293 cells, co-expression of a Rab11 dominant negative (DN) construct and RGS4 resulted in decreased translocation of RGS4 to the plasma membrane, along with decreased RGS4 function (Bastin and Heximer, 2013). Moreover, we hypothesize that there may be an interaction between RGS2 and Rab11. It is possible that knocking down RGS2 would result in increased Rab11 activity which could account for the significantly potentiated D2R recycling upon quinpirole stimulation, as seen in our study. Previous literature details that Rab activity and GDP/GTP exchange play a critical regulatory role in Rab protein translocation. The GTPase activity of Rab proteins and their translocation to respective subcellular compartments is necessary for their function, including endocytosis and recycling (Dorn 2nd and Mochly-Rosen, 2002). Therefore, evaluating RGS2 knockdown effects on Rab5

and Rab11 translocation could provide novel insight to the interactions between these two proteins and their regulation of D2R trafficking.

In future studies we hope to better characterize the functional interactions between RGS2 and Rab proteins in our neuronal N2A cell culture model. To do so, we will evaluate the translocation of Rab4, Rab5 and Rab11 proteins in both control and RGS2 knockdown cells to evaluate how RGS2 knockdown impacts the activity of these Rab proteins in particular. Similar immunocytochemistry techniques to those demonstrated in this study can be utilized to quantitate the translocation of Rab4, Rab5 or Rab11 both in basal and quinpirole stimulated conditions. We hypothesize that if RGS2 knockdown increases Rab11 activity thus potentiating D2R recycling then will observe increased Rab11 translocation. Additionally, we hypothesize that RGS2 knockdown could cause a decrease in Rab5 activity thus decreasing D2R internalization then we will observe a decrease in translocation of Rab5.

To gain a comprehensive understanding of the functional interactions between Rab4, Rab5 or Rab11 and RGS2, we plan to evaluate how altered Rab function effects RGS2 activity. Specifically, dominant negative or constitutively active Rab4 or Rab11 constructs can be co-expressed to evaluate alterations in RGS2 translocation as well as D2R trafficking. To do so, a series of experiments will be conducted to quantitate RGS2 translocation using immunocytochemistry

and confocal microscopy. From these experiments, we will be able to characterize the functional implications of constitutively active or dominant negative Rab protein manipulation on RGS2 function.

To evaluate the effect of Rab4, Rab5 or Rab11 on D2R trafficking patterns, particularly the internalization and recycling patterns observed in RGS2 knockdown cells, RGS2 knockdown will be performed in addition to overexpression of Rab4 or Rab11 constructs. Both D2R internalization and recycling will be evaluated using many of the techniques mentioned in Chapter 2. We hypothesize that if Rab4 or Rab11 function is disrupted then the potentiated D2R recycling trends observed in RGS2 knockdown cells will be abolished. Additionally, if Rab5 function is disrupted then the D2R internalization observed in control cells will be abolished. This collection of experiments will elucidate the potential functional interaction between RGS2 and Rab4, Rab5 or Rab11 thus providing detailed insight on regulation of D2R internalization and recycling by small modulatory proteins.

These studies as a whole, have provided insight into the range of regulatory control that RGS2 has on D2R trafficking. Due to its critical control of D2R trafficking and function, it is imperative to delineate a more detailed understanding of RGS2 function. Previous literature has demonstrated that RGS2 localization at or near the plasma membrane, where G proteins and other

effectors are also located, is vital to RGS2 function (Chatterjee and Fisher, 2000). RGS2 translocation from the nucleus to the plasma membrane occurs in a signal-dependent manner and is also vital to RGS2 function (Heximer *et al.*, 2001). From this evidence, one of our future goals is to characterize RGS2 translocation in our neuronal N2A cell culture model. Previous literature has shown that the RGS2 N-terminus is necessary for RGS2 translocation to the plasma membrane after agonist-stimulation (Heximer et al., 2001). Additionally, the N-terminal of RGS2 has also been demonstrated vital to intracellular targeting motifs, localization, degradation and signaling (Chatterjee and Fisher, 2000; Bodenstein et al., 2007). One of our future goals is to characterize RGS2 translocation and verify specifically which region of the N-terminus is necessary for RGS2 translocation. Moreover, we hypothesize that disrupting RGS2 translocation thus disruption RGS2 function will result in differential D2R trafficking from both control and full RGS2 knockdown cells.

Due to this critical role of the N-terminus in RGS2 activity and function, we aim to evaluate the specific effects of two truncated RGS2 N-terminus mutants on RGS2 translocation and activity, as well as D2R function. To do so, the cDNA of two RGS2 mutants were developed with truncated N-termini: 1) a partial truncation of the N-terminus (aa 33-69) and 2) a full truncation of the N-terminus (aa 1-79). To further study the effect of truncating the N-terminus, we will use immunocytochemistry and confocal microscopy to evaluate 1) RGS2

translocation, 2) D2R surface expression and 3) D2R signaling, in particular phosphorylation of ERK.

To quantify RGS2 translocation, two approaches will be utilized. N2A-D2R cells will be transiently transfected with either the full or partial N-terminus truncated RGS2 constructs for each experiment. RGS2 translocation will then be evaluated in both basal and quinpirole-stimulated conditions. Firstly, RGS2 translocation in and out of the nucleus will be addressed by evaluating RGS2 and nuclear stain, DAPI, colocalization. Secondly, RGS2 translocation towards and away from the plasma membrane will be quantified using the segmentation techniques described in Chapter 2. Together, the combination of these two approaches should provide a detailed overview of how truncation of the N-terminus may alter RGS2 translocation in neuronal N2A cells.

Altering RGS2 translocation by truncating the N-terminus could potentially mediate RGS2 negative modulatory control on various aspects of D2R function. Next, to evaluate the effect of RGS N-terminus truncation on D2R trafficking, immunocytochemistry and confocal microscopy will also be used to evaluate quinpirole-stimulated D2R surface expression. These studies will expand upon findings previously reported in our study, which demonstrated a unique effect of RGS2 knockdown on D2R surface expression as D2R surface expression was decreased upon quinpirole-stimulation. The primary goal of our future study is to

investigate if different truncations of the RGS2 N-terminus will result in varied D2R surface expression, in comparison to control and full RGS2 knockdown cells. The findings from these experiments will determine a more specific mechanism of how RGS2 exerts regulatory control on D2R trafficking.

Finally, to investigate the functional implications of specific regions of the RGS2 N-terminal on D2R signaling, basal and quinpirole-stimulated phosphorylation of ERK will be evaluated. N2A-D2R cells will be transiently transfected with the full or partial N-terminus truncated RGS2 constructs and cells will be stimulated with quinpirole at various time points. Western blot analysis will then be used to probe for phosphorylated ERK and total ERK to evaluate the impact of each RGS2 N-terminus mutant on D2R function. Together, the use of these RGS2 N-terminus mutant constructs will provide a detailed mechanism for RGS2 function: both by characterizing RGS2 translocation and RGS2 regulatory control of D2R trafficking and signaling.

Conclusions:

Together these studies begin to elucidate the molecular mechanisms underlying D2R trafficking, specifically internalization and recycling. Our findings implicate RGS2 in particular as a novel modulatory protein of D2R trafficking. We hope that through future investigation we can develop a more detailed understanding of D2R function and its modulation by small proteins, like RGS2.

Better understanding the regulatory roles of RGS2 in the dopaminergic system and on D2R function could potentially provide insight to the pathophysiology of numerous mental health disorders and movement disorders, as well as serve as a novel target for therapeutics to treat diseases and disorder impacting the dopaminergic system and dopamine neurotransmission.

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PUBLICATIONS

Luessen DJ, Hinshaw TP, Sun H, Howlett AC, Marrs G, and Chen R. RGS2 modulation of dopamine D2 receptor trafficking. Molecular Pharmacology. (Submitted Aug 2015)

Calipari ES, Sun H, Eldeeb K, **Luessen DJ**, Feng X, Howlett AC, Jones SR, and Chen R (2014) Amphetamine self-administration attenuates dopamine D2

autoreceptor function. Neuropsychopharmacology 39:1833–42, American College of Neuropsychopharmacology.

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Institutional / Departmental

Luessen, DJ (2015, April). RGS2 Modulates Dopamine D2 Receptor Trafficking and Signaling. Program in Physiology and Pharmacology Seminar, Wake Forest School of Medicine, Winston-Salem, NC.

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Funding

2014-2015 Center for Molecular Communication and Signaling Research Grant
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PROFESSIONAL REFERENCES: Available upon request