Regulation of the D2-Like Dopamine Autoreceptor by the Dopamine Transporter

by

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DEDICATION

I dedicate this thesis to my parents, Bob and Lori, and my siblings, Rob and Ali, Lauryn, and Emily. Thank you all for your love and support over the years, as well as your many, many funny text messages. To my husband Mike, thank you so much for keeping me going throughout this process. I am looking forward to our next adventures together as we move on to new places and opportunities. Finally, to my grandpa, Bob Luderman, Sr., your constant fascination with how the world works is an inspiration. I have appreciated every article you have shared with me and if I stay half as curious about life as you are, I will be lucky indeed.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

4AP: 4-aminopyridine, potassium channel blocker

AGG-D_{2S}: T225A/S228G/S229G-D_{2S} phosphomutant

COMT: catechol-*o*-methyl transferase

DOPAC: 3,4-dihydroxylphenylacetic acid

D_1-like: D_1 dopamine receptor family

D_2-like: D_2 dopamine receptor family

D_1R: D_1 dopamine receptor

D_2R: D_2 dopamine receptor

D_2 autoreceptor: D_2R functioning as an autoreceptor

D_{2S}: short variant of D_2R

D_{2L}: long variant of D_2R

D_3R: D_3 dopamine receptor

D_4R: D_4 dopamine receptor

D_5R: D_5 dopamine receptor

DAT: dopamine transporter

ERK: extracellular signal-regulated kinase

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

GIRK: G protein inwardly rectifying potassium channel

HVA: homovanillic acid

L-DOPA: 3,4-dihydroxyl-l-phenylalanine

LY: LY379196, PKCβ inhibitor

MAO: monoamine oxidase

PKA: cAMP-stimulated protein kinase

PKC: protein kinase C

PTX: pertussis toxin

QP: quinpirole, D_2R agonist

VMAT2: vesicular monoamine oxidase transporter 2

VTA: ventral tegmental area

ABSTRACT

Despite its relatively low abundance in the brain, the neurotransmitter dopamine is vitally important for controlling motor coordination, motivation, reward, and cognition, among other processes. The amount of dopamine in the extracellular space determines the amount of dopamine signaling and is primarily controlled by two presynaptic proteins: the dopamine transporter (DAT), which removes dopamine from the extracellular space, and the D2-like dopamine autoreceptor (D_2 autoreceptor). D_2 autoreceptor decreases extracellular dopamine by inhibiting dopamine synthesis, decreasing dopamine exocytosis, and increasing dopamine reuptake by DAT. My thesis focuses on understanding the regulation of D_2 autoreceptor and I determined that D_2 autoreceptor regulation changes depending on its context in the membrane. D_2 autoreceptor activation increases surface DAT localization, particularly in times of high neuronal stimulation, such as in response to natural rewards or abused drugs. I investigated the converse, DAT regulation of the D_2 autoreceptor and found that co-expression of DAT with D_2R in a heterologous cell system transforms the regulation of D_2R through a novel D_2R-DAT context. Within this context, less D_2R was on the surface as compared to expression without DAT, an effect dependent on protein kinase Cβ (PKCβ) activity. The D_2R-DAT context was disrupted by removing PKC phosphorylation sites from D_2R and DAT, suggesting PKC stabilizes this context. Normally, PKC causes internalization and desensitization of D_2R; using PKCβ knockout mice and specific PKCβ inhibitors, I found that PKCβ decreases D_2 autoreceptor activity. Furthermore, in the presence of DAT, agonist stimulation of D_2R increased surface D_2R localization, reminiscent of the D_2 autoreceptor-mediated increase in

surface DAT localization. Interaction with DAT increases D_2R signaling through ERK, perhaps through an arrestin-mediated mechanism. Because the D_2 autoreceptor stimulated increase of dopamine uptake only occurs during neuronal burst firing, I propose that the D_2 autoreceptor-DAT context is a mechanism to quickly decrease the extracellular dopamine concentration following burst firing through increased dopamine reuptake. During tonic dopamine release, D_2 autoreceptor regulates extracellular dopamine by suppressing dopamine synthesis and exocytotic release. My results identify a novel, DAT-mediated mechanism for regulation of D_2 autoreceptor and further our understanding of D_2R regulation.

CHAPTER ONE

INTRODUCTION

The Dopaminergic System

Despite its relatively low abundance in the brain, the neurotransmitter dopamine is a critical regulator of many important physiological processes, including motor function, cognition, motivation, and pituitary function. Dopamine is a precursor to the other catecholamines norepinephrine and epinephrine, though dopamine has its own separate neurons for signaling. Dopamine cell bodies are primarily located in the substantia nigra and the ventral tegmental area in the mesencephalon area of the brain, or midbrain. These cells project to other regions of the brain through three main pathways (Figure 1-1). The nigrostriatal pathway connects the cell bodies in the substantia nigra with the dorsal striatum. This projection is involved in controlling voluntary motor function and is implicated in neurological diseases such as Parkinson's and Tourette's syndromes. The dopaminergic cell bodies in the ventral tegmental area (VTA) form two dopamine projections. The mesocortical pathway connects the VTA with the frontal cortex. This pathway is integral for motivation, emotion, and cognitive control and is implicated in schizophrenia and attention deficit hyperactivity disorder. The second projection emanating from the VTA is the mesolimbic pathway, which terminates in the limbic structures of the brain, including the nucleus accumbens, olfactory tubercles, amygdala, and hippocampus. This pathway is involved in incentive salience, reinforcement, learning and desire and thus is thought to play a central role in addiction (Berridge, 2007). Several smaller projections exist, such as

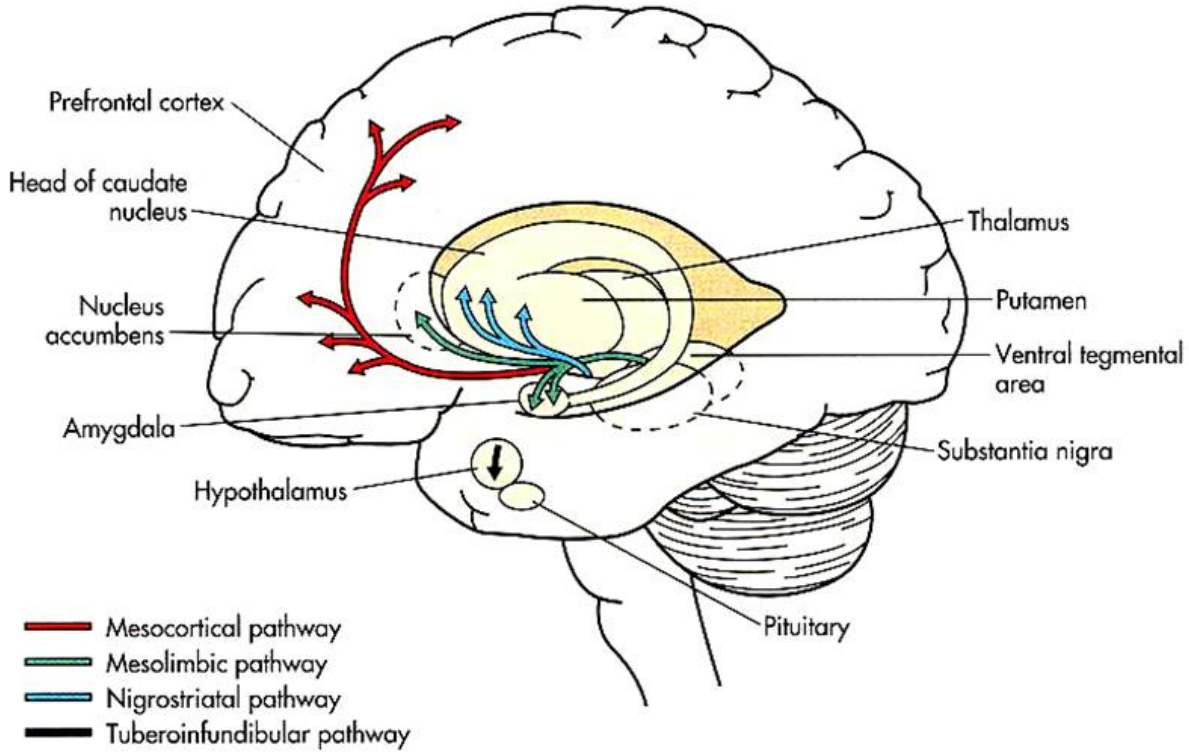

Figure 1-1: Dopamine Neuron Projections (Brody et al., 1998)

those in the hypothalamus and pituitary gland which control prolactin secretion. Dopamine is also found in the periphery and is involved in blood pressure regulation in the heart and kidney.

The synthetic pathway for the catecholamines is shown in Figure 1-2. The first step of this synthesis is the conversion of the amino acid tyrosine to 3, 4-dihydroxyl-l-phenylalanine (L-DOPA) by tyrosine hydroxylase. This enzyme is the rate-limiting step in the catecholamine synthesis pathway. L-DOPA is then decarboxylated by DOPA decarboxylase (aromatic amino acid decarboxylase) to form dopamine. In other cells, both within and outside the central nervous system, dopamine can be converted to norepinephrine and epinephrine. Once synthesized, dopamine is stored in vesicles to protect the neurotransmitter from degradation. These vesicles use the vesicular monoamine transporter 2 (VMAT2), which is coupled to a proton pump to provide the energy to concentrate dopamine inside the vesicle. The turnover of dopamine in the vesicles in the brain is very rapid due to leaky vesicles (Floor et al., 1995). Dopamine must be constantly synthesized to maintain stable dopamine levels in the brain.

Following action potential stimulation, the dopamine neuron depolarizes. Rising intracellular calcium concentrations stimulate the fusion of vesicles containing dopamine to the plasma membrane and dopamine is released in the extracellular synaptic space. From there, dopamine can bind to and activate receptors to propagate neuronal signaling. The amount of dopamine in the extracellular space determines the amount of dopaminergic signaling. Dopamine signaling is primarily terminated via reuptake of dopamine into the presynaptic neuron by the dopamine transporter. This removal of dopamine is more efficient than degradation by metabolizing enzymes or simple diffusion of dopamine away from the synapse.

Dopamine is metabolized primarily by two enzymes: monoamine oxidase (MAO) and catechol-*o*-methyl transferase (COMT). MAO is located inside the neuron on the exterior of

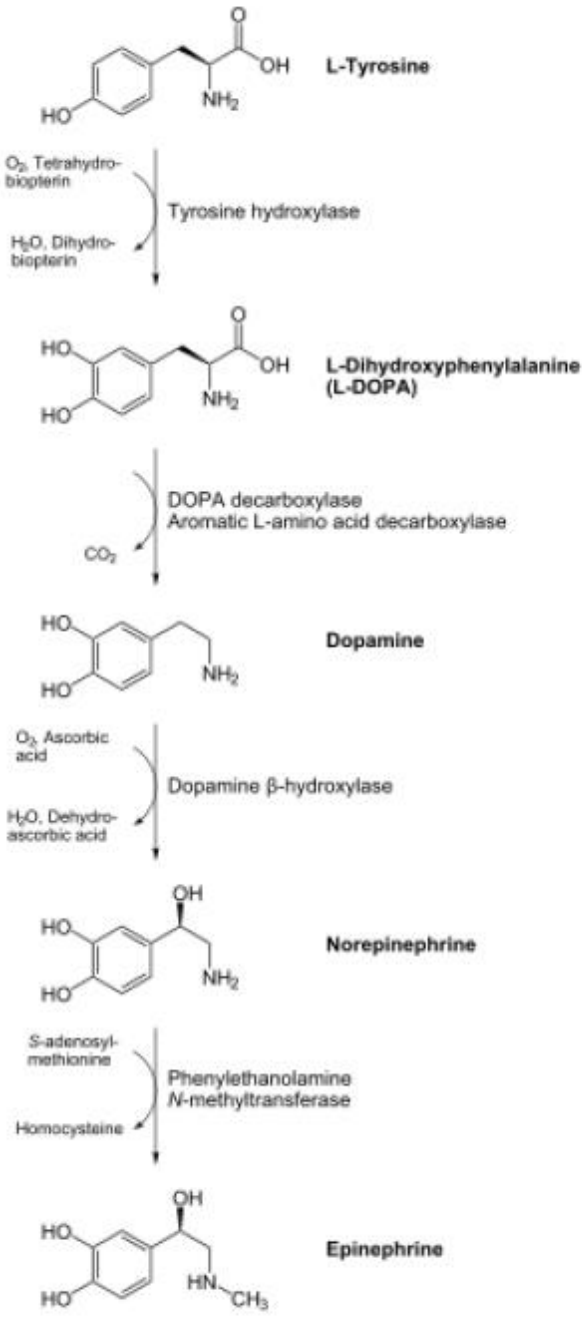

Figure 1-2: Synthetic Pathway for Dopamine (Gnegy, 2012)

mitochondria. It oxidizes the amine group on cytosolic dopamine, forming 3, 4-dihydroxy-phenylacetic acid, or DOPAC. MAO inhibitors have been used clinically to increase monoamine concentrations for the treatment of depression, obsessive-compulsive disorder, and Parkinson's disease. When on these drugs, the patient must not consume foods containing tyramine, which can cause an unsafe increase in monoamines in the body, leading to a hypertensive crisis. COMT is positioned on post-synaptic neurons and glia. Within cells, it is localized to the plasmalemmal membrane, the outer mitochondrial membrane, and rough endoplasmic reticulum. COMT metabolizes released dopamine and DOPAC by adding a methyl group to a hydroxyl group on the catechol ring, forming 3-methoxytyramine. MAO and COMT can further metabolize each other's metabolites, forming homovanillic acid (HVA). DOPAC and HVA are the major metabolites of dopamine. Measurement of these metabolites from cerebral spinal fluid or the bloodstream can be used to assess dopamine signaling in the patient.

The D_2 Dopamine Receptor

Identification and Classification

Dopamine signals through dopamine receptors. In the late 1970s, two different types of dopamine receptors, the D_1 and D_2 receptors, were identified using pharmacological methods (Cools and Van Rossum, 1976). The D_1 receptors stimulated adenylyl cyclase activity and had lower affinity for the butyrophenone and substituted benzamide classes of dopamine receptor ligands. The D_2 receptors, on the other hand, had high affinity for the butyrophenones and substituted benzamides. Unlike the D_1 receptors, D_2 receptors had either no effect on or inhibited adenylyl cyclase (Kebabian and Calne, 1979). Using molecular cloning techniques, five separate dopamine receptors were identified in the late 1980s. These five receptors were classified into two subfamilies according to activity. The D_1-like family contains the D_1 and D_5

receptors and is coupled to the stimulatory G_s protein for signaling. The D_2-like family comprises the D_2, D_3, and D_4 receptors and signals through the inhibitory G proteins $G_{i/o}$. The D_2 receptor is the focus of this thesis and will be discussed in greater detail.

Structure

The D_2 receptor (D_2R) is a seven transmembrane receptor and a member of the class A GPCR family. It is translated from the gene *DRD2*. D_2R was first cloned using a β_2 adrenergic receptor probe to screen a rat genomic library (Bunzow et al., 1988). In humans, this gene is approximately 52 kb long and contains 8 exons, the first of which is non-coding (Gandelman et al., 1991). Similar gene structures have been found for rat and mouse (Mack et al., 1991; O'Malley et al., 1990).

The human *DRD2* gene is translated to form a 414-443 amino acid protein containing seven transmembrane domains. The amino acid sequence and topology of D_2R is shown in Figure 1-3. The N-terminus is extracellular and contains three consensus sites for N-linked glycosylation. The receptor has a long third intracellular loop and short intracellular C-terminus tail, both of which are characteristic of receptors coupled to inhibitory G proteins (Sibley et al., 1993). Additionally, the receptor contains consensus sites for phosphorylation by various kinases, generally in the second and third intracellular loops, including cAMP-dependent protein kinase (protein kinase A) (Elazar and Fuchs, 1991), protein kinase C (PKC) (Morris et al., 2007; Namkung and Sibley, 2004), and G protein receptor kinases (Namkung et al., 2009a; Namkung et al., 2009b). Alternative splicing of the sixth exon of *DRD2* leads to the expression of short and long D_2R isoforms (D_{2S}, short; D_{2L}, long). The short isoform of D_2R lacks 29 amino acids in the third intracellular loop (Usiello et al., 2000). Because G proteins bind in this region of the

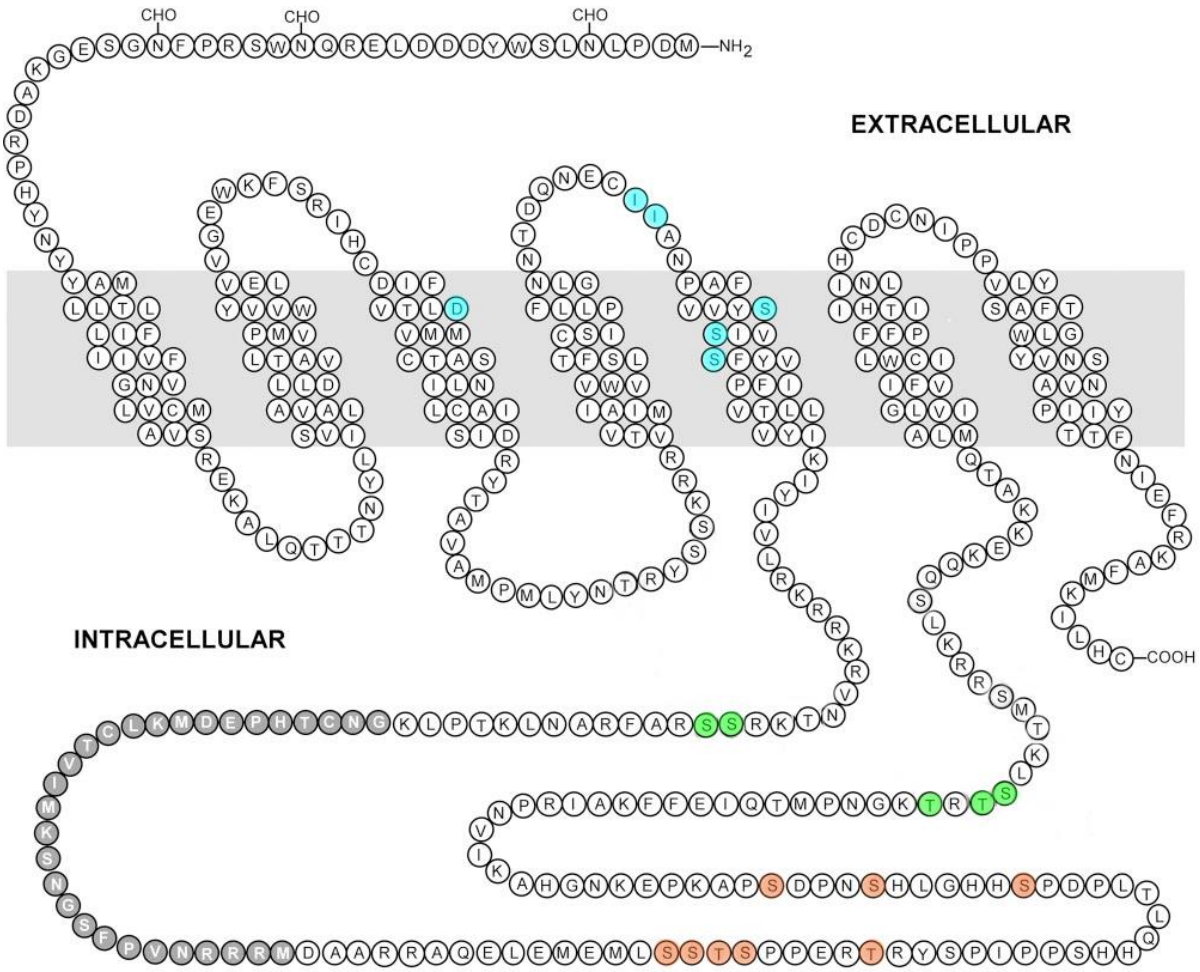

Figure 1-3: Amino acid sequence and topology of D_2R. Blue residues are involved in ligand binding. Green residues are protein kinase C (PKC) phosphorylation sites. Orange residues are G protein-coupled receptor kinases (GRK) phosphorylation sites. The 29 amino acids present in D_{2L} but not D_{2S} due to alternative splicing are indicated by the grey residues. Modified from (Namkung and Sibley, 2004).

receptor, this splice variant is reported to alter G protein interaction between the two receptors (Guiramand et al., 1995; Montmayeur et al., 1993).

The binding pocket in D_2R has been identified using several experimental approaches as well as molecular modeling. These studies found that dopamine and other ligands bind in a pocket formed by transmembrane domains three and five. Ionic interactions between the protonated amine and Asp114 in TM3 and hydrogen bonds between Ser193, 194, and 197 in TM5 and the catechol ring coordinate dopamine binding. The second extracellular loop also interacts with the ligand binding pocket such that Ile 183 and 184 form hydrophobic interactions with the ligand (Moreira et al., 2010). In addition to the ligand binding pocket, D_2R activity is influenced by ions such as Na^+, Mg^{2+}, and H^+. Sodium and lowering pH increase ligand affinity, while magnesium increases B_{max} (Neve, 1991; Sibley and Creese, 1983; Watanabe et al., 1985). These findings suggest that ions change the conformation of D_2R, altering the affinity and binding states of the receptor.

The other members of the D_2-like family, D_3R and D_4R, have the same basic receptor structure, with a long third intracellular loop and a short C-terminus. Homology between the receptors is highest within the transmembrane segments, with approximately 75% homology between D_2R and D_3R and 53% homology between D_2R D_4R (Gingrich and Caron, 1993). The D_3R contains 400-446 amino acids and also has several splice variants of the D_3R receptor. Splice variants of the third and fifth transmembrane domains and the second intracellular loop have no dopaminergic ligand binding activity (Giros et al., 1991). Two splice variants of D_3R that have receptor activity were identified in mouse, resulting in 21 additional amino acids in the third intracellular loop (Fishburn et al., 1993). The longer isoform shared high homology with the rat D_3R, while the short isoform more closely resembled the human D_3R. The D_4R normally

contains 387 amino acids, however this receptor has a 48 base pair variable-number tandem repeat in exon three. D_4R expressing 2-11 repeats have been found, resulting in 32-176 extra amino acids in the third intracellular loop (Grady et al., 2003). Associations between the 7-repeat allele of D_4R and novelty seeking and attention deficit hyperactivity disorder (ADHD) have been found (Ebstein et al., 1996; Faraone et al., 1999).

Distribution and Cellular Localization

D_2R expression throughout the brain was determined using autoradiography and ligand binding studies as well as through mRNA detection. Within the central nervous system, the receptor has the highest expression in the caudate putamen, nucleus accumbens, and olfactory tubercule, while lower expression has been detected in the substantia nigra and ventral tegmental area. Outside of the central nervous system, D_2R is expressed in the pituitary, retina, and kidney. The D_3R is expressed to a smaller extent than D_2R and is primarily found in the limbic regions of the brain, including the olfactory tubercle and nucleus accumbens, as well as the substantia nigra and ventral tegmental area (Gingrich and Caron, 1993). D_4R is also expressed at lower levels than D_2R and is primarily expressed in the frontal cortex, medulla, and amygdala (Sibley et al., 1993). Peripherally, both D_3R and D_4R are expressed in the kidney. Additionally, D_4R is highly expressed in the heart (O'Malley et al., 1992).

Differences in expression and localization of D_{2S} and D_{2L} are not fully understood. The mRNA for D_{2S} and D_{2L} are both found in the brain regions that express D_2R, though the ratio of D_{2S} to D_{2L} differs between regions (Neve et al., 1991). Some regions, including pituitary, striatum, and the midbrain were found to express more D_{2L} than D_{2S}. Other regions, including substantia nigra and cortex, express more equivalent amounts of the two splice variants. Khan and colleagues attempted to identify differences in cellular localization of D_{2L} in D_{2S}. In primate brain, D_{2S} co-

stained with tyrosine hydroxylase in dopaminergic neurons in the substantia nigra and ventral tegmental area (Khan et al., 1998). In the rhesus monkey striatum, D_{2L} was primarily found on GABAergic and cholinergic neurons. This promoted the thinking that D_{2S} was located presynaptically on dopaminergic neurons and functioned as an autoreceptor while D_{2L} was located postsynaptically. However, studies using single-cell RT-PCR from dopaminergic neurons isolated from rat substantia nigra found that these neurons are capable of expressing both D_{2S} and D_{2L} either singly or together (Jang et al., 2011). Further work is needed to determine if a difference in localization between D_{2S} and D_{2L} truly exists and what this may mean functionally.

G protein coupling

The members of the D_2-like dopamine receptor family are coupled to an inhibitory G protein heterotrimer of the $G_{\alpha i/o}$ family for signaling. This family of G proteins is characterized by inhibition of adenylyl cylase signaling and inactivation by pertussis toxin treatment. The D_2R has expressed promiscuity in coupling to G proteins. Several groups have found that D_2R can couple effectively to both $G_{\alpha o}$ and $G_{\alpha i}$ (Gazi et al., 2003; Lledo et al., 1992) and that agonists may induce selectivity for one G protein subtype over another (Cordeaux et al., 2001; Gazi et al., 2003). Studies using $G_{\alpha z}$ knockout mice found that D_2R couples to this pertussis toxin-insensitive G protein (Leck et al., 2006). The other members of the D_2-like family, D_3R and D_4R, activate multiple G proteins. D_3R can also couple to G_z and G_q, which activate phospholipase C (Lane et al., 2008; Sidhu and Niznik, 2000) in addition to G_o and G_i. D_4R activates G_z, G_o, and G_t (transducin) (Sidhu and Niznik, 2000). D_2-like family receptor coupling to specific G_β or G_γ members of the G protein hetermotrimer have not been determined.

The G protein heterotrimer binds to a receptor at the third intracellular loop of the receptor. As previously stated, the alternative splice variant of D_2R contains 29 additional amino acids in the third intracellular loop of D_{2L}. The secondary structure of this third intracellular loop appears to confer G protein selectivity, which may be affected by the amino acid insert in D_{2L} (Guiramand et al., 1995). D_{2L} and D_{2S} preferentially couple to different G proteins, with D_{2S} favoring coupling to G_o and D_{2L} preferring coupling to G_i (Lane et al., 2008; Liu et al., 1994; Montmayeur et al., 1993; Nickolls and Strange, 2003).

Signaling

By way of its coupling to G proteins, D_2R mediates downstream signaling through a variety of pathways. Many of the D_2R signaling pathways are summarized in Figure 1-4. Inhibition of adenylyl cyclase was the first signaling pathway identified for $G_{\alpha i/o}$ proteins coupled to D_2R (Neve et al., 2004). Activation of D_2R triggers a $G_{\alpha i/o}$ protein to inhibit adenylyl cyclase, decreasing in cAMP production and, in some cases, opposing the action of the stimulatory D_1Rs. The decrease in cAMP mediated by D_2R elicits other signaling changes, such as decreases in DARP32 (Lindgren et al., 2003) and tyrosine hydroxylase phosphorylation (Lindgren et al., 2001).

D_2R activation of $G_{\beta\gamma}$ subunits regulates the intracellular concentrations of Na^+ and Ca^{2+} ions. D_2R decreases neuron excitability by hyperpolarizing the cells via activation of potassium channels by $G_{\beta\gamma}$ (Fulton et al., 2011; Leaney and Tinker, 2000). Depending on cellular localization, the D_2 autoreceptor can activate different potassium channels. Somatodendritic D_2 autoreceptors activate G protein inwardly rectifying potassium (GIRK) channels (Inanobe et al., 1999; Pillai et al., 1998) At the terminal, D_2 autoreceptors couple with voltage gated potassium

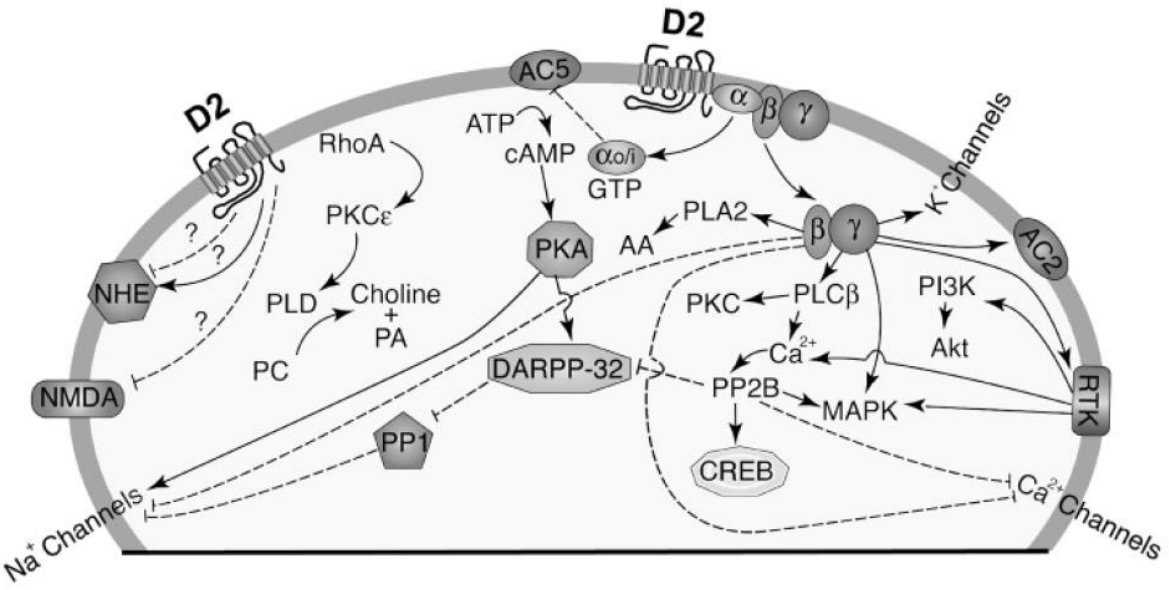

Figure 1-4: D_2R Signaling Pathways. Stimulatory pathways are indicated with solid arrows and inhibitory pathways are indicated with dashed bars. Signaling pathways are simplified with intermediate steps omitted. AA, arachidonic acid; AC, adenylyl cyclase; CREB, cAMP response element binding protein; DARPP-32, dopamine and cAMP regulated phosphoprotein, 32 kDA; MAPK, mitogen-activated protein kinase; NHE, Na^+/H^+ exchanger; PA, phophatidic acid; PC, phosphatidylcholine; PI3K, phophatidylinositol 3 kinase; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PP1 or PP2A, protein phosphatase 1 or 2A; RTK, receptor tyrosine kinase. (Neve et al., 2004)

channels, particularly those containing the Kv1.2 subunits (Cass and Zahniser, 1991; Congar et al., 2002; Fulton et al., 2011). D_2R can also inhibit L, N, and P/Q-type calcium channels to decrease neuron activity (Lledo et al., 1992; Neve et al., 2004). This leads to a decrease in intracellular calcium and inhibition of exocytosis of neurotransmitters such as acetylcholine (Dunlap et al., 1995), glutamate (Koga and Momiyama, 2000), and GABA (Momiyama and Koga, 2001). D_2R does not have a consistent effect on sodium channels, perhaps due to localization and interaction with other receptors, including D_1Rs (Neve et al., 2004). By activating the Na^+/H^+ exchanger, D_2R can increase the pH of the neuron to modulate signaling (Neve et al., 1992). Alterations to the sodium and pH balance of the neuron will also affect D_2R ligand affinity (Neve, 1991).

Activation of D_2R stimulates MAP kinases, including extracellular signal-regulated kinase (ERK). ERK activation regulates many cellular processes, including growth and differentiation. D_2R activates ERK through either $G_{\beta\gamma}$ or arrestin-mediated signaling, though there is some evidence that D_2R can activate ERK through $G_{\alpha i}$ (Beom et al., 2004; Kim et al., 2004; Lan et al., 2009). As will be discussed below, D_2R activation of ERK leads to an increase in surface DAT localization, resulting in greater dopamine reuptake from the extracellular space (Bolan et al., 2007). Although activation of phospholipase C activity is often associated with G_q-coupled signaling, D_2R can activate phospholipase C β via $G_{\beta\gamma}$. This leads to increased intracellular calcium concentrations and activation of protein kinase C (Hernandez-Lopez et al., 2000).

Desensitization and Internalization

D_2R signaling is terminated via desensitization and internalization of the receptor. Desensitization can be either homologous or heterologous, depending on if desensitization is mediated by the receptor's agonist (homologous) or if the desensitization is triggered by another

receptor type (heterologous). D_2R can undergo both types of desensitization. When undergoing homologous desensitization, agonist stimulation of D_2R triggers phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs). This phosphorylation decreases the receptor-G protein coupling, as well as increase the recruitment of arrestin and subsequent internalization and sequestration of the receptor. Using a heterologous cell system, six serine and two threonine (serines 285, 286, 288, 311, 317, and 321; threonines 287 and 293) residues in the third intracellular loop of D_{2L} were identified as GRK phosphorylation sites (Namkung et al., 2009a). Phosphorylation of these sites was increased by agonist treatment and overexpression of GRK2 and GRK3, but not by protein kinase C activation. Though this study was done using $D_{2L,}$ the identified GRK phosphorylation sites are present in both D_{2L} and $D_{2S.}$ For many GPCRs, agonist-stimulated phosphorylation of the receptor by GRK recruits arrestins to the receptor, triggering internalization of the receptor. D_2R preferentially associates with arrestin2 in neostriatal neuron cultures, though both arrestin2 and arrestin3 interact with the third intracellular loop of D_2R in striatal brain homogenates and heterologous cell systems (Macey et al., 2004). Once internalized and sequestered, GPCRs can either be recycled back to the surface of the cell or degraded (Ferguson et al., 1996). The internalization of D_{2L} and D_{2S} is differentially regulated. In a heterologous cell system, dopamine stimulated sequestration of D_{2S} at a faster rate than D_{2L} (Itokawa et al., 1996). Additionally, internalized D_{2S} receptors recycled back to the cell surface following dopamine washout faster than did D_{2L} receptors.

Interestingly, removal of the GRK phosphorylation sites did not alter the desensitization, internalization, or arrestin recruitment of D_2R in a heterologous cell system (Namkung et al., 2009a). Instead, the lack of GRK phosphorylation resulted in less recycling of the receptor back to the cell surface. This suggests that GRK phosphorylation determines the fate of the receptor

once it is internalized. Further, GRK2 suppressed D_2R surface localization and coupling to G proteins in a phosphorylation-independent manner (Namkung et al., 2009b). These findings suggest that GRKs can regulate receptors through mechanisms other than desensitization. Similar findings have been described for the β-adrenergic receptor where distinct phosphorylation of the receptor by two different GRKs leads to different signaling responses by the receptor (Nobles et al., 2011).

Heterologous desensitization occurs when activation of one receptor type causes the desensitization of a second. D_2R undergoes homologous desensitization via protein kinase C (PKC) phosphorylation (Morris et al., 2007; Namkung and Sibley, 2004). Mutation studies identified several PKC phosphorylation sites in the third intracellular loop of D_{2L}. Phosphorylation of these sites (serines 228, 229, and 355; threonines 352 and 354) caused internalization and desensitization of the receptor. These residues are also present in D_{2S}. Others have reported that D_{2L} is resistant to PKC-mediated desensitization due to a PKC pseudosubstrate domain in the third intracellular loop of the receptor (Morris et al., 2007). A D_{2L} mutant lacking the pseudosubstrate domain was regulated by PKC phosphorylation similarly to D_{2S}. Though D_2R can activate PKC via a non-canonical $G_{\beta\gamma}$-mediated phospholipase Cβ pathway, agonist treatment of D_2R does not stimulate PKC-stimulated phosphorylation of the receptor (Namkung and Sibley, 2004). The precise mechanism for PKC activation leading to regulation of D_2R is unknown. PKC is classically activated through G_q protein-mediated signaling. Stimulation of the G_q-coupled neurotensin receptor results in PKC-mediated internalization and desensitization of D_2R (Thibault et al., 2011), though more investigation is needed to determine if this is the mechanism responsible for PKC activation.

The D_2-Like Dopamine Autoreceptor

Because dopamine signaling is integral for so many normal physiological functions, as noted at the start of the Introduction, synaptic dopamine must be carefully regulated. The amount of dopamine in the extracellular space determines the amount of signaling and is controlled primarily by two proteins, the dopamine transporter and the D_2-like dopamine autoreceptor (D_2 autoreceptor).

Many D_2Rs are located postsynaptically and act as heteroreceptors on other neuron types that receive dopaminergic input, such as GABAergic and cholinergic cells (Khan et al., 1998). Some D_2Rs are located on dopaminergic cells and act as autoreceptors to decrease the amount of dopamine released by that cell. These receptors are located on the dendrites, soma, axons, and nerve terminals of dopaminergic neurons (Bello et al., 2011). The D_2 autoreceptors located on the dendrites and soma mainly decrease neuron firing rate (Bunney et al., 1973), while autoreceptors at the terminals inhibit dopamine synthesis by regulating tyrosine hydroxylase, decreasing dopamine exocytosis, and increasing reuptake through the dopamine transporter, discussed later in this section. Dopamine autoreceptors were identified as belonging to the D_2-like family rather than the D_1-like family due to the ability of selective D_2-like agonists and antagonists to alter stimulated dopamine release (Cubeddu et al., 1989). Due to receptor distributions, it was determined that D_2R and D_3R, but not D_4R can act as autoreceptors (Gingrich and Caron, 1993; Jang et al., 2011; Sibley et al., 1993).

D_2R and not D_3R was found to be the predominant autoreceptor in mice (Bello et al., 2011; L'Hirondel et al., 1998) In experiments measuring the release of dopamine *ex vivo* from wild type mice, the D_2R favoring agonist *R*(-)-propylnorapomorphine suppressed dopamine release but the selective D_3R agonist PD-128,907 could not (L'Hirondel et al., 1998). Additionally, in

D_2R knockout mice neither D_2R nor D_3R agonists could suppress dopamine release suggesting that the autoreceptor in mice is strictly D_2R. This conclusion is reinforced by the development of autoreceptor-selective knockout mice. These mice were generated by crossing *Drd2*$^{loxP/loxP}$ mice with *Dat*$^{+/IRES\text{-}cre}$ mice resulting in loss of D_2R only in those neurons also expressing the dopamine transporter (Bello et al., 2011). Again, the lack of presynaptic D_2R resulted in a loss of dopaminergic autoreceptor function. Together, these findings strongly suggest that D_2R is the predominant autoreceptor in mice. However, a role of D_3R as an autoreceptor cannot be ruled out in other animals (Jang et al., 2011).

Activation of D_2 autoreceptor results in hyperpolarization of the neuron and a decrease in cell firing (Anzalone et al., 2012; Bunney et al., 1973). While this autoreceptor activation occurs in response to exogenous agonists *in vitro*, *in vivo* it occurs in response to released dopamine (Paladini et al., 2003). D_2 autoreceptors influence neuron excitability and dopamine release by interacting with ion channels, as discussed above. The D_2 autoreceptor hyperpolarizes cells and decreases excitability by activating GIRK potassium channels (Cass and Zahniser, 1991; Fulton et al., 2011; Leaney and Tinker, 2000) or inhibiting calcium channels (Lledo et al., 1992; Neve et al., 2004). Both types of channels are involved in the release of dopamine (Phillips and Stamford, 2000). Not all dopaminergic neurons express GIRK channels (Lammel et al., 2008), thus the exact channel type(s) that D_2 autoreceptors interact with is unknown. The time course of the autoreceptor inhibition of dopamine release is estimated to last for milliseconds to seconds, depending on the experimental system and measurement used (Schmitz et al., 2003).

A second mechanism by which the D_2 autoreceptor controls dopamine signaling is by inhibiting tyrosine hydroxylase, the first and rate-limiting enzyme in the synthesis of dopamine from tyrosine. Phosphorylation of tyrosine hydroxylase at serine 40 by cAMP-stimulated protein

kinase A (PKA) increases the activity of the enzyme, increasing dopamine synthesis. Dopamine storage in vesicles is not very stable, with a half-life of minutes (Floor et al., 1995). Thus, the amount of dopamine synthesis is an important determinant of dopamine signaling and continuous synthesis of dopamine is required. Activation of the D_2 autoreceptor decreases phosphorylation of tyrosine hydroxylase at serine 40, decreasing dopamine synthesis (Lindgren et al., 2001). The decreased phosphorylation is thought to be through decreased cAMP concentrations in the cell which would lower PKA activity; however increased activity of phosphatases cannot be ruled out. Lack of D_2R activity, such as in the D_2 autoreceptor knockout mice, results in an increase in tyrosine hydroxylase activity, as measured by increased accumulation of the tyrosine hydroxylase product L-DOPA (Bello et al., 2011).

The third way the D_2 autoreceptor regulates the amount of extracellular dopamine is through interaction with the dopamine transporter. The dopamine transporter (DAT) is a presynaptically located transmembrane protein primarily responsible for removing dopamine from the extracellular space and thus terminating dopamine signaling. DAT will be introduced more fully in the next section of this chapter. In striatal synaptosomes from rat, treatment with the D_2R agonist quinpirole significantly increased the dopamine uptake rate through DAT (Meiergerd et al., 1993). This effect was blocked by co-treatment with the D_2R antagonist sulpiride, demonstrating involvement of the D_2 autoreceptor. The D_2 autoreceptor-stimulated increase in dopamine uptake via DAT is accompanied by an increase in surface DAT localization (Bolan et al., 2007). $D_2R^{-/-}$ mice, which lack all D_2Rs, have decreased DAT activity, but no change in DAT expression (Dickinson et al., 1999). $D_2R^{-/-}$ mice have slower dopamine clearance and lack the D_2 autoreceptor modulation of DAT observed in wild type mice. Studies using the D_2 autoreceptor knockout mice reported no difference in DAT activity from wild type, though the

D_2 autoreceptor-mediated increase in DAT activity was not directly assessed (Bello et al., 2011). Wu and colleagues determined that the apparent increase in dopamine release following treatment with a D_2R antagonist is due to decreased dopamine reuptake through DAT (Wu et al., 2002). As a result, the authors suggested that two populations of D_2 autoreceptor exist, one to control dopamine release and one to control dopamine reuptake via DAT. Furthermore, they found that the D_2 autoreceptor control of dopamine release predominated at lower stimulation frequencies, with autoreceptor control of reuptake becoming prominent at higher stimulation frequencies. Benoit-Marand and colleagues also found that dopamine reuptake is increased only in times of high stimulation *in vivo* (Benoit-Marand et al., 2011), suggesting the D_2 autoreceptor-mediated increase in DAT is a mechanism to decrease high extracellular dopamine during times of burst firing. The mechanism linking the D_2 autoreceptor and DAT involves ERK signaling stimulated by G protein activation (Bolan et al., 2007). Pretreatment with either pertussis toxin or the ERK inhibitor PD980059 inhibited the D_2R agonist quinpirole-mediated increase in dopamine uptake. The PI3K inhibitor LY294002 had no effect on the D_2 autoreceptor-stimulated increase in dopamine uptake, suggesting the Akt pathway is not involved. The signaling pathway leading to increased reuptake following D_2 autoreceptor stimulation involves PKCβ; PKCβ$^{-/-}$ mice lack the coordination between the D_2 autoreceptor and DAT (Chen et al., 2013). A physical interaction between the N-terminus of DAT and the third intracellular loop of D_2R has been reported, resulting in increased surface DAT localization and dopamine uptake (Lee et al., 2007).

The Dopamine Transporter

The dopamine transporter (DAT) is another presynaptic protein that predominantly controls the amount of dopamine in the extracellular space. DAT is a member of the family of Na^+/Cl^-

dependent transporters and mRNA for DAT is expressed in dopaminergic neurons. In primate brain, DAT colocalizes with tyrosine hydroxylase in many, but not all dopaminergic neurons (Lewis et al., 2001). In electron microscopy studies DAT was located outside of the active zone in the synapse, suggesting that dopamine must diffuse away from the site of release to be taken up by DAT. DAT is made of 620 amino acids and has intracellular N- and C-termini with twelve transmembrane domains, separated by alternating extracellular and intracellular loops (Giros and Caron, 1993). The second extracellular loop is particularly large and has several sites for glycosylation. The amino acid sequence and topology of DAT is depicted in Figure 1-5. The crystal structure for DAT has not been solved, but DAT structural analysis has been based on the crystal structure of the bacterial homolog leucine transporter (Yamashita et al., 2005). This structure indicated that substrates bind in a pocket formed by transmembrane domains one and six. The model for substrate uptake involves dopamine and two sodium ions binding to the outward-facing DAT. Once the substrate and ions are bound, DAT transitions to an inward conformation, where dopamine and sodium are released to the interior of the cell (Krishnamurthy et al., 2009).

DAT contains several consensus sites for phosphorylation by kinases such as PKA, PKC, and calcium/calmodulin-dependent protein kinase II (CaMKII), which can alter DAT activity. For example, phosphorylation by PKC at N-terminal serines significantly impairs amphetamine-stimulated dopamine efflux through DAT, but has no effect on dopamine uptake (Foster et al., 2002; Khoshbouei et al., 2004). The N-terminus of DAT interacts with several proteins, including syntaxin 1A, RACK1, and synuclein (Torres, 2006).

The critical role DAT plays in regulating dopaminergic signaling was demonstrated using $DAT^{-/-}$ mice (Giros et al., 1996). These mice were unable to remove dopamine from the extracellular

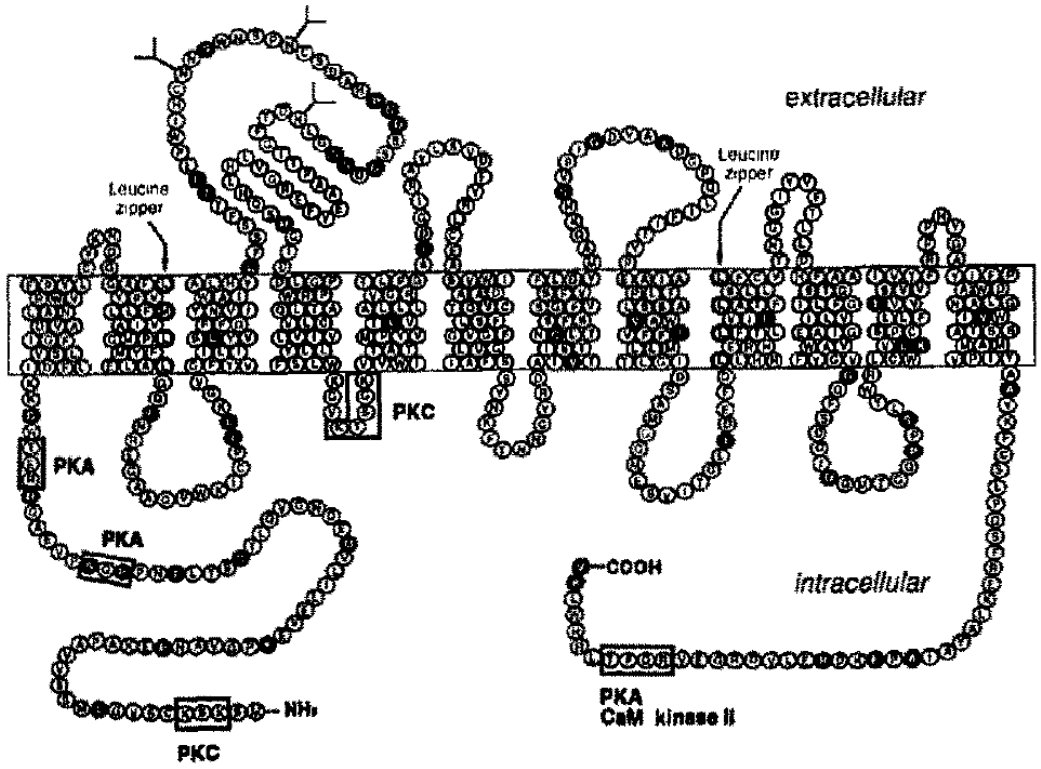

Figure 1-5: Amino acid sequence and topology of DAT (Giros and Caron, 1993)

space, resulting in a profound increase in basal locomotor activity. Additionally, these mice did not respond to the abused drugs cocaine and amphetamine, indicating that these drugs act at DAT. Interestingly, the $DAT^{-/-}$ mice had a 50% reduction in D_2R mRNA in the substantia nigra and VTA, further indicating the close relationship between these two proteins.

Protein Kinase C

PKC is a member of the larger group of serine/threonine kinases that contain protein kinases G and A, among others and is widely expressed throughout the body. The family of PKCs is made up of ten different isoforms, classified into three groups based on their regulatory domains. The conventional PKCs (α, βI, βII, and γ) require diacylglycerol, calcium, and phospholipids for activation. Novel PKCs (δ, ε, θ, and η) do not require calcium for activation, but do have a higher affinity for diacylglycerol. Finally, the atypical PKCs (ζ, ι/λ) require anionic phospholipids for activation instead of either calcium or diacylglycerol (Wu-Zhang and Newton, 2013).

Within the brain, PKC interacts with neurotransmitters via several mechanisms, including increasing SNARE complex formation, interacting with ion channels, and increasing the vesicle pool (Leenders and Sheng, 2005; Majewski and Iannazzo, 1998; Tanaka and Nishizuka, 1994) As a result, PKC activity increases the release of many neurotransmitters, including dopamine (Cubeddu et al., 1989), norepinephrine (Huang et al., 1989), and glutamate (Barrie et al., 1991; Tibbs et al., 1989). PKC regulates both D_2R and DAT through phosphorylation. As stated earlier, the N-terminus of DAT contains a series of serines that are phosphorylated by PKC (Foster et al., 2002). Removal of these serines either via mutation to non-phosphorylatable alanines or truncation of the N-terminus abolishes the amphetamine-stimulated efflux of dopamine through DAT without altering the normal uptake process (Khoshbouei et al., 2004).

D_2R is phosphorylated by PKC on the third intracellular loop causing internalization and desensitization of the receptor (Morris et al., 2007; Namkung and Sibley, 2004).

Our lab demonstrated that PKCβ activity is required for amphetamine-stimulated dopamine efflux through the use of PKCβ-specific inhibitors (Johnson et al., 2005b). Additionally, we found that PKCβ is involved in the rapid trafficking of DAT to the neuron surface in response to substrates and that PKCβ is expressed in dopaminergic neurons along with DAT (Chen et al., 2009; Furman et al., 2009; O'Malley et al., 2010). Finally, we determined that PKCβ is in the signaling cascade that links the D_2 autoreceptor and DAT (Chen et al., 2013).

Thesis Summary

The aim of this thesis is to better understand the signaling and regulation of the D_2 autoreceptor, particularly with regard to PKCβ and DAT. Together, the D_2 autoreceptor and DAT control the amount of dopamine in the extracellular space, and thus control the amount of dopamine signaling in the brain. Therefore, understanding how the D_2 autoreceptor is regulated is crucial to comprehend the control of the dopamine system.

This thesis project began with the observation that mice lacking PKCβ did not display the D_2 autoreceptor-mediated increase in surface DAT localization observed in PKCβ$^{+/+}$ mice (Chen et al., 2013). To determine if the D_2 autoreceptor is functional in these PKCβ$^{-/-}$ mice, I developed an assay to measure the D_2 autoreceptor control of exocytosis. I found that in the absence of PKCβ activity, the activation of the D_2 autoreceptor suppresses dopamine release to a greater extent than in the presence of PKCβ. The increased D_2 autoreceptor activity was not due to compensatory changes in overall D_2R expression or other PKC isoforms in the knockout mice; D_2 autoreceptor control of dopamine release was increased following acute inhibition of

PKCβ in synaptosomes from wild type mice. Our collaborator, using fast-scan cyclic voltammetry, which measures electro-stimulated dopamine release with precise spatial and temporal resolution, confirmed that acute inhibition of PKCβ enhances D_2 autoreceptor control of dopamine exocytosis. Mechanistically, I found that inhibition of PKCβ increases surface localization of D_2 autoreceptor. The increase in surface D_2R likely leads to greater D_2 autoreceptor activity, resulting in a reduction in extracellular dopamine and dopamine signaling. This mechanism was demonstrated behaviorally as increased locomotor suppression following treatment with the D_2R agonist quinpirole in the PKCβ$^{-/-}$ mice. The results are presented in Chapter Two.

A protein's environment can have profound effects on its activity. Phosphorylation at specific residues (Namkung et al., 2009a; Namkung et al., 2009b; Nobles et al., 2011) or binding of different agonists (Gazi et al., 2003) alter G protein selectivity, signaling pathways, or receptor downregulation of G-protein coupled receptors. Local ion concentrations or pH can regulate agonist binding to the receptor (Neve, 1991). We know that D_2 autoreceptor influences DAT surface localization and activity through signaling and/or physical interaction (Bolan et al., 2007; Lee et al., 2007). I posed the novel hypothesis that DAT alters D_2 autoreceptor surface localization and activity. This investigation into the DAT-specific context of D_2R is presented in Chapter Three. For this study, I used confocal microscopy and immunofluorescence to measure changes in surface localization of D_{2S} in the presence or absence of DAT. These experiments were performed in N2A neuroblastoma cells transfected with FLAG-D_{2S} with or without HA-DAT. I found that the presence of DAT significantly affects the regulation of D_{2S} by agonist or PKCβ. When D_{2S} is expressed in the absence of DAT, its surface localization is regulated similarly to other GPCRs, so that treatment with the agonist quinpirole internalizes the receptor.

However, when D_{2S} is co-expressed with DAT, D_{2S} is in a dissimilar, DAT-specific context, leading to different regulation. In this context, D_{2S} is in a state that is susceptible to internalization by PKCβ, manifested as decreased surface localization. PKCβ inhibition in this context therefore increases surface localization of D_{2S}. This increase in surface D_{2S} localization following PKCβ inhibition matches my findings in Chapter Two. In that study, I determined that PKCβ inhibition increases surface D_2R localization in mouse striatal synaptosomes, which express both D_2 autoreceptor and DAT. PKCβ appears to have no effect on basal surface D_{2S} localization in the absence of DAT. Removal of three PKC phosphorylation sites on the third intracellular loop of D_{2S} or truncation of the DAT N-terminus disrupts the interaction of D_{2S} and DAT, thus allowing D_{2S} to be regulated more similarly to a D_{2S} outside of the D_{2S}-DAT context. The D_2R agonist quinpirole differentially interacts with D_{2S} receptors alone or D_{2S} receptors interacting with DAT. When quinpirole activates D_{2S} receptors in the absence of DAT, the receptors internalize. However, when D_{2S} is interacting with DAT, quinpirole elicits an increase in the surface localization of both the D_{2S} receptor and DAT. This D_{2S}-DAT context also extends to ERK signaling, but not cAMP signaling, demonstrating a bias in the coupling of the receptor to effector proteins.

The final chapter of this thesis discusses the implications of the context-dependent regulation of D_2 autoreceptor, as well as the outstanding questions regarding D_2 autoreceptor regulation. Because the D_2 autoreceptor is one of two presynaptic proteins that controls extracellular dopamine, fully understanding its regulation will add to our comprehension of both normal dopamine signaling and the changes in that signaling that accompany various neurological and psychiatric disorders.

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CHAPTER TWO

PROTEIN KINASE C BETA REGULATES THE D2-LIKE DOPAMINE AUTORECEPTOR

Abstract

Protein Kinase C (PKC) regulates neuronal signaling by interacting with neurotransmitter release via several mechanisms, including interacting with ion channels and the structures involved in vesicular release. In addition, PKC desensitizes autoreceptors to increase the release of several different neurotransmitters. The focus of this study was the regulation of the D_2-like dopamine autoreceptor (D_2 autoreceptor) by PKCβ. Together with the dopamine transporter, the D_2 autoreceptor regulates the amount of extracellular dopamine and thus dopaminergic signaling. Here, using both PKCβ$^{-/-}$ mice and specific PKCβ inhibitors, we determined that lack of PKCβ activity increased the D_2 autoreceptor-stimulated decrease in dopamine release following both chemical and electrical stimulations. Inhibition of PKCβ resulted in an increase of D_2R on the surface of mouse striatal synaptosomes. The increase in active, surface D2Rs could underlie the increased sensitivity to quinpirole following inhibition of PKCβ. Finally, inhibition of PKCβ increased the sensitivity to the quinpirole-induced suppression of locomotor activity, demonstrating that this regulation of the D_2 autoreceptor by PKCβ is physiologically significant. Overall, we have found that PKCβ desensitizes the D_2 autoreceptor, providing an additional layer of regulation for dopaminergic signaling. We propose that in the absence of PKCβ

activity, surface D_2 autoreceptor localization and thus D_2 autoreceptor signaling is increased, leading to less dopamine in the extracellular space and lower dopaminergic signaling.

Introduction

Tight regulation of extracellular dopamine is crucial for normal dopaminergic signaling and is primarily achieved presynaptically by both the dopamine transporter (DAT) and the D_2-like dopamine autoreceptor (D_2 autoreceptor). The primary function of the DAT is to remove dopamine from the extracellular space, terminating dopaminergic signaling (Giros et al., 1996). The D_2 autoreceptor regulates extracellular dopamine levels by inhibiting further dopamine release upon agonist stimulation (L'Hirondel et al., 1998). Both D_2R and DAT are substrates for the widely expressed serine/threonine kinase protein kinase C (PKC) (Foster et al., 2002; Namkung and Sibley, 2004).

PKC is involved in many cellular processes, including neurotransmitter exocytosis. Activation of PKC by phorbol esters increases the release of various neurotransmitters following a depolarizing stimulus, including dopamine (Cubeddu et al., 1989; Huang et al., 1989; Barrie et al., 1991). PKC can affect exocytosis through several different mechanisms, including interacting with potassium or calcium channels, increasing the size and replenishing rates of vesicle pools, and increasing availability of the SNARE complex proteins involved in vesicle fusion [see reviews (Leenders and Sheng, 2005; Majewski and Iannazzo, 1998; Tanaka and Nishizuka, 1994)]. PKC can also alter exocytosis by interacting with presynaptic autoreceptors. Cubeddu and colleagues (1989) demonstrated that PKC activation with a phorbol ester reduced the activity of the D_2 autoreceptor, leading to a decrease in D_2R agonist-dependent inhibition of dopamine release. PKC activation phosphorylates D_2R to cause internalization and desensitization of the receptor (Namkung and Sibley, 2004; Morris et al., 2007). While there is

evidence showing that PKC affects the regulation of extracellular dopamine and D_2R, it has yet to be determined which of the ten mammalian PKC isoforms interacts with D_2R to cause these changes.

We previously reported that the PKCβ isoform regulates DAT trafficking and activity in response to amphetamine (Johnson et al., 2005; Furman et al., 2009; Chen et al., 2009). More recently, we determined that PKCβ is crucial for coordinating the interaction between the D_2 autoreceptor and DAT (Chen et al., 2013). Because of these findings, we hypothesized that PKCβ also regulates the D_2 autoreceptor. In the present study we used mice genetically lacking PKCβ along with specific PKCβ inhibitors to determine the impact of this kinase on D_2R activity. D_2 autoreceptor activity was assessed by measuring dopamine exocytosis following chemical stimulation of synaptosomes or electrical stimulation in brain slices, as well as measuring D_2R-mediated changes in locomotor activity. We determined that PKCβ interacts with the D_2 autoreceptor to regulate its surface localization and activity. Coupled with our findings regarding PKCβ regulation of DAT, this work identifies a role for PKCβ as a key regulator of extracellular dopamine levels and thus dopaminergic signaling.

Materials and Methods

Animals. All animal use and procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines. Wild type C57BL/J6 mice were obtained from an in-house breeding program and Jackson Laboratories. The generation of $PKC\beta^{+/+}$ and $PKC\beta^{-/-}$ mice was previously described (Leitges et al., 1996) and included backcrossing with C57BL/6J mice at least ten times. Mice had free access to water and

standard laboratory chow. Experimental mice were gender matched and were used between two and four months of age.

Chemicals. LY379196 was a generous gift from Eli Lilly (Indianapolis, Indiana). Enzastaurin was purchased from LC Labs (Woburn, MA). The [^{3}H]-sulpiride for radioligand binding studies was from PerkinElmer (Waltham, MA). Complete Mini protease inhibitor was purchased from Roche Diagnostics (Indianapolis, IN). All other chemicals, including 4-aminopyridine, quinpirole, sulpiride, and butaclomol, were purchased from Sigma Aldrich (St. Louis, MO).

Striatal dopamine release via suprafusion. Synaptosomes from whole striata were prepared as described previously (Chen et al, 2009). Briefly, mice were sacrificed by cervical dislocation. Striata were dissected on ice and homogenized in 0.32 M sucrose containing Complete Mini protease inhibitor cocktail. Homogenates were centrifuged at 4°C (800xg, 10 minutes) to remove cellular debris. The supernatant was centrifuged again (12,000xg, 15 minutes, 4°C). The pellet containing synaptosomes was resuspended in oxygenated Kreb-Ringer's Buffer (KRB) (145 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $MgCl_2$, 10 mM glucose, 24.9 mM $NaHCO_3$, 0.05 mM ascorbic acid, 0.05 mM pargyline, pH 7.4). Synaptosomes were loaded into the chambers of a Brandel suprafusion apparatus (Brandel Inc., Gaithersburg, MD). The samples were perfused with oxygenated KRB at approximately 800 µl/min. Following a 60 minute wash to achieve a steady baseline, 14 fractions were collected at one-minute intervals. Exocytotic dopamine release was stimulated at fractions seven and eight with 50 µM 4-aminopyridine (4AP). When present, quinpirole and sulpiride treatments were included with the 4AP stimulation. Treatment with the PKCβ inhibitor LY379196 began during the 60 minute wash period and continued throughout fraction collection. An internal standard solution

composed of 50 mM perchloric acid, 25 μM EDTA, and 10 nM 2-aminophenol was added to each fraction. Dopamine content in each fraction was measured using HPLC with electrochemical detection (Thermo Scientific/esa, Sunnyvale, CA).

Striatal dopamine release via electrical stimulation. Brain slices were prepared as described previously (Mateo et al., 2005). Briefly, mice were decapitated, brains rapidly removed, and coronal brain slices (400 μm thick) containing the nucleus accumbens were prepared using a vibrating tissue slicer. Slices were maintained at 32 °C in oxygen-perfused (95% O_2–5% CO_2) modified Kreb's buffer, which consisted of (in mM): NaCl, 126; $NaHCO_3$, 25; D-glucose, 11; KCl, 2.5; $CaCl_2$, 2.4; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; L-ascorbic acid, 0.4; pH adjusted to 7.4. A capillary glass-based carbon-fiber electrode (active area ~100 μm long, 7 μm wide) was positioned approximately 75 μm below the surface of the slice in the nucleus accumbens core. Dopamine release was evoked every 5 min by a 4-ms, one-pulse stimulation (monophasic, 300 μA) from a bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) placed 100–200 μm from the carbon-fiber electrode.

Fast-scan cyclic voltammetry recordings were performed and analysed using locally written software (Demon Voltammetry and Analysis; Yorgason et al., 2011). The electrode potential was linearly scanned as a triangular waveform from −0.4 to 1.2 V and back to −0.4 V (Ag vs. AgCl) using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon-fiber electrode every 100 ms by means of a potentiostat (Dagan, Minneapolis, MN, USA). Once the stimulated dopamine response was stable for at least three successive collections, baseline measurements were taken. Evoked extracellular concentrations of dopamine were assessed by comparing the current at the peak oxidation potential for dopamine with electrode calibrations of known concentrations of dopamine (1–3 μM). Data were modeled using Michaelis-Menten kinetics to

determine DA released and V_{max} (Yorgason et al., 2011).

The selective D2-type receptor agonist (–)-quinpirole hydrochloride was used to induce autoreceptor activation. Quinpirole-induced decreases in electrically stimulated dopamine release were compared with pre-drug values (each animal served as its own control) to obtain a percent change in stimulated dopamine release. Treatment with the PKCβ inhibitor enzastaurin (200 nM) began after stable baselines were obtained, 60 minutes before quinpirole was added, and continued throughout the experiment. Quinpirole dose–response curves were plotted as log concentration (*M*) of quinpirole vs. percent of control dopamine response.

D2 Receptor Binding. Striatal synaptosomes were prepared as described above and were resuspended in KRB. To measure surface D_2R binding, synaptosomes were treated for 5 minutes at 37°C with vehicle or the PKCβ inhibitor enzastaurin. Following treatment, the synaptosomes were incubated with 10 nM [^{3}H]-sulpiride, a hydrophilic D2 receptor antagonist, for 3.5 hours on ice. Non-specific binding was determined by including 10 μM (-)-butaclamol. Binding was terminated by filtering over GF/B Whatman filters and washing 3X with ice cold KRB and was quantified by scintillation counting. Overall D_2R expression was determined using a membrane preparation from the striatal synaptosomes prepared above. These synaptosomes were resuspended in 50 mM Tris-HCl (pH 7.4) and centrifuged at 40,000 x g for 15 minutes. The resulting membrane fraction was resuspended in KRB and then treated with vehicle or enzastaurin for 5 minutes at 37°C. Membranes were incubated with 10 nM [^{3}H]-sulpiride $\pm$ 10 μM (-)-butaclamol for 90 minutes at room temperature. Binding was terminated by filtering over GF/B Whatman filters and washing 3X with ice cold KRB and was quantified by scintillation counting.

Locomotor suppression by acute quinpirole treatment. Locomotor suppression following quinpirole treatment in a novel environment was measured using radiotransmitter implantation (Mini Mitter Co., Bend, OR) as previously described (Chen et. al, 2007). Briefly, a radiotransmitter was implanted into the peritoneal cavity of each mouse. Following recovery, $PKC\beta^{+/+}$ and $PKC\beta^{-/-}$ mice were injected with saline and quinpirole (0.03, 0.1, or 0.3 mg/kg i.p.). Locomotor activity (gross activity count) was recorded immediately after the injection for 15 minutes.

Statistical Analysis. Results were analyzed using GraphPad Prism 6 software (San Diego, CA) and are plotted as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$. Comparisons between multiple groups or treatments were made using one-, two- or three-way ANOVA with appropriate post-test. Three-way ANOVA was performed using Systat (Chicago, IL). When only two groups were compared, a paired, two-tailed Student's *t*-test was used.

Results

Suppression of PKCβ activity increases D_2 autoreceptor control of dopamine release.
The primary function of the D_2 autoreceptor is to control the amount of dopamine in the extracellular space and thus the amount of dopamine signaling. Activation of the D_2 autoreceptor inhibits further dopamine exocytosis. We developed a suprafusion assay to measure this D_2 autoreceptor control of dopamine exocytosis. In this assay, striatal synaptosomes are prepared from mice and perfused with KRB to achieve steady basal release of endogenous dopamine and then several fractions are collected. The amount of dopamine in each fraction is determined using an HPLC coupled with electrochemical detection. Dopamine

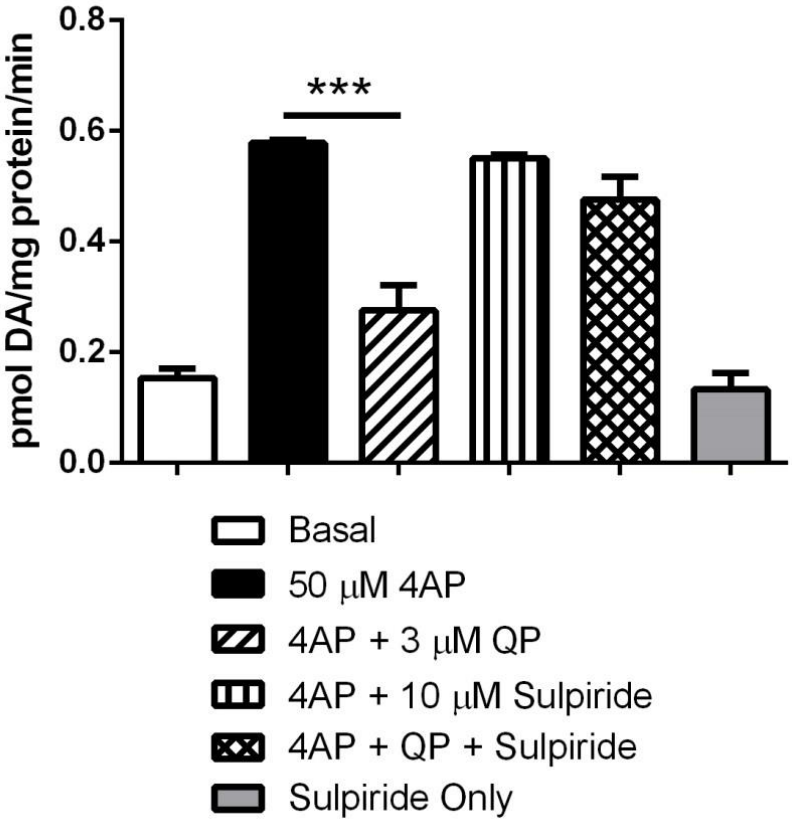

Figure 2-1: Stimulation of the D_2 autoreceptor inhibits dopamine exocytosis. Striatal synaptosomes from wild type mice were perfused with KRB and one minute fractions were collected for 14 minutes. Dopamine release was stimulated with 50 μM 4AP at fractions seven and eight + 3 μM quinpirole (QP) + 10 μM sulpiride. The amount of dopamine in each fraction collected was determined using HPLC-EC and normalized to protein concentration. N = 3, *** $p < 0.0001$ vs. 4AP control via one-way ANOVA with Tukey's post-hoc anaylsis.

exocytosis is stimulated using the potassium channel blocker 4-aminopyridine (4AP, 50 μM) to depolarize the synaptosomes (L'Hirondel et al., 1998). This stimulation increases dopamine release 2-3 fold over basal (Figure 2-1). D_2 autoreceptor control of dopamine exocytosis is determined by adding the D_2R agonist quinpirole simultaneously with 4AP. Agonist activation of D_2R reduces 4-AP-stimulated exocytotic dopamine release. In Figure 2-1, treatment with 3 μM quinpirole, a maximally effective concentration, inhibits 4AP-stimulated dopamine release. A one-way ANOVA found a significant effect of quinpirole treatment (N = 3, $F(5, 12) = 46.28$, $p < 0.0001$). To demonstrate D_2R specificity for the quinpirole suppression of dopamine release, we included the D_2R antagonist sulpiride. Sulpiride had no effect on either basal release or 4AP-stimulated dopamine release. The sulpiride treatment blocked the quinpirole suppression of dopamine release, demonstrating D_2R specificity of quinpirole suppression.

To determine if PKCβ influences the D_2 autoreceptor activity, we measured the 4AP-stimulated dopamine exocytosis in to the presence and absence of quinpirole in striatal synaptosomes prepared from $PKC\beta^{+/+}$ and $PKC\beta^{-/-}$ mice (Figure 2-2). Addition of 100 nM quinpirole decreased dopamine release in $PKC\beta^{+/+}$ mice, as expected. 4AP-stimulated dopamine release was not stastically different in $PKC\beta^{-/-}$ mice as compared to $PKC\beta^{+/+}$ controls (N = 4). There was, however, an enhanced suppression of dopamine release in response to quinpirole. A three-way ANOVA with repeated measures yielded a significant main effect of genotype, $F(1,12) = 8.998$, $p < 0.05$, and drug, $F(1,12) = 7.23$, $p < 0.05$, and a significant interaction between time and genotype, $F(12,144) = 2.44$, $p < 0.05$.

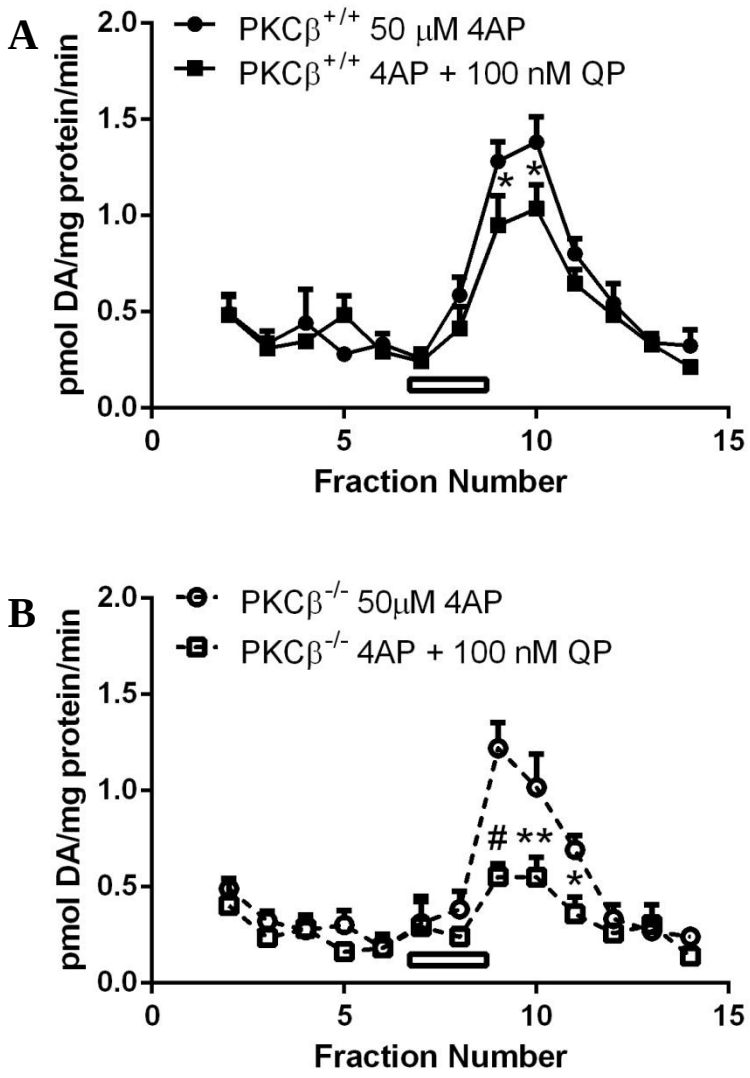

Figure 2-2: Quinpirole (QP)-induced suppression of 4AP-stimulated dopamine release is enhanced in PKCβ$^{-/-}$ mice. Striatal synaptosomes from PKCβ$^{+/+}$ (A) and PKCβ$^{-/-}$ mice (B) were perfused with KRB and one minute fractions were collected for 14 minutes. Dopamine release was stimulated with 50 μM 4AP at fractions seven and eight ± 100 nM QP. The amount of dopamine in each fraction collected was determined using HPLC-EC and normalized to protein concentration. N = 4, * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$ vs. 4AP control via three-way ANOVA with Bonferonni post-hoc analysis.

The results shown in Figure 2-2 were generated using mice constitutively lacking PKCβ. To ensure any differences observed were not due to compensatory changes in the genetic PKCβ knockout, we inhibited PKCβ activity in wild type mice using specific inhibitors. We repeated the dopamine exocytosis experiment using the PKCβ-specific inhibitor LY379196 (IC_{50} = 30 nM, Jirousek et. al., 1996). Striatal synaptosomes from wild type mice were pretreated with vehicle or 200 nM LY379196 for 60 minutes prior to addition of 50 μM 4AP and 30 nM quinpirole. The lower concentration of quinpirole was used to better detect potential increases in sensitivity due to PKCβ inhibition. 4AP-stimulated dopamine release following quinpirole treatment in the presence and absence of LY379196 is shown in Figure 2-3. In the vehicle-treated control samples, 30 nM quinpirole did not significantly decrease stimulated dopamine release. Acute inhibition of PKCβ by LY379196 increased the D_2 autoreceptor reactivity to quinpirole, causing a significant suppression of the 4AP-stimulated dopamine release (two-way ANOVA, interaction $F(1, 8) = 1.683$, $p = 0.2307$; LY379196 treatment $F(1, 8) = 0.0048$, $p = 0.9464$; quinpirole treatment $F(1, 8) = 6.861$, $p = 0.0307$, N = 5). The increased sensitivity to quinpirole following acute PKCβ inhibition mimics the increased quinpirole reactivity measured in the PKCβ$^{-/-}$ mice. Acute PKCβ inhibition had no effect on 4AP-stimulated dopamine release in the absence of quinpirole, again replicating the results obtained with the PKCβ$^{-/-}$ mice.

Lack of PKCβ activity increases D_2 autoreceptor control of dopamine release in the nucleus accumbens.

The suprafusion experiments described above use a depolarizing stimulus to trigger dopamine release. We then used fast-scan cyclic voltammetry to determine if PKCβ inhibition would increase the D_2 autoreceptor control of dopamine release using electrical stimulation.

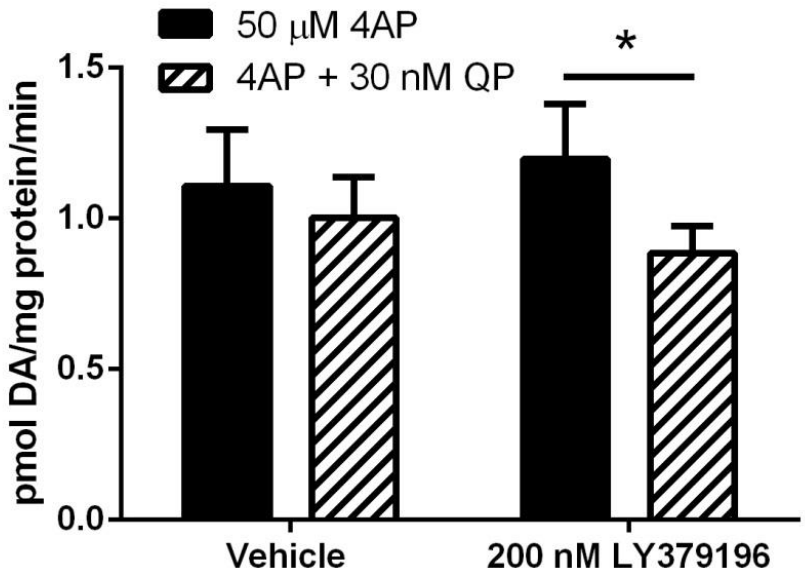

Figure 2-3: Acute PKCβ inhibition increases dopamine release suppression in response to quinpirole (QP). Striatal synaptosomes from wild type mice were perfused with vehicle control or 200 nM LY379196 for 60 minutes and one minute fractions were collected for 14 minutes. Dopamine release was stimulated using 50 μM 4AP ± 30 nM QP at fractions seven and eight. A lower concentration of QP was used here to better detect potential increases in sensitivity due to PKCβ inhibition. Dopamine content was determined via HPLC-EC and was normalized to protein concentration and is shown here as peak stimulated dopamine release. N = 5, * $p < 0.05$ via two-way ANOVA with Sidak post-hoc analysis.

Dopamine release was evoked from mouse nucleus accumbens core in striatal slices with single 300 μA stimulations. The slices were pretreated for 60 minutes with either vehicle or the PKCβ inhibitor enzastaurin (200 nM, IC_{50} = 6 nM, Graff, 2005) and a concentration-response curve was generated for dopamine release suppression in response to quinpirole. Enzastaurin treatment had no effect on baseline stimulated dopamine release (Figure 2-4A; vehicle treatment: 1067.24 ± 80.23 nM, N = 10; enzastaurin treatment: 1058.55 ± 88.11 nM, N = 8). Similar to the suprafusion results described above, inhibition of PKCβ using enzastaurin increased the effectiveness of the D_2R agonist quinpirole, leading to increased suppression of dopamine release (Figure 2-4B). A two-way ANOVA revealed a significant effect of pretreatment group as well as quinpirole, but no significant interaction (Interaction F (5, 82) = 1.00 , p = 0.4231; Quinpirole F (5, 82) = 109.4, $p < 0.0001$; treatment group F (1, 82) = 16.00, p = 0.0001; N = 8-10). PKCβ inhibition also had no effect on the reuptake of dopamine via DAT (Vmax, Vehicle treatment: 2335 ± 308.3 nM/sec, N = 11; enzastaurin treatment: 2291 ± 260.9 nM/sec, N = 8).

PKCβ inhibition increases surface localization of the D_2 autoreceptor

We then investigated the mechanism by which PKCβ inhibition could increase the activity of the D_2 autoreceptor. Previous reports found that PKC phosphorylation elicits internalization and desensitization of D_2R in heterologous cells (Namkung and Sibley, 2004; Morris et al., 2007). We therefore hypothesized that inhibition of PKCβ would increase D_2R surface localization. For this experiment, striatal synaptosomes from wild type mice were treated with vehicle or 200 nM enzastaurin for 5 minutes prior to using the hydrophilic antagonist [^{3}H]-sulpiride to bind the D_2 autoreceptor located on the surface of the synaptosomes. To ensure PKCβ inhibition was not altering overall D_2R expression or binding, receptor binding was repeated using lysed

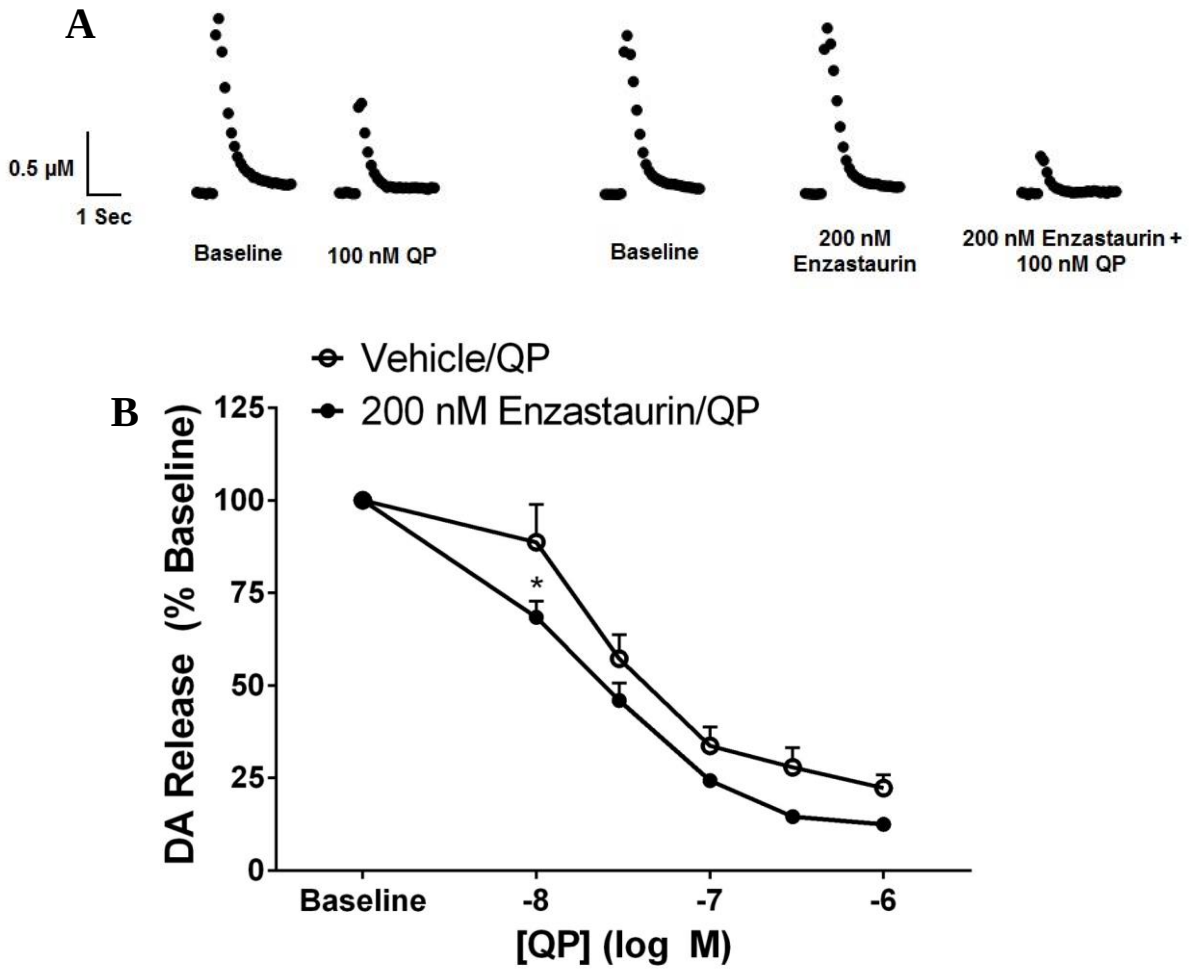

Figure 2-4: Acute PKCβ inhibition increases D_2R agonist quinpirole (QP) effectiveness. (A) Representative traces of electrically stimulated dopamine release in nucleus accumbens core in brain slices was inhibited by QP in a dose-responsive manner. Pretreatment (60 min) of slices with 200 nM enzastaurin significantly decreased the QP dose-response curve (B), normalized to % pre-QP baseline values to the left, indicating supersensitivity of the D_2 autoreceptor (two-way ANOVA F (1, 82) = 16.00, p = 0.0001). N = 8-10, * $p < 0.05$ via two-way ANOVA in post-hoc Bonferroni.

membranes prepared from striatal synaptosomes. PKCβ inhibition significantly increased D_2 autoreceptor surface binding in intact synaptosomes but did not affect D_2R binding in lysed membranes (Figure 2-5, N = 3 with 4-5 replicates per N, paired t-test, $p < 0.01$; specific [^{3}H]-sulpiride binding in vehicle treated samples: Intact synaptosomes: 79.08 $\pm$ 0.01 fmol/mg protein, Lysed membranes: 82.6 $\pm$ 0.01 fmol/mg protein). Thus the increase in D_2 autoreceptor surface localization following inhibition of PKCβ may underlie the increased D_2 autoreceptor sensitivity to quinpirole.

PKCβ$^{-/-}$ mice have increased quinpirole-induced locomotor suppression.

To determine the physiological relevance of PKCβ inhibition on D_2 autoreceptor activity, we measured locomotor activity suppression in response to the D2R agonist quinpirole in both PKCβ$^{+/+}$ and PKCβ$^{-/-}$ mice. Studies using mice with a deletion of the D_2 autoreceptor (*Drd2$^{loxP/loxP}$*; *Dat$^{+/IRES\text{-}cre}$*) in midbrain dopamine neurons have concluded that this locomotor suppression is primarily mediated by the D_2 autoreceptor (Bello et al., 2011). Here, the locomotor activity following an injection of either saline or an increasing dose of quinpirole in PKCβ$^{+/+}$ and PKCβ$^{-/-}$ mice was measured and the results are shown plotted as the total locomotor activity in fifteen minutes, normalized to the saline control (Figure 2-6). Quinpirole dose-dependently suppressed locomotor activity in both genotypes (N = 6-15). The PKCβ$^{-/-}$ mice showed a significantly greater locomotor suppression to quinpirole than PKCβ$^{+/+}$ controls. A two-way ANOVA indicated a significant main effect of quinpirole dose, $F(4,77) = 81.73$, $p < 0.0001$, and genotype, $F(1, 77) = 12.02$, $p < 0.001$, as well as a significant interaction, $F(4, 77) = 2.73$, $p < 0.05$. The results of this experiment demonstrate that the PKCβ regulation of the D_2 autoreceptor measured in our suprafusion assays is physiologically relevant.

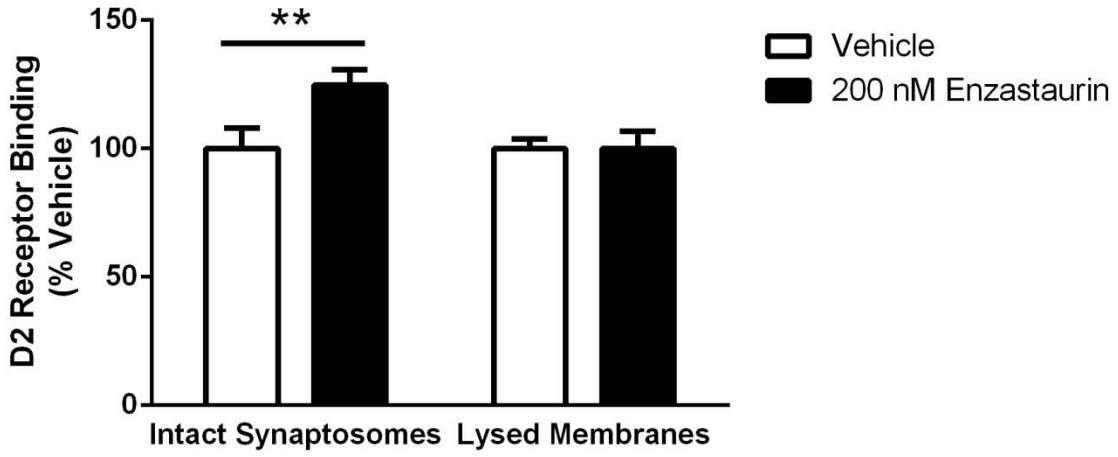

Figure 2-5: PKCβ inhibition increases D_2 autoreceptor surface localization. Striatal synaptosomes from wild type mice were prepared. Half of the synaptosomes were lysed and membranes were isolated. The tissue preparations were incubated with vehicle or 200 nM enzastaurin for 5 minutes at 37° C. Following treatment, intact synaptosomes were incubated with [^{3}H]-sulpride on ice for 3.5 hours to measure surface D_2 autoreceptor binding. Lysed membranes were incubated with [^{3}H]-sulpride for 1.5 hours at room temperature to measure total D_2 autoreceptor binding. Binding was terminated by filtering samples and radioligand binding was determined via scintillation counting. N = 3, with 3-5 replicates per N, ** $p < 0.01$ via paired t-test.

Discussion

In the present study, we determined that PKCβ regulates presynaptic D_2 autoreceptor surface location and activity. Previous reports demonstrated that PKC activation can regulate D_2R (Cubeddu et al., 1989; Namkung and Sibley, 2004; Morris, 2007). Our results identify the PKCβ isoform specifically as a PKC-mediated regulator of the D_2 autoreceptor. Using both PKCβ$^{-/-}$ mice and specific PKCβ inhibitors, we found that reduced PKCβ activity increased D_2 autoreceptor function, likely by increasing receptor surface localization. We are the first to demonstrate the physiological relevance of the PKC regulation by demonstrating greater quinpirole suppression of locomotor behavior in PKCβ$^{-/-}$ as compared to PKCβ$^{+/+}$ mice. Our model for this finding is that under normal conditions, PKCβ activity enhances phosphorylation of the D_2 autoreceptor, causing internalization and desensitization of the receptor, leading to a blunting of D_2 autoreceptor-mediated control of extracellular dopamine. However, when PKCβ is inhibited, more D_2 autoreceptor is left on the neuronal surface, leading to increased signaling and a greater D_2 autoreceptor-mediated inhibition of dopamine exocytosis. This would result in lower extracellular dopamine and less dopaminergic signaling, causing increased suppression of locomotor activity in response to the D_2R agonist quinpirole, as we observed in PKCβ$^{-/-}$ mice.

PKC appears to increase the release of neurotransmitters through many different mechanisms. By either inhibiting potassium channels (Colby and Blaustein, 1988) or preventing G protein blockade of calcium channels (Barrett and Rittenhouse, 2000), PKC can cause cell depolarization and an increase in neurotransmitter release. PKC can also interact with vesicular release machinery. Munc18, a PKC substrate, dimerizes with syntaxin, a SNARE complex member. Phosphorylation by PKC breaks the dimer between Munc18 and syntaxin, resulting in increased

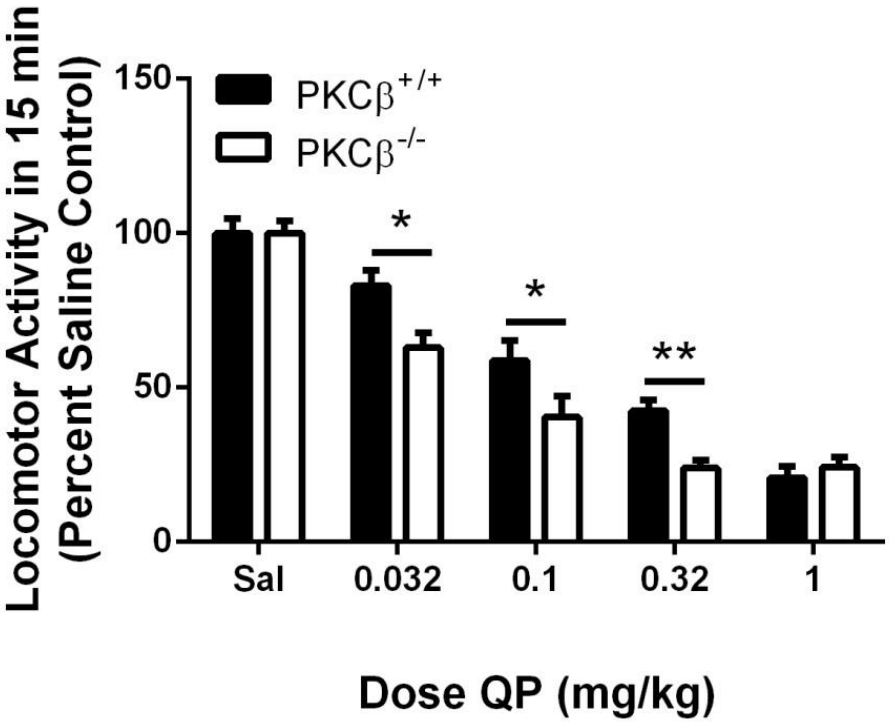

Figure 2-6: Quinpirole (QP) suppression of locomotor activity is increased in PKCβ$^{-/-}$ mice. PKCβ$^{+/+}$ and PKCβ$^{-/-}$ mice were implanted with Mini-mitter tracking devices and placed in a novel environment. Mice were injected IP with saline or increasing doses of QP (0.032 – 1 mg/kg). Locomotor activity was measured for 15 minutes following injection and total activity normalized to saline control is shown. N = 5-15, * $p < 0.05$, ** $p < 0.01$ via two-way ANOVA with Bonferonni post-hoc analysis.

vesicle fusion and neurotransmitter release (de Vries et al., 2000). PKC can also interact with autoreceptors to alter neurotransmitter release, which is most pertinent to this study. Activation of PKC by phorbol esters increased 4AP-stimulated [^{3}H]-norepinephrine release from rabbit hippocampus slices (Huang et al., 1989). In that study, PKC activation increased 4AP-stimulated norepinephrine release and blocked the α_2-adrenergic agonist-mediated inhibition of norepinephrine release. PKC activation also increased electrical stimulation of [^{3}H]-dopamine from rabbit striatal and prefrontal cortex slices (Cubeddu et al., 1989, but see (Iannazzo et al., 1997)). PKC activation antagonized the activity of several D_2R agonists, including quinpirole, leading the authors to hypothesize that PKC activation could decrease the surface localization of D_2 autoreceptors. Both of these reports agree with our findings. These authors activated PKC and saw decreased autoreceptor control of neurotransmitter exocytosis. We found that inhibition of PKCβ increased D_2 autoreceptor control of dopamine exocytosis.

Extracellular dopamine levels, and thus dopaminergic signaling, are tightly controlled by both DAT and the D_2 autoreceptor. PKC has been found by our lab and others to regulate both of these proteins through phosphorylation. PKC phosphorylates the D_2R receptor to change surface localization and receptor activity. Several PKC phosphorylation sites have been identified on the receptor using mutagenesis studies, particularly on the third intracellular loop. Phosphorylation at these sites causes internalization and desensitization of the receptor (Namkung & Sibley, 2004; Morris et al., 2007; Thibault et al., 2011). Overexpression of PKCβ in HEK cells suggests that this isoform specifically phosphorylates D_2R (Namkung & Sibley, 2004). In addition to inhibiting dopamine exocytosis, the D_2 autoreceptor can increase the surface localization and uptake of dopamine through DAT to control extracellular dopamine levels (Bolan et al., 2007). This coordination between DAT and the D_2 autoreceptor allows for more precise control of

dopaminergic signaling and may occur through a separate population of autoreceptors (Wu et al., 2002). Using PKCβ $^{-/-}$ mice and PKCβ-specific inhibitors we found that PKCβ signaling is required for this D_2 autoreceptor-DAT coordination (Chen et al., 2013). Additionally, the D_2 autoreceptor and DAT are reported to physically interact between the third intracellular loop of D_2R and the N-terminus of DAT (Lee et al., 2007). Both the D_2R third intracellular loop (Morris et al., 2007; Namkung and Sibley, 2004) and the DAT N-terminus (Foster et al., 2002) contain PKC phosphorylation sites, suggesting that PKC phosphorylation may be involved in this D_2 autoreceptor-DAT interaction and thus the regulation of extracellular dopamine. Studies are currently underway to further understand how PKC regulates this D_2 autoreceptor-DAT interaction.

In the present study, we used the potassium channel blocker 4-aminopyridine (4AP) to stimulate dopamine exocytosis in our suprafusion assay. Some studies have reported that 4AP or other potassium channel blockers interfere with autoreceptor regulation of neurotransmitter release (norepinephrine: Hu & Fredholm, 1989; acetylcholine: Drukarch et al., 1989; dopamine: Cass & Zahniser, 1991 and Fulton et al., 2011). Unlike our assay, these studies did not use 4AP stimulation alone, but combined potassium channel blockade with electrical stimulation. This combined stimulation appears to cross an upper threshold of neurotransmitter release above which autoreceptors are no longer able to regulate the release. This threshold is crossed following prolonged depolarization, such as that seen with high concentrations of KCl which is not subject to autoreceptor regulation (Tibbs et al., 1989; L'Hirondel et al., 1998). Additionally, the similarity of our findings using 4AP as a stimulus to those with electrical stimulation in the fast-scan cyclic voltammetry experiments suggest that our findings are physiologically relevant.

Our findings demonstrate that PKCβ regulates the D_2 autoreceptor *in* vivo. Further work is needed to understand the conditions under which PKCβ is activated, leading to the modulation of the D_2 autoreceptor. It does not appear that agonist-induced phosphorylation of D_2R occurs though PKC (Namkung & Sibley, 2004). PKC is canonically activated by diacylglycerol and IP_3 generated by phospholipase through the G_q or G_i-protein signaling cascades. Activation of G_q-coupled GPCRs such as the neurotensin receptor cause internalization and desensitization of D_2R through a PKC-dependent mechanism (Thibault et al., 2011). However, the specific mechanisms for the *in vivo* activation of PKCβ leading to the phosphorylation and subsequent regulation of the D_2 autoreceptor remain unknown.

This study focused on the interaction of PKCβ with the presynaptic D_2 autoreceptors; however, the majority of D_2Rs are located postsynaptically. In mice lacking the D_2 autoreceptor, quinpirole treatment no longer suppresses locomotor activity, indicating that this locomotor response is mediated by the presynaptic D_2R population (Bello et al., 2011). Our results showing that PKCβ$^{-/-}$ mice have increased suppression of locomotor activity following quinpirole treatment strongly suggest that we are measuring regulation of the D_2 autoreceptor population by PKCβ. Otherwise, we are unable to definitively differentiate experimentally between postsynaptic and presynaptic receptor populations. The D_2R is also expressed as two splice variants, short and long, with the long D_{2L} having an additional 29 amino acids in the third intracellular loop (Usiello et al., 2000). Conflicting reports indicate that PKC may or may not differentially regulate the long variant of D_2R compared to the short (Namkung & Sibley, 2004; Morris et al., 2007). Future work is needed to understand if PKCβ regulation is the same for both populations of D_2R and if this regulation is the same for other D_2-like family members, such as the closely related D_3R receptor.

In conclusion, we have found that PKCβ regulates the D_2 autoreceptor *in vivo*, adding an additional layer of regulation to the control of dopamine signaling. We demonstrated that loss of PKCβ activity increases the sensitivity of the D_2 autoreceptor to the agonist quinpirole, as measured by the increased suppression of dopamine release. Mechanistically, PKCβ inhibition increased the surface localization of the D_2 autoreceptor, increasing receptor signaling. Finally, we demonstrated that this PKCβ regulation of the D_2 autoreceptor is physiologically relevant, as loss of PKCβ activity increased the sensitivity of mice to quinpirole suppression of locomotor activity. These findings increase our understanding of how the D_2 autoreceptor is regulated and may aid in the development of therapeutics targeting disorders and disease states associated with dopamine signaling.

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CHAPTER THREE

THE D2-LIKE DOPAMINE AUTORECEPTOR IS REGULATED BY A NOVEL, DOPAMINE TRANSPORTER-MEDIATED CONTEXT

Abstract

The function of GPCRs such as the D_2-like dopamine autoreceptor (D_2 autoreceptor) is regulated by the milieu at the plasmalemmal membrane. This regulation can occur through local ion concentrations, phosphorylation, or interaction with other proteins. In this study, we investigate the regulation of the D2 receptor (D_2R) by the dopamine transporter (DAT). Together, D_2 autoreceptor and DAT regulate the amount of extracellular dopamine and dopaminergic signaling. It is well established that activation of the D_2 autoreceptor increases dopamine uptake by increasing surface DAT localization. Using a heterologous cell system, we found that co-expression of DAT with the short isoform of D_2R (D_{2S}) induces a different, DAT-dependent regulatory context for D_{2S}. When co-expressed with DAT, the proportion of baseline D_{2S} on the surface was decreased as compared to empty vector control, but the surface levels were increased following either agonist treatment or inhibition of protein kinase Cβ (PKCβ). PKCβ appears to stabilize the D_{2S}-DAT context. Removal of PKC phosphorylation sites from D_{2S} or DAT disrupted the context, allowing D_{2S} to be regulated more similarly to other GPCRs. Within the D_{2S}-DAT context there is decreased G protein activation by D_{2S} and increased D_{2S}-mediated increases the ERK signaling. We propose that the D_{2S}-DAT context may ultimately lead to increased surface DAT localization, increased dopamine reuptake, and consequently less dopaminergic signaling.

Introduction

The function of the D_2-like dopamine receptor (D_2R) is regulated by the milieu at the plasmalemmal membrane. The activity and cellular location of this G protein-coupled receptor are sensitive to ions, ligands, receptor modifications such as phosphorylation and protein binding partners. For instance, increasing intracellular Na^+ or H^+ concentrations decreases the D_2R affinity for agonists, but increases the affinity of the substituted benzamide class of D_2R antagonists (Neve, 1991; Watanabe et al., 1985). Diverse agonists preferentially promote D_2R coupling to distinct members of the inhibitory G protein family (Cordeaux et al., 2001; Gazi et al., 2003). Phosphorylation is an important mechanism of regulation of trafficking and signaling through GPCRs, including D_2R. Protein Kinase C (PKC) phosphorylates D_2R to cause heterologous desensitization and internalization (Namkung and Sibley, 2004). G protein-coupled receptor kinases (GRK) phosphorylate receptors including D_2R to stimulate internalization and desensitization. Additionally, GRKs are able to regulate the D_2R even in the absence of GRK phosphorylation sites (Namkung et al., 2009a; Namkung et al., 2009b). By interacting with D_2R in the absence of phosphorylation, GRK regulates surface localization and recycling of the receptor.

There is a growing appreciation for the variety of proteins with which both pre- and postsynaptic D_2-like receptors can interact, resulting in a modulation of activity [see (Hazelwood et al., 2010)]. In the brain, D2Rs on dopamine neurons function as autoreceptors and serve the specialized function of regulating the release of dopamine and thus its extracellular concentration. A striking example of an interaction of the D_2 autoreceptor with a complementary protein that may or may not be closely within its milieu is the interaction of the D_2 autoreceptor with the dopamine transporter (DAT). Both presynaptic proteins function to reduce levels of

extracellular dopamine: the D_2 autoreceptor by inhibiting the release of vesicular dopamine and DAT by transporting dopamine into the cell. Both proteins are regulated by PKCβ; PKCβ promotes substrate-induced reverse transport of DAT and inhibits D_2 autoreceptor-mediated suppression of vesicular dopamine release (Chapter 2). Stimulation of the D_2 autoreceptor by agonists increases the surface localization of DAT and consequently reuptake of dopamine, ultimately leading to a decrease in extracellular dopamine content (Bolan et al., 2007; Lee et al., 2007; Meiergerd et al., 1993). The D_2 autoreceptor-mediated enhancement of DAT activity is mediated by extracellular signal-regulated kinase ERK and PKCβ signaling (Bolan et al., 2007; Chen et al., 2013) and may be facilitated by a direct interaction between D_2R and DAT (Lee et al., 2007). While this D_2 autoreceptor-mediated regulation of DAT has been extensively investigated, there have been few investigations as to whether or not DAT regulates the D_2 autoreceptor.

In this study, we investigated the possibility of a reciprocal regulation of the D_2 autoreceptor by DAT. Because the D_2 autoreceptor exists both pre- and post-synaptically, we used a heterologous cell system transfected with the D_2R in the absence and presence of DAT. We analyzed cell surface localization of both proteins as well as changes in downstream G-protein-mediated signaling and the role of phosphorylation in the regulation. Our results demonstrate that when DAT and D_2R are present in the same cell, D_2R is in a new, DAT-specific context that changes the regulation of D_2R by agonists and PKCβ.

Materials and Methods

Cell Culture and Transfection N2A neuroblastoma cells were cultured in Opti-MEM I media (Life Technologies) supplemented with FBS and penicillin/streptomycin. For confocal microscope experiments, cells were seeded on poly-D-lysine (Sigma Aldrich) coated glass

coverslips at a density of 300,000 cells/mL. For signaling experiments, cells were seeded onto uncoated 10 cm dishes. The following day, cells were transfected using Lipofectamine 2000 (Life Technologies). Cells were transfected with the short isoform of human D_2R (D_{2S}) with an N-terminal FLAG (DYKDDDDK) tag (FLAG-D_{2S}, a gift from Dr. David Sibley) with either hemagglutinin-tagged human DAT (HA-DAT, tag in second extracellular loop, a gift from Dr. Jonathan Javitch) or empty vector control. T225A/S228G/S229G-D_{2S} (FLAG-AGG-$D2_S$) was generated by mutagenesis using FLAG-D_{2S} cDNA as a template. Mutant DNA was generated by PCR using Pfu ultra polymerase (Agilent Technologies, Santa Clara, CA) and sense and antisense primers containing the desired mutations, followed by digestion of parental DNA by DpnI enzyme and transformation into XL10-Gold competent cells (Strategene, La Jolla, CA). Mutations were analyzed by DNA sequencing. HA-ΔN22-DAT truncation was made by deleting the first 22 amino acids of HA-DAT by PCR using Pfu ultra polymerase and confirmed by sequencing. Experiments were performed 48 hours post-transfection.

D_{2S}, which lacks 29 amino acids in the third intracellular loop due to alternative splicing, was used for all experiments. We used D_{2S} because it is known to have a presynaptic location, although presynaptic D_2 autoreceptors need not be exclusively D_{2S} (Khan et al., 1998; Jang et al., 2011). The abbreviation D_{2S} will be used throughout this manuscript when discussing the short variant of the D_2R specifically, such as when discussing our experimental results. D_2R or D_2 autoreceptor will be used to when discussing the receptor when not discussing the specific variants.

Immunofluorescence Labeling. Changes in surface localization of FLAG-D_{2S} and HA-DAT were determined using immunofluorescence labeling of both surface and intracellular populations. 48 hours post-transfection, cells were incubated with vehicle, the D_2R agonist quinpirole, or a

specific PKCβ inhibitor LY379196 for either 5 or 30 minutes. Treatment was stopped by washing cells with ice cold phosphate buffered saline with calcium and magnesium (155 mM NaCl, 2.97 mM $Na_2HPO_4 \cdot 7H_2O$, 1.1 mM KH_2PO_4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) (PBS/Ca/Mg). All immunofluorescence labeling was done on ice. Non-specific binding was blocked with 4% normal goat serum prepared in PBS/Ca/Mg (Vector Laboratories, Burlingame, CA). Surface populations of FLAG-D_{2S} were labeled by incubating with a primary mouse anti-FLAG antibody (Sigma Aldrich) for 1 hour, followed by secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (Life Technologies) for 45 minutes. Surface populations of HA-DAT were labeled by incubating with a primary mouse anti-HA antibody (Covance, Princeton, NJ) for 1 hour, followed by secondary goat anti-mouse antibody conjugated to Alexa Fluor 594 (Life Technologies) for 45 minutes. Antibody solutions were prepared in PBS/Ca/Mg with 4% normal goat serum. Either surface FLAG-D_{2S} or HA-DAT were labeled on a given cell to decrease steric hindrance from antibody labeling. Following surface labeling, cells were fixed and permeabilized for 10 minutes each with 4% paraformaldehyde and 0.1% Triton X-100. Cells were then incubated with rabbit anti-FLAG primary antibody (Sigma Aldrich) for 1 hour followed by goat anti-rabbit conjugated to Alexa Fluor 405 secondary antibody (Life Technologies) for 45 minutes to label intracellular populations of FLAG-D_{2S}. Following labeling for intracellular FLAG-D_{2S}, intracellular HA-DAT was then labeled in cells expressing that protein using rabbit anti-HA antibody (Covance) and goat anti-rabbit conjugated to Alexa Fluor 647 (Life Technologies). Once all labeling was completed, coverslips were mounted to glass slides using ProLong Gold anti-fade reagent (Life Technologies).

Confocal Microscopy and Quantification. Fluorescent signals from the labeled cells were imaged using a Nikon A1R confocal microscope (Nikon Instruments, Inc., Melville, NY) with a

60x1.4 numerical aperture oil objective. Cells were imaged by taking a z-series with 0.5 μm sections. The laser configuration was as follows: Alexa Fluor 405 was excited by a 405 nm laser and passed through a 450/50 nm filter; Alexa Fluor 488 was excited by a 488 nm laser and passed through a 525/50 nm filter; Alexa Fluor 594 was excited by a 561 nm laser and passed through a 595/50 nm filter; and Alexa Fluor 647 was excited by a 638 nm laser and passed through a 700/75 nm filter. Sequential scan was used to minimize bleed through of signals. Image quantification was performed using Image J software (NIH, Bethesda, MD). Surface and intracellular signal intensities were determined and background was subtracted individually for both FLAG-D_{2S} and HA-DAT. The fraction of FLAG-D_{2S} or HA-DAT on the surface of the cell was determined by dividing the surface label intensity by the sum of the surface and intracellular label intensities.

ERK Activity. Cells were harvested 48 hours post transfection and treated in suspension with vehicle or the D_2R agonist quinpirole for 5 minutes at 37°C in Krebs Ringer's HEPES buffer (KRH, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2 \cdot 2\ H_20$, 1.2 mM $MgSO_4 \cdot 7\ H_20$, 5.6 mM glucose, 25 mM HEPES, pH 7.4). Treatment was terminated by washing with ice cold KRH. Cells were lysed with solubilization buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, pH 7.4) containing Complete Mini protease inhibitor and PhosStop phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN) for one hour and protein concentration for each sample was determined. Total and phosphoERK were quantified via Western blotting using antibodies against phosphoERK (Cell Signaling Technologies, Danvers, MA) and total ERK (Santa Cruz Biotechnology, Dallas, TX). Band density was measured using Image J software and ERK activity was determined by dividing the optical density for phosphoERK by the optical density for total ERK.

Cyclic AMP Assay. Cells were incubated with KRH containing 30 μM forskolin (adenylyl cyclase activator, Sigma Aldrich), 1 mM IBMX (phosphodiesterase inhibitor, Sigma Aldrich), and a concentration-response curve of quinpirole for 15 minutes at 37°C. Treatment was terminated by replacing treatment solution with ice cold 3% perchloric acid (Sigma Aldrich) and incubating samples for 30 minutes at 4°C. Samples were neutralized with 2.5 M $KHCO_3$. cAMP accumulation was determined using a cyclic AMP EIA kit obtained from Cayman Chemical (Ann Arbor, MI). Results are expressed as percent of forskolin-stimulated control in the absence of quinpirole treatment.

Agonist-Stimulated [^{35}S]GTPγS-Binding Assays. To measure agonist-mediated activation of G proteins, binding of the slowly hydrolysable GTP analog guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) was measured. N2A cells were harvested 48 hours post transfection. Membrane homogenates were prepared as previously described and stored at -80C (Clark et al., 2003). Briefly, membrane homogenates (10μg protein) were incubated with 0.1nM [^{35}S]GTPγS in the presence or absence of various concentrations of quinpirole for 60 minutes at 25°C in the following buffer: 50 mM Tris base, pH 7.4, 5mM MgCl2, 100 mM NaCl, 1mM EDTA, and 30 μM GDP. Binding reaction was terminated by rapid filtration onto GF/C filters (Whatman, Kent, UK) using a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD). Filters were washed 6-8 times with ice-cold wash buffer (50mM Tris base, pH 7.4, 5 mM MgCl2, and 100 mM NaCl). Filters were dried, saturated with EcoLume scintillation cocktail (MP Biomedicals, Solon, OH), and bound radioactivity was measured using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Statistical Analysis Results were analyzed using GraphPad Prism 6 software (San Diego, CA) and are plotted as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$. Comparisons

between two groups were done using paired Student's *t* test. Comparisons between multiple groups were performed using one or two-way ANOVA with Dunnett's or Tukey's post-test.

Results

Co-expression with DAT Changes D_{2S} Regulation

To test the hypothesis that DAT can alter the regulation of D_{2S}, we used a homologous cell system. To validate the cell system, we initially established that we could measure the D_{2S}-mediated increase in surface DAT localization, a well-documented phenomenon in both heterologous cells and brain tissue. Cells were transfected with FLAG-D_{2S} and HA-DAT. Following a five minute treatment with 1 μM quinpirole, a D_2R agonist, or 200 nM LY39196, a specific PKCβ inhibitor, surface HA-DAT localization was determined using immunofluorescence and confocal microscopy (Figure 3-1). In agreement with previous studies (Bolan et al., 2007; Chen et al., 2013), stimulation of FLAG-D_{2S} by quinpirole significantly increased surface HA-DAT localization, (one-way ANOVA, $F(2, 228) = 19.61$, $p < 00001$, N = 32-118 cells per treatment group). As we found in mouse striatal synaptosomes, PKCβ inhibition had no effect on surface HA-DAT (Chen et al., 2013).

We next queried if the presence of DAT affects surface FLAG-D_{2S} regulation. Initially, cells expressing FLAG-D_{2S} without HA-DAT were treated for 30 minutes with 1 μM quinpirole and

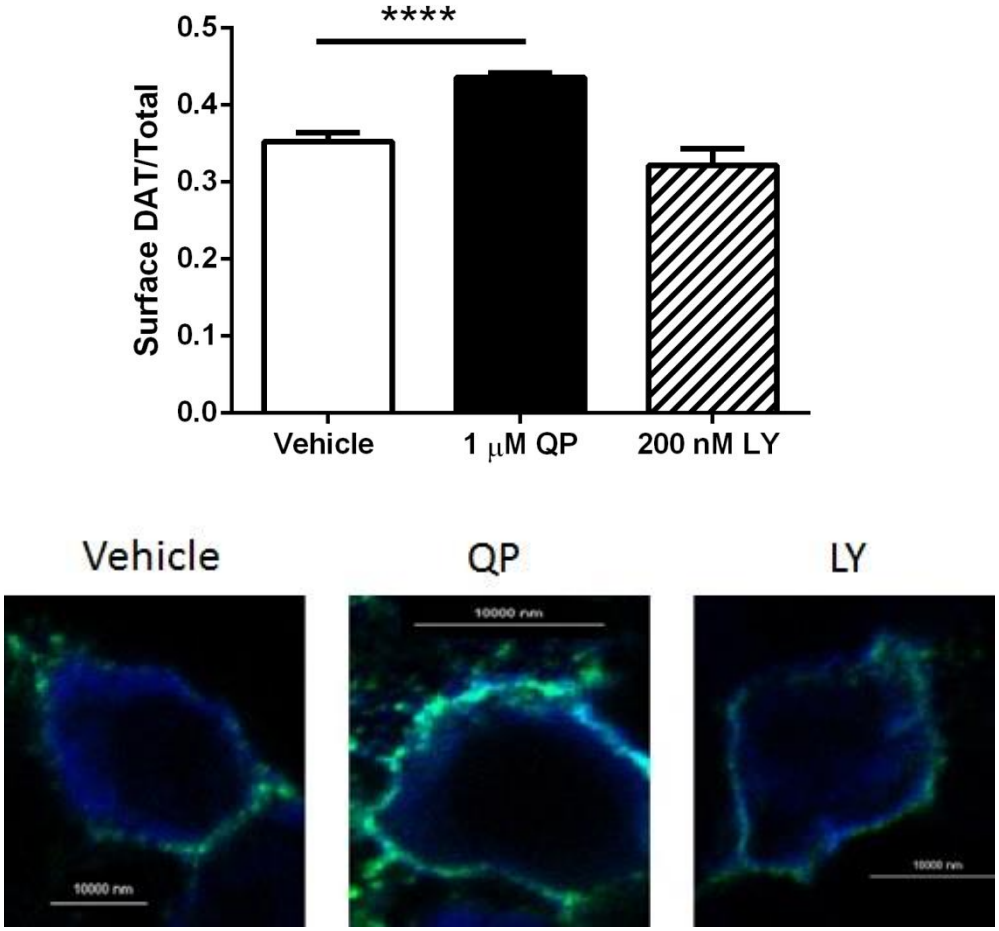

Figure 3-1: FLAG-D_{2S} stimulation increases surface HA-DAT localization. N2A cells transfected with FLAG-D_{2S} and HA-DAT were treated for 5 minutes with vehicle, 1 μM quinpirole (QP), or 200 nM LY379196 (LY). Surface HA-DAT was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface HA-DAT is green, intracellular is blue. N = 32-118 cells. **** $p < 0.0001$ vs. vehicle control by one-way ANOVA with Dunnett's post-hoc analysis.

surface FLAG-D_{2S} was measured (Figure 3-2). As expected for a GPCR, treatment with quinpirole internalized FLAG-D_{2S}. In cells co-expressing FLAG-D_{2S} and HA-DAT, the baseline fraction of surface FLAG-D_{2S} was reduced as compared to cells expressing FLAG-D_{2S} alone, despite equivalent total amounts of FLAG-D_{2S} between the two transfection conditions as determined by total immunofluorescence labeling (FLAG-D_{2S}/Vector vehicle: 9.62 $\pm$ 0.817, N = 43; FLAG-D_{2S}/HA-DAT vehicle: 10.09 $\pm$ 1.008, N = 54). Surprisingly, quinpirole treatment in the FLAG-D_{2S}/HA-DAT-N2A cells increased surface localization of FLAG-D_{2S} (one-way ANOVA $F(3, 196) = 13.02$, $p < 0.0001$). The quinpirole-stimulated increase in surface FLAG-D_{2S} was reminiscent of the D_{2S}-mediated increase in surface HA-DAT, and indicated that DAT can alter the regulation of D_{2S}.

DAT Regulation of D_{2S} Requires PKCβ Activity and DAT N-Terminus

We previously demonstrated that PKCβ is upstream of ERK in the signaling cascade linking D_{2S} stimulation to increased surface DAT (Chen et al., 2013). To further investigate the DAT-mediated regulation of D_{2S}, we now interrogated if PKCβ is also involved in the contextual regulation of D_{2S}. Cells transfected with FLAG-D_{2S} without or with HA-DAT were treated for 5 minutes with 200 nM LY39196, a specific PKCβ inhibitor (IC_{50} = 30 nM, Jirousek et al., 1996) (Figure 3-3). In cells expressing only FLAG-D_{2S}, inhibition of PKCβ had no effect on surface localization of FLAG-D_{2S} (vehicle: 0.510 $\pm$ 0.015, N = 43; 200 nM LY379196: 0.469 $\pm$ 0.0144, N = 44). However, in cells co-expressing FLAG-D_{2S} and HA-DAT, inhibition of PKCβ significantly increased surface localization of FLAG-D_{2S} (one-way ANOVA $F(3, 252) = 26.61$, $p < 0.0001$). PKC, including PKCβ, phosphorylates the D_2R to cause internalization and desensitization of the receptor (Namkung and Sibley, 2004). Our results suggest that when DAT regulates D_{2S}, the receptor is in a state that is receptive to phosphorylation by PKCβ, leading to

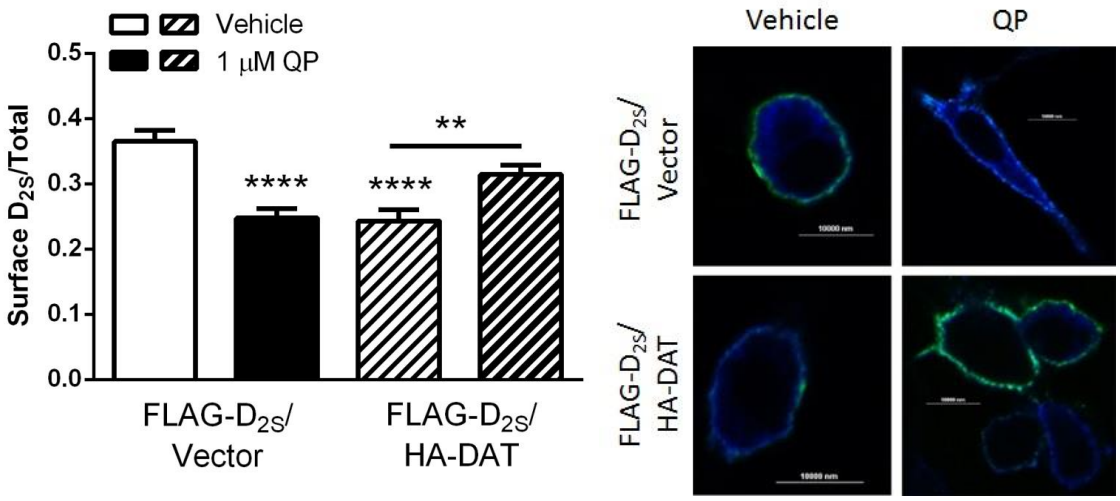

Figure 3-2: Quinpirole (QP) stimulation increases surface FLAG-D_{2S} localization in the presence of HA-DAT. N2A cells transfected with FLAG-D_{2S} ± HA-DAT were treated for 30 minutes with vehicle or 1 μM QP. Surface FLAG-D_{2S} localization was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface FLAG-D_{2S} is green, intracellular is blue. N = 43-56 cells. **** $p < 0.0001$ vs FLAG-D_{2S}/Vector Vehicle, ** $p < 0.01$ by one-way ANOVA with Tukey's post-hoc analysis.

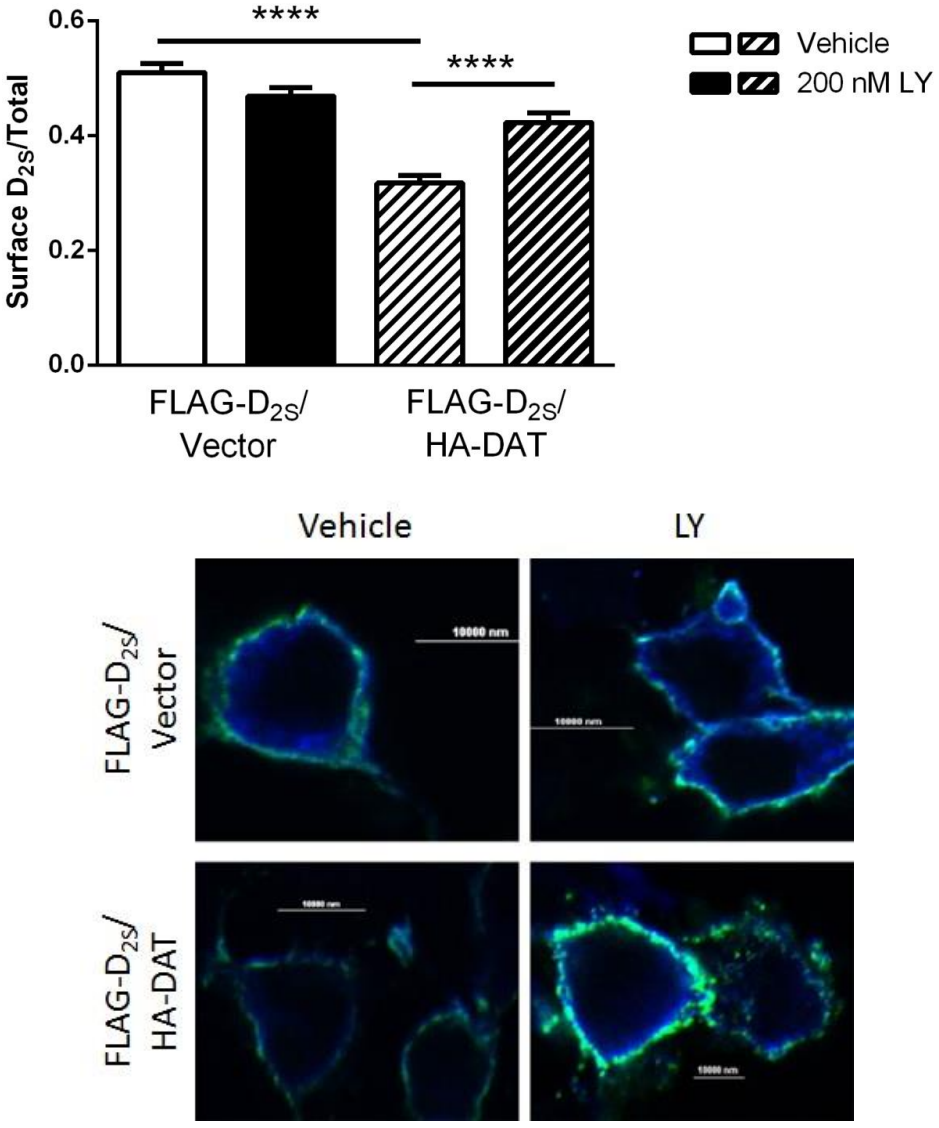

Figure 3-3: LY379196 (LY) treatment increases surface FLAG-D_{2S} localization in the presence of HA-DAT. N2A cells transfected with FLAG-D_{2S} ± HA-DAT were treated for 5 minutes with vehicle or 200 nM LY. Surface FLAG-D_{2S} localization was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface FLAG-D_{2S} is green, intracellular is blue. N = 43-86 cells. **** $p < 0.0001$ by one-way ANOVA with Tukey's post-hoc analysis.

the decreased basal surface localization of FLAG-D_{2S} in our cell system. Following PKCβ inhibition, this phosphorylation-driven internalization of FLAG-D_{2S} is blocked, leading to increased surface localization. To test this notion, we used a mutant FLAG-D_{2S} that abrogated putative PKCβ phosphorylation sites. PKC phosphorylates D_2R on several residues in the third intracellular loop of the receptor. Phosphorylation of three residues in particular, threonine 225 and serines 228 and 229, triggers the internalization of D_2R (Morris et al., 2007; Namkung and Sibley, 2004). These three residues were mutated to non-phosphorylatable alanine and glycine resides (FLAG-T225A/S228G/S229G-D_{2S}, FLAG-AGG-D_{2S}). Cells were transfected with HA-DAT plus either wild type FLAG-D_{2S} or the phosphomutant FLAG-AGG-D_{2S} and were treated with vehicle or LY379196 (50 or 200 nM, 5 minute, Figure 3-4A). In the FLAG-D_{2S}/HA-DAT cells, LY379196 concentration-dependently increased surface localization of FLAG-D_{2S}. A two-way ANOVA yielded a significant effect of LY379196 ($F(2,332) = 6.883$, $p = 0.0012$), FLAG-AGG-D_{2S} ($F(1, 332) = 67.25$, $p < 0.0001$), and a significant interaction between the two ($F(2, 332) = 4.150$, $p = 0.017$). In cells expressing the FLAG-AGG-D_{2S} phosphomutant and HA-DAT, LY379196 had no effect on surface localization of FLAG-AGG-D_{2S}, suggesting that PKCβ phosphorylates the receptor at these residues to cause internalization (vehicle: 0.511 $\pm$ 0.015, N = 44, 50 nM LY379196: 0.544 $\pm$ 0.0159, N = 55, 200 nM LY379196: 0.525 $\pm$ 0.018, N = 56). However, a greater fraction FLAG-AGG-D_{2S} was localized on the cell surface as compared to wild type FLAG-D_{2S}, supporting our model that during the DAT regulation of D_{2S}, the decreased fraction of surface FLAG-D2S is due to increased phosphorylation by PKCβ (FLAG-D_{2S}: 0.380 $\pm$ 0.015, N = 57, FLAG-AGG-D_{2S}: 0.511 $\pm$ 0.015, N = 44).

We next tested the hypothesis that the T225/S228/S229 PKC phosphorylation sites within D_{2S} are required for agonist activation of D_{2S} to increase surface DAT. Cells were

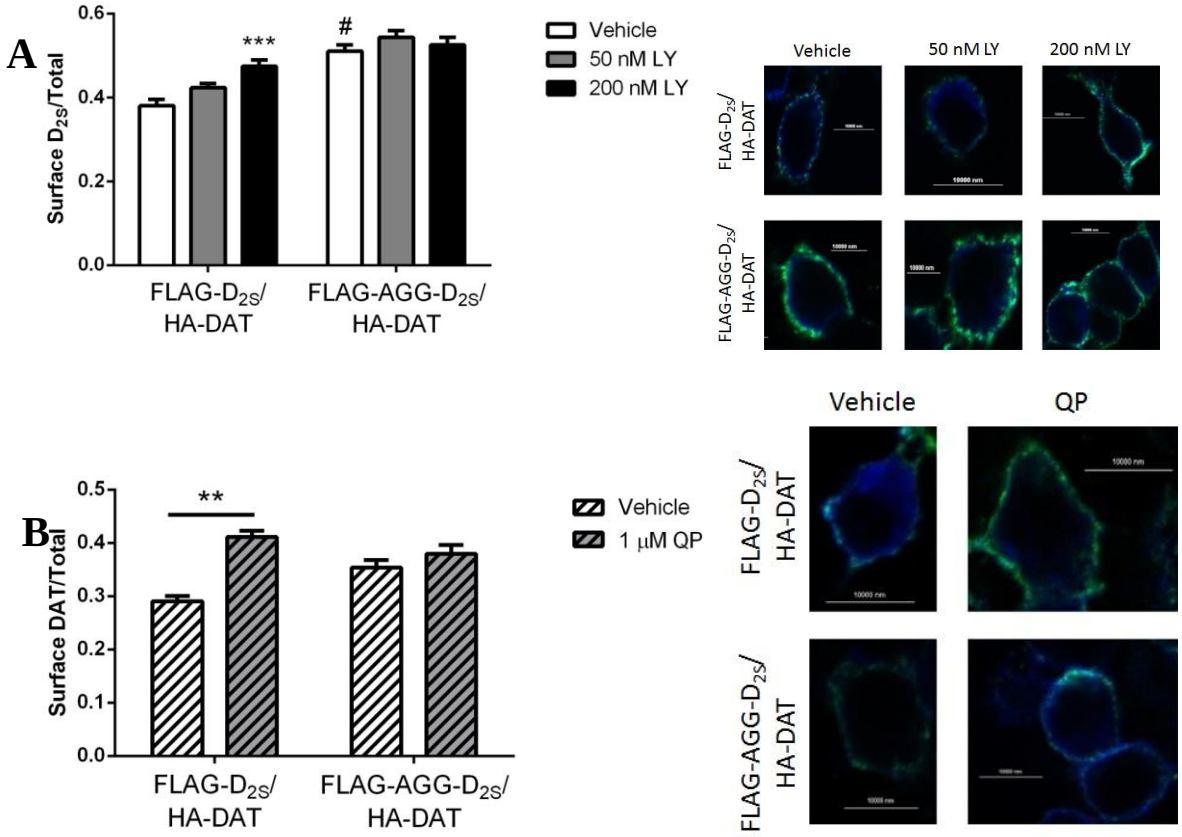

Figure 3-4: FLAG-AGG-D_{2S} disrupts DAT regulation of D_{2S}. (A) N2A cells transfected with FLAG-D_{2S} or FLAG-AGG-D_{2S} and HA-DAT were treated for 5 minutes with vehicle, 50nM, or 200 nM LY. Surface FLAG-D_{2S} or FLAG-AGG-D_{2S} localization was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface FLAG-D_{2S} is green, intracellular is blue. N = 44-72 cells. *** $p < 0.001$, # $p < 0.0001$ vs. FLAG-D_{2S}/HA-DAT vehicle by two-way ANOVA with Tukey post-hoc analysis. (B) N2A cells transfected with FLAG-D_{2S} or FLAG-AGG-D_{2S} and HA-DAT were treated for 5 minutes with vehicle or 1 μM quinpirole (QP). Surface HA-DAT localization was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface HA-DAT is green, intracellular is blue. N = 21-80 cells. ** $p < 0.01$, by two-way ANOVA with Tukey post-hoc analysis.

transfected with HA-DAT and either wild type FLAG-D_{2S} or the FLAG-AGG-D_{2S} phosphomutant and treated with vehicle or 1 μM quinpirole for 5 minutes (Figure 3-4B). HA-DAT surface localization was significantly increased following quinpirole treatment in cells co-expressing FLAG-D_{2S} but not the FLAG-AGG-D_{2S} phosphomutant (two-way ANOVA, interaction $F(1,171) = 6.228$, $p = 0.014$; quinpirole $F(1, 171) = 14.94$, $p = 0.0002$; FLAG-AGG-D_{2S} $F(1, 171) = 0.6891$, $p = 0.408$). Thus without the possibility of phosphorylation at three sites, D_{2S} is unable to stimulate the increase in surface DAT localization (vehicle: 0.354 ± 0.014, N = 80, quinpirole: 0.379 ± 0.017, N = 48). The disruption of this regulation did not affect baseline levels surface HA-DAT like it did for basal surface FLAG-D_{2S} localization (see Figure 3-4A). This matches previous findings that PKCβ inhibition does not affect basal surface DAT localization (see Figure 3-1 and Chen et al., 2013).

DAT and D_2R are reported to form a physical complex, interacting at the third intracellular loop of D_2R and the N-terminus of DAT (Lee et al., 2007). The interaction was disrupted by including a peptide against the first 15 amino acids of DAT. This region of DAT contains several PKC phosphorylation sites (Foster et al., 2002). To determine if the DAT N-terminus is required for the DAT regulation of D_{2S}, we measured changes in surface localization of FLAG-D_{2S} in cells co-expressing FLAG-D_{2S} and either full-length HA-DAT or a truncation HA-DAT mutant lacking the first 22 amino acids (HA-ΔN22-DAT). Surface FLAG-D_{2S} was determined following a 5 minute treatment with vehicle or increasing concentrations of LY379196 to ascertain if phosphorylation of DAT by PKCβ influences the DAT regulation of D_{2S} (Figure 3-5). As before, PKCβ inhibition increases surface FLAG-D_{2S} localization in cells expressing full-length HA-DAT (two-way ANOVA, Interaction $F(2, 333) = 2.250$, $p < 0.1070$, DAT $F(1, 333) = 57.67$, $p < 0.0001$, LY379196 $F(2, 333) = 8.692$, $p = 0.0002$). The fraction of surface FLAG-D_{2S}

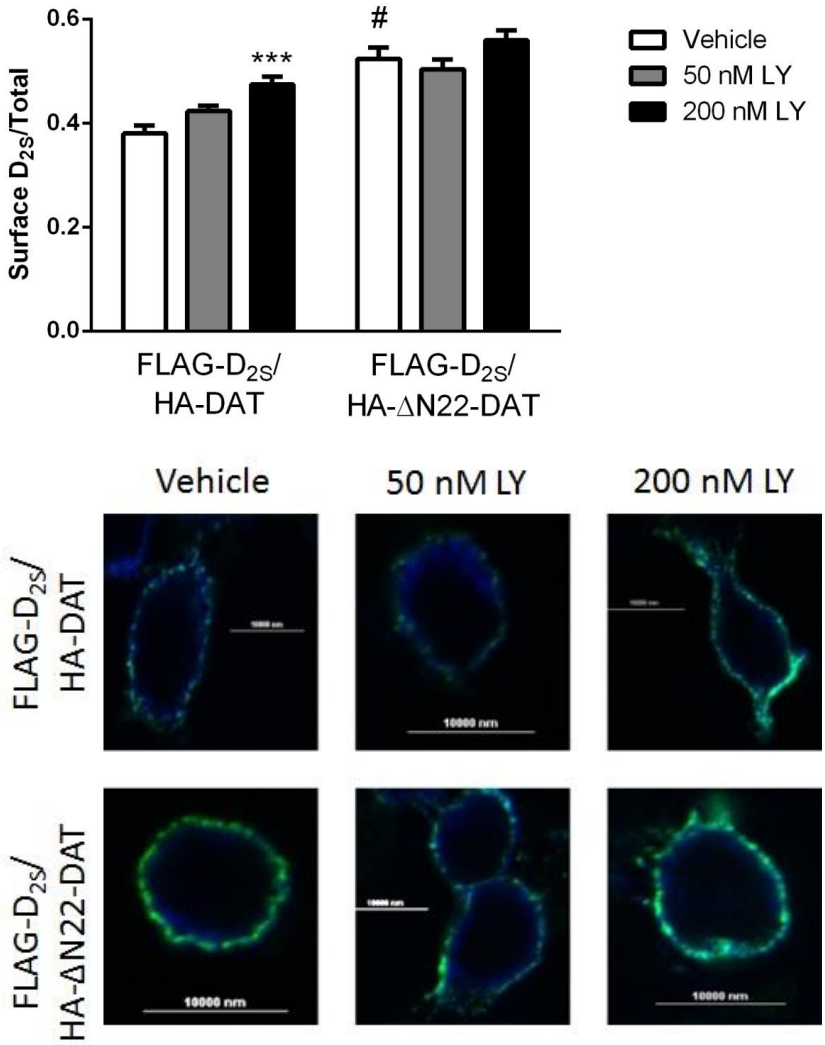

Figure 3-5: DAT N-terminus is required for DAT regulation of D_{2S}. N2A cells transfected with FLAG-D2S and HA-DAT or HA-ΔN22-DAT were treated for 5 minutes with vehicle, 50 nM, or 200 nM LY. Surface FLAG-D_{2S} localization was determined by immunofluorescence labeling and confocal microscopy. In representative images, surface FLAG-D_{2S} is green, intracellular is blue. N = 49-72 cells. *** $p < 0.001$, # $p < 0.0001$ vs. FLAG-D2/HA-DAT vehicle by two-way ANOVA with Tukey post-hoc analysis.

when co-expressed with HA-ΔN22-DAT is greater than with full-length HA-DAT following vehicle treatment, but is not further changed following inhibition of PKCβ (vehicle: 0.524 ± 0.022, N = 49, 50 nM LY379196: 0.503 ± 0.019, N = 58, 200 nM LY379196: 0.559 ± 0.019, N = 49). This result is similar to the increased surface localization and PKCβ insensitivity demonstrated by FLAG-AGG-D_{2S} (see Figure 3-4A). Together, these results indicate that the DAT N-terminus as well as the T225/S228/S229 PKC phosphorylation sites are required for the D_{2S}-DAT regulation.

DAT Regulation of D_{2S} Changes D_{2S}-Mediated Signaling

If the presence of DAT changes cellular localization and agonist responsivity of D_{2S}, there should be an impact on D_{2S} signaling. Through its coupling to inhibitory G proteins, D_2R initiates activation or inhibition of several second-messenger signaling pathways [see review (Neve et al., 2004)]. We chose to measure inhibition of cAMP accumulation, ERK activation and G protein activation by [^{35}S]-GTPγS binding. D_2R primarily inhibits adenylyl cyclase to decrease cAMP formation through G_α subunits, while ERK can be activated either by $G_{\beta\gamma}$ or arrestin-mediated signaling (Kim et al., 2004). N_2A cells express members of both the G_o and G_i inhibitory G protein family (Zhang et al., 2006), and D_2Rs can couple to both to elicit downstream signaling (Gazi et al., 2003; Lledo et al., 1992).

To measure the D_{2S}-stimulated inhibition of cAMP formation, cells expressing FLAG-D_{2S} with and without HA-DAT were stimulated with 30 μM forskolin to activate adenylyl cyclase in the absence and presence of various concentrations of quinpirole. No difference was found in inhibition of cAMP formation between the two cell types (IC_{50}, FLAG-D_{2S}/Vector 17.10 ± 1.43 nM, FLAG-D_{2S}/HA-DAT 20.09 ± 1.92 nM), suggesting that G_α signaling via D_{2S} is not altered by the presence of DAT (Figure 3-6A).

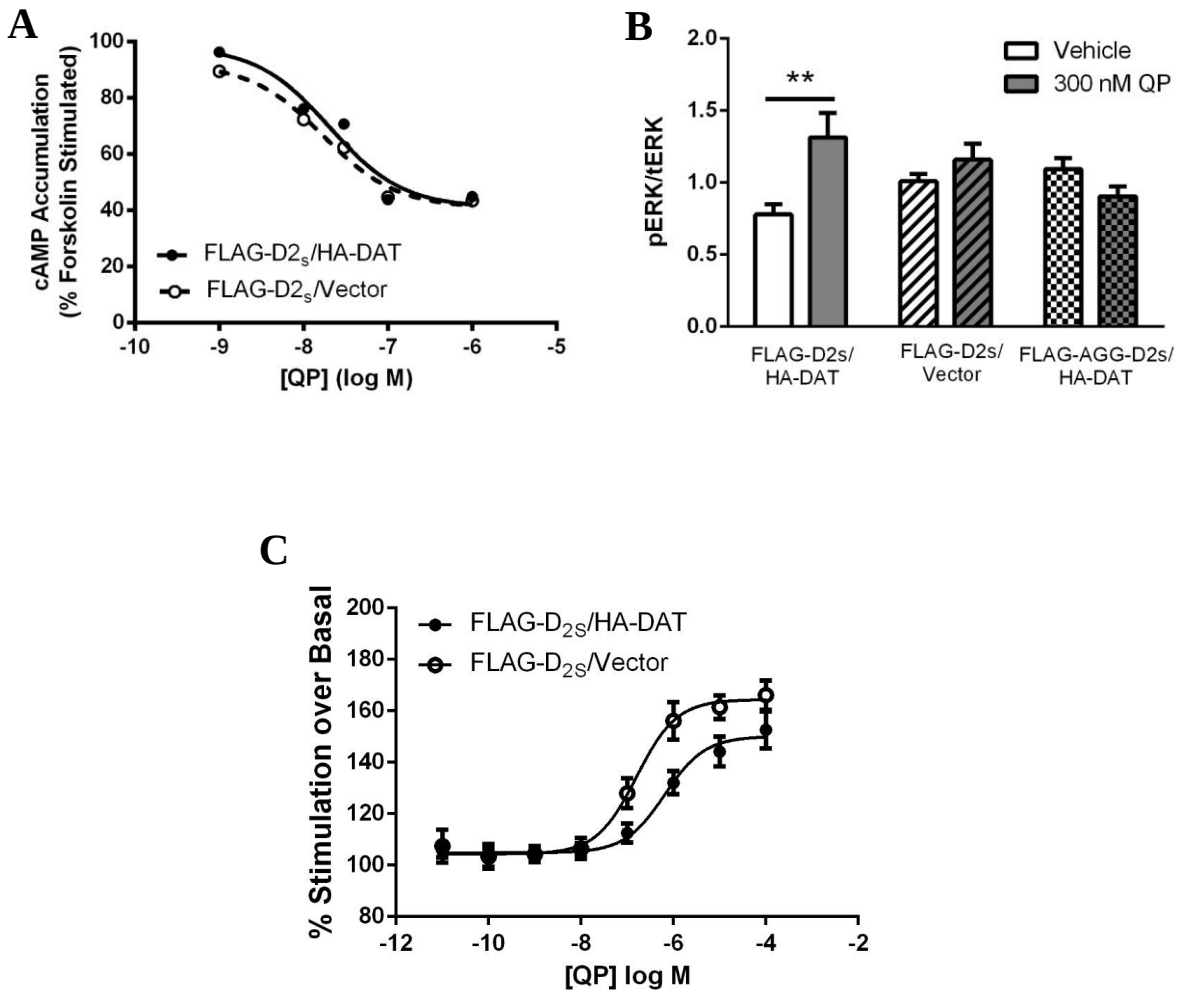

Figure 3-6: D_{2S}-DAT interaction increases $D2_S$ signaling through the ERK pathway. (A) Inhibition of cAMP accumulation was measured in N2A cells transfected with FLAG-D2S ± HA-DAT following treatment with a quinpirole (QP) concentration-response curve. N = 3. (B) Stimulation of ERK in N2A cells expressing FLAG-D2S ± HA-DAT or FLAG-AGG-D2S + HA-DAT was determined via Western blot following 5 minute treatment with 300 nM QP. ** $p < 0.01$, by two-way ANOVA with Sidak post-hoc analysis, N = 5. (C) G protein activation via [^{35}S]-GTPγS binding in cells expressing FLAG-D2S ± HA-DAT.

Next, we determined ERK activation following treatment with quinpirole by measuring phosphoERK formation. Cells expressing FLAG-D_{2S} ± HA-DAT or FLAG-AGG-D_{2S} and HA-DAT were treated for 5 minutes with 300 nM quinpirole. Only cells expressing wild type FLAG-D_{2S} with HA-DAT showed an increase in ERK activation (Figure 3-6B). A two-way ANOVA indicated a significant interaction of quinpirole and DAT presence ($F(2,24) = 6.739$, $p = 0.0048$). We next measured D_{2S}-stimulated G protein activation in the presence or absence of DAT (Figure 3-6C). Cells co-expressing FLAG-D_{2S} and HA-DAT displayed decreased effectiveness for G protein activation following quinpirole treatment, as measured by [^{35}S]-GTPγS binding (EC_{50}: FLAG-D_{2S}/HA-DAT 652.63 ± 1.54 nM, FLAG-D_{2S}/Vector 159.59 ± 1.45 nM, $p = 0.039$ by unpaired t-test; Top: FLAG-D_{2S}/HA-DAT 150.1 ± 3.3%, FLAG-D_{2S}/Vector 164.4 ± 3.3%, $p = 0.016$, N = 5). Taken together, these data indicate that the DAT regulation of D_{2S} impacts D_{2S} signaling, shifting D_{2S} signaling towards the ERK pathway.

Discussion

In this study, we made the original observation that D_{2S} activity and localization are regulated by DAT. The D_2 autoreceptor-mediated increase in DAT trafficking to the cell surface has been described previously (Bolan et al., 2007; Lee et al., 2007; Meiergerd et al., 1993) but a systematic exploration of the effects of DAT on D_2 autoreceptor localization and signaling has not been done. D_{2S}, which lacks 29 amino acids in the third intracellular loop due to alternative splicing, was used for all experiments. This variant was thought to be predominantly expressed in presynaptic dopaminergic neurons and thus functions as the dopamine autoreceptor (Khan et al., 1998; Usiello et al., 2000), however RT-PCR work in cells isolated from rat substantia nigra indicate that both the short and long form of D_2R as well as D_3R are expressed in these cells and can function as an autoreceptor (Jang et al., 2011). Our results demonstrate that the regulation of

D_{2S} by receptor agonist and by PKCβ is profoundly changed by the co-expression of DAT. We therefore propose that DAT regulates D_{2S} via a D_{2S}-DAT context. The model for our findings is summarized in Figure 3-7. When D_{2S} is not in the presence of DAT, its surface localization is regulated similarly to other GPCRs in that agonist treatment elicits internalization of the receptor. When D_{2S} interacts with DAT, D_{2S} enters into a different, DAT-specific context, which changes the regulation of the receptor. By our model, this D_{2S}-DAT context results in a reduced surface localization of D_{2S} by changing the conformation of the receptor such that it is more susceptible to phosphorylation by PKCβ, leading to increased internalization of the D_{2S}. This notion is supported by our data showing that inhibition of PKCβ leads to increased surface localization of D_{2S}. The D_{2S}-DAT context was disrupted either through removal of three PKC phosphorylation sites on the third intracellular loop of D_{2S} or by removal of the first 22 amino acids of the DAT N-terminus. Disruption of this complex allows D_{2S} to be regulated more similarly to a D_{2S} outside of the D_{2S}-DAT context. The effect of the D_2R agonist quinpirole on localization and signaling of D_{2S} differs whether within or outside of the D_{2S}-DAT context. Quinpirole, acting on D_{2S} in the absence of DAT, elicits internalization of the receptor. When D_{2S} interacts with DAT, quinpirole treatment brings D_{2S} as well as DAT to the surface. The second messenger signaling through D_{2S} similarly reflects the DAT-dependent regulation. In the D_{2S}-DAT context, there is a more pronounced activation of ERK by quinpirole, reflective of the increase in surface D_{2S} by quinpirole in the D_{2S}-DAT context. Because quinpirole stimulation of ERK is involved in the D_2 autoreceptor-mediated increase in surface DAT (Bolan et al., 2007; Chen et al., 2013), this D_{2S}-DAT context may ultimately lead to increased surface DAT localization, increased dopamine reuptake, and consequently less dopaminergic signaling.

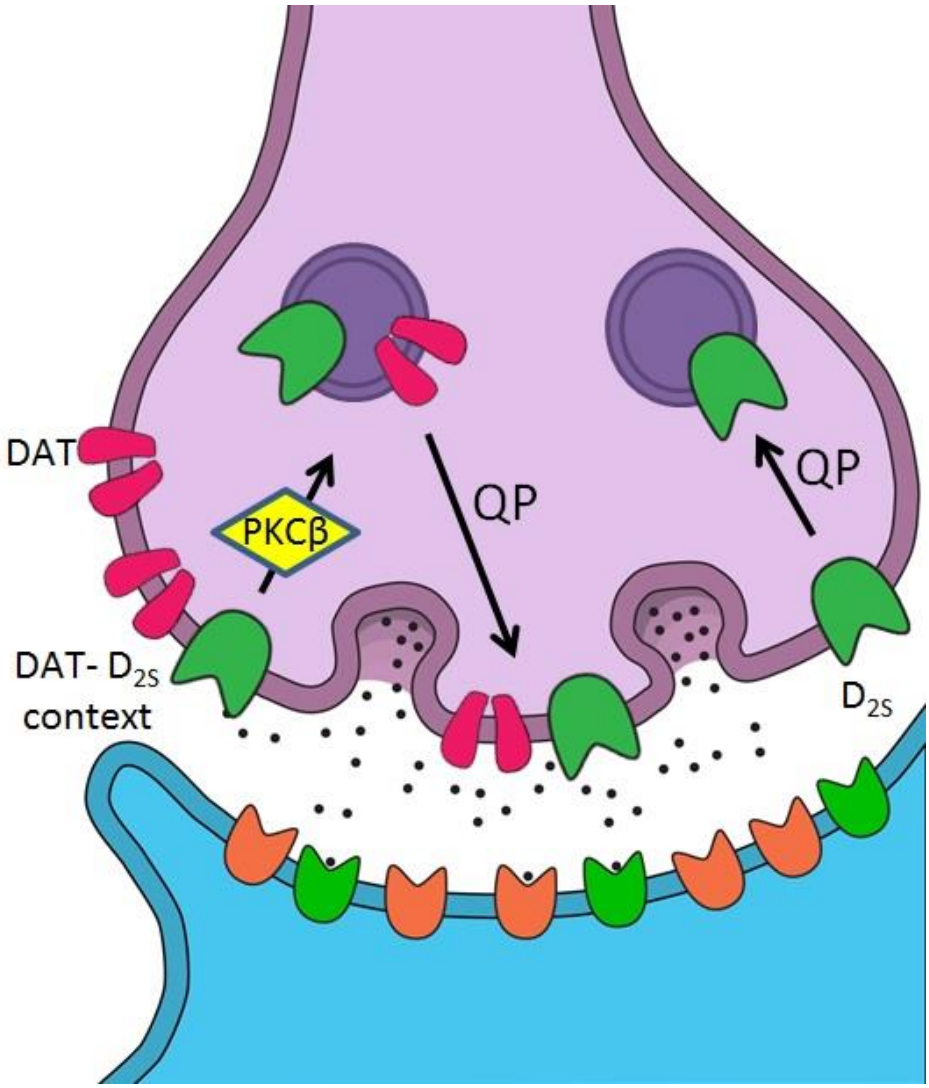

Figure 3-7: Model of the D_{2S}-DAT context. When in the D_{2S}-DAT context, D_{2S} is in a conformation that increases PKCβ-mediated internalization basally. Treatment with quinpirole (QP) increases surface localization of both D_{2S} and DAT. When D_{2S} is outside of the D_{2S}-DAT context, it is regulate more like a GPCR, with QP treatment causing internalization of the receptor.

The context by which a receptor is surrounded is important for determining the function and regulation of that protein, be it the concentration of ions altering ligand affinity (Neve, 1991; Watanabe et al., 1985) or a specific ligand that biases signaling of the receptor through a particular pathway (Cordeaux et al., 2001; Gazi et al., 2003; Urban et al., 2007). There is a growing understanding of how phosphorylation or interaction with kinases changes the context and regulation of the receptor. Phosphorylation by G protein-coupled receptor kinases (GRKs) is associated with desensitization and internalization of receptors (Moore et al., 2007). Removal of the GRK phosphorylation sites from D_2R, however, did not change the sensitivity of the receptor to desensitization or internalization. Instead, GRK phosphorylation determined the propensity of the receptor to be recycled back to the surface of the cell or degraded (Namkung et al., 2009a). Through a novel mechanism, GRK2 physically interacts with D_2R to decrease receptor expression and signaling without phosphorylation by the kinase (Namkung et al., 2009b). Likewise, phosphorylation of discrete residues on the β_2-adrenergic receptor directed the second messenger signaling pathway of the receptor as well as its response to ligands (Nobles et al., 2011). Together, these findings and ours indicate that phosphorylation can regulate receptors, including D_2R, beyond simple desensitization and internalization.

The finding that prevention of PKCβ phosphorylation either through inhibition or removal of phosphorylation sites disrupts the D_{2S}-DAT context suggests that PKCβ is involved in the regulation of this context. PKCβ mediates the signaling cascade that leads to increased surface DAT localization following D_2 autoreceptor stimulation (Chen et al., 2013). In that study, mice genetically lacking PKCβ or wild type mice treated with a PKCβ inhibitor lacked the D_2 autoreceptor-stimulated increase in surface DAT. Further experimental work found that PKCβ activation is upstream of ERK activation. Our results suggest that PKCβ activity may be driving

the formation or stabilization of the D_{2S}-DAT context prior to activation of the ERK signaling pathway. In this study, we used mutant D_{2S} lacking three PKC phosphorylation sites (FLAG-AGG-D_{2S}) and found that loss of these residues disrupted the D_{2S}-DAT context. PKC can also phosphorylate DAT on several serines residues on its N-terminal tail (Foster et al., 2002). These residues are located within the 22 amino acids deleted in the truncation mutant of DAT we used in this study (HA-ΔN22-DAT), which also disrupted the D_{2S}-DAT complex. So while we posit that PKCβ phosphorylation is important for the D_{2S}-DAT interaction, we cannot definitively say if D_{2S} or DAT or both are phosphorylated by PKCβ for the interaction.

The D_{2S}-DAT context also preferentially changes D_{2S}-mediated signaling. While there was no change in the $G_{\alpha i/o}$-coupled decrease in cAMP accumulation, activation of ERK was increased in cells expressing FLAG-D_{2S} and HA-DAT compared with cells expressing FLAG-D_{2S} in the absence of HA-DAT or FLAG-AGG-D_{2S} with HA-DAT. D_2R activates ERK signaling via $G_{\beta\gamma}$ (Beom et al., 2004) and arrestin (Kim et al., 2004). In cells expressing both D_{2S} and DAT, quinpirole was less efficacious in activating G proteins as compared to those cells expressing D_{2S} without DAT. These findings suggest that within the D_{2S}-DAT context, ERK is activated via the arrestin signaling pathway.

While we have identified a specific D_{2S}-DAT context, several questions remain concerning its regulation. First, we do not know if the context involves a physical interaction between D_{2S} and DAT. A physical coupling between these two proteins was reported, with the interaction occurring between the third intracellular loop of D_{2S} and the N-terminus of DAT (Lee et al., 2007) though another study found no involvement of the DAT N-terminus (Bolan et al., 2007). We attempted to determine if the D_{2S}-DAT complex involves a physical interaction between the two proteins with bioluminescence resonance energy transfer (BRET). Using this technique to

measure interactions between two membrane proteins such as D_{2S} and DAT is challenging because the technique has difficulties separating true, specific interactions from false signals generated from random interactions at the cell surface (Gavalas et al., 2013). Therefore, we abandoned this line of experiments and reached no conclusion concerning a physical complex.

We also do not currently understand the physiological circumstances under which this D_{2S}-DAT context is present. Complete ablation of the context using $DAT^{-/-}$ mice cannot help us answer this question as these have a mice have a 50% decrease in D_2R mRNA and lack D_2 autoreceptor activity consequent to their severe hyperdopaminergia (Giros et al., 1996; Jones et al., 1999). *In vitro*, $D_2R^{-/-}$ mice display decreased DAT function without a change in overall DAT expression and no change in basal or K^+-stimulated dopamine release, confirming that D_2 autoreceptor modulates DAT activity (Dickinson et al., 1999). *In vivo*, the D_2 autoreceptor-mediated increase in dopamine uptake via DAT only occurs at high stimulation frequencies (Benoit-Marand et al., 2011). Additionally, the method by which the D_2 autoreceptor controls extracellular dopamine shifts from decreasing dopamine exocytosis to increasing DA reuptake at these high frequencies of stimulation (Wu et al., 2002). In human cerebrocortical synaptosomes, stimulation using 4-aminopyridine, a potassium channel blocker, increased cytosolic calcium concentrations and increased PKC activity (Moe et al., 2002). Given these findings, we could speculate that high frequencies of stimulation in dopamine neurons would increase PKCβ activity, increasing the number of D_2 autoreceptors in the D_{2S}-DAT context. This would cause increased surface DAT localization and increased dopamine reuptake, resulting in decreased extracellular dopamine and dopamine signaling. More studies are needed to prove this hypothesis.

In conclusion, we have determined that DAT is able to regulate the D_2R, specifically the D_{2S} splice variant, through a DAT-specific context. Within this context, treatment with the D_2R

agonist quinpirole does not trigger internalization of D_{2S}, but instead stimulates increased surface localization. The D_{2S}-DAT context also alters D_{2S} signaling, increasing activation of the ERK signaling pathway following agoinst treatment. PKCβ phosphorylation and the DAT N-terminus are required for the formation of the D_{2S}-DAT interaction, as removal of either allows D_{2S} to be regulated more like other GPCRs. The identification of this DAT-mediated regulation of D_2R increases our knowledge of how dopaminergic signaling and D_2R are regulated. This may aid in the understanding of diseases involving D_2R and the dopaminergic system and the development of better therapeutics to treat these disorders.

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CHAPTER FOUR

DISCUSSION

This thesis highlights the importance of phosphorylation and domain milieu on the regulation of the D_2 dopamine receptor (D_2R) within the dopamine neuron. Together with DAT, the D_2-like dopamine autoreceptor (D_2 autoreceptor) regulates the amount of extracellular dopamine and thus dopaminergic signaling to maintain homeostasis. The D_2 autoreceptor decreases extracellular dopamine through three mechanisms: inhibiting dopamine exocytosis, inhibiting tyrosine hydroxylase to decrease dopamine synthesis, and increasing dopamine reuptake by interacting with DAT. Two of these mechanisms, per our current knowledge, involve PKCβ. My thesis expands our understanding of how the D_2 autoreceptor is regulated.

Model

The results of my thesis indicate that the activity of the D_2 autoreceptor is regulated differently when it is in a context with DAT than when it is not. When interacting with DAT, the D_2 autoreceptor will, upon stimulation, increase surface DAT and the amount of dopamine removed from the extracellular space will concomitantly be increased. The D_2 autoreceptor regulation of DAT mainly occurs during high frequency stimulation of dopamine neurons, whereas the D_2R autoreceptor inhibition of dopamine exocytosis happens during low frequency stimulations (Benoit-Marand et al., 2011; Wu et al., 2002). Therefore, I propose that the D_2 autoreceptor-DAT interaction that changes the regulation of the receptor mainly occurs at high frequency

stimulations. This D_2 autoreceptor-DAT coordination serves as an additional layer of regulation for the amount of dopamine in the extracellular space.

At stimulations occurring at low frequencies, such as with tonic neuronal firing, the D_2 autoreceptor does not interact with DAT (Figure 4-1). Here, activation of the D_2 autoreceptor inhibits both dopamine exocytosis and synthesis of dopamine at tyrosine hydroxylase to decrease the amount of extracellular dopamine. During these times of tonic firing, the D_2 autoreceptor would signal through both G_α and $G_{\beta\gamma}$ pathways, with G_α signaling decreasing cAMP formation and tyrosine hydroxylase activity, while $G_{\beta\gamma}$ interaction with potassium or calcium channels would hyperpolarize the neuron to inhibit the release of dopamine. DAT would also remove dopamine from the extracellular space via reuptake. D_2 autoreceptor regulation would occur through GRK-mediated pathways, similar to other GPCRs (Namkung et al., 2009a). PKC-mediated heterologous desensitization of D_2R (Namkung and Sibley, 2004) is possible but likely does not occur without PKC activation, such as through G_q coupled signaling (Thibault et al., 2011). I demonstrated that PKCβ inhibition has no effect on surface D_{2S} localization in the absence of DAT (Chapter 3).

At higher neuronal firing frequencies, such as during burst firing, the D_2 autoreceptor control of extracellular dopamine shifts from decreasing dopamine exocytosis to increasing dopamine uptake through DAT (Wu et al., 2002). D_2 autoreceptor stimulation increases surface DAT localization and increases the amount of dopamine uptake (Bolan et al., 2007; Dickinson et al., 1999; Meiergerd et al., 1993), though this only occurs during high frequency stimulations and not during tonic firing (Benoit-Marand et al., 2011). This D_2 autoreceptor-mediated increase in surface DAT involves both the ERK and PKCβ signaling pathways (Chen et al., 2013). By my model, this high firing neuronal firing rate would drive the D_2 autoreceptor and DAT to

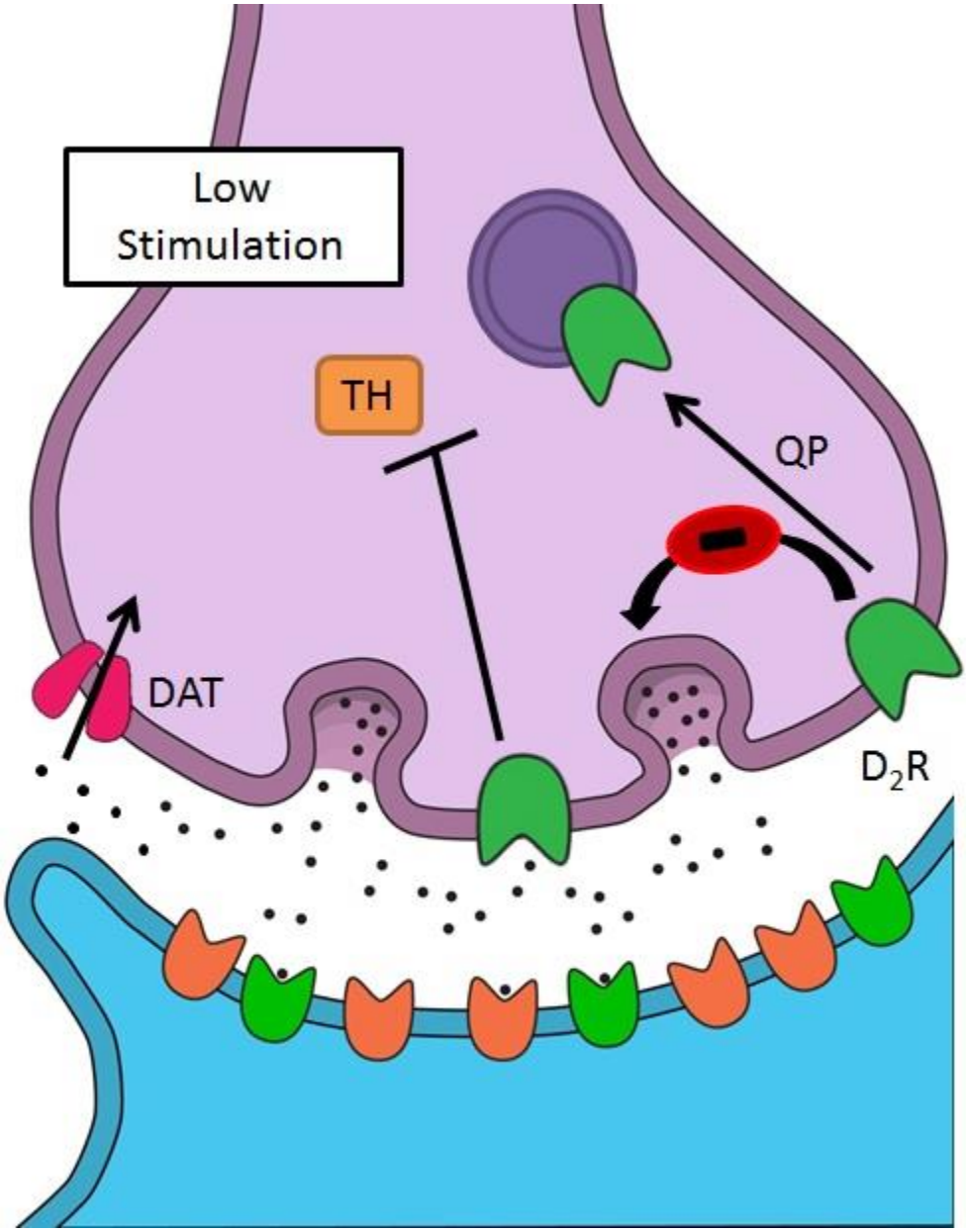

Figure 4-1: D_2 autoreceptor regulation of extracellular dopamine during low frequency stimulation. QP, quinpirole; TH, tyrosine hydroxylase.

interact, causing the D_2 autoreceptor to be regulated under the D_2 autoreceptor-DAT context (Figure 4-2). D_2 autoreceptor and DAT appear to physically couple between the third intracellular loop of D_2R and the N-terminus of DAT (Lee et al., 2007). The D_2 autoreceptor-DAT interaction may be stabilized by PKCβ. I found that substitution of three PKC phosphorylation sites in the third intracellular loop of D_{2S} or truncation of the DAT N-terminus disrupts the D_2 autoreceptor-DAT regulation (Chapter 3). PKC can be activated following stimulation of human cerebrocortical synaptosomes with the potassium channel blocker 4-aminopyridine (Moe et al., 2002) or by the DAT substrate amphetamine (Giambalvo, 2004). Both mechanisms depolarize the plasmalemmal membrane. Once the D_2 autoreceptor interacts with DAT, I hypothesize that it adopts a conformation that makes it more susceptible to phosphorylation by PKC. This increases the internalization of the receptor, perhaps to a recycling endosome.

In Chapter 2, I investigated the regulation of the D_2 autoreceptor by PKCβ using both PKCβ$^{-/-}$ mice and specific PKCβ inhibitors. I found that without PKCβ activity, mice had increased suppression of both dopamine release and locomotor activity following autoreceptor stimulation by the D_2-like agonist quinpirole. PKCβ$^{-/-}$ mice are unable to increase surface DAT localization following D_2 autoreceptor activation (Chen et al., 2013). These data suggest that without PKCβ activity, the D_2 autoreceptor is regulated under the low stimulation paradigm.

As discussed in Chapter 3, agonist treatment of the D_2 autoreceptor-DAT complex increases the surface localization of both the D_2 autoreceptor and DAT. This would serve to further increase the mechanisms to reduce extracellular dopamine through increased reuptake and increased D_2 autoreceptor signaling. The increased D_2R signaling includes a shift towards ERK signaling,

which would stimulate a further increase in surface DAT localization. Overall, the D_2 autoreceptor-DAT interaction and changed D_2 autoreceptor regulation is a mechanism

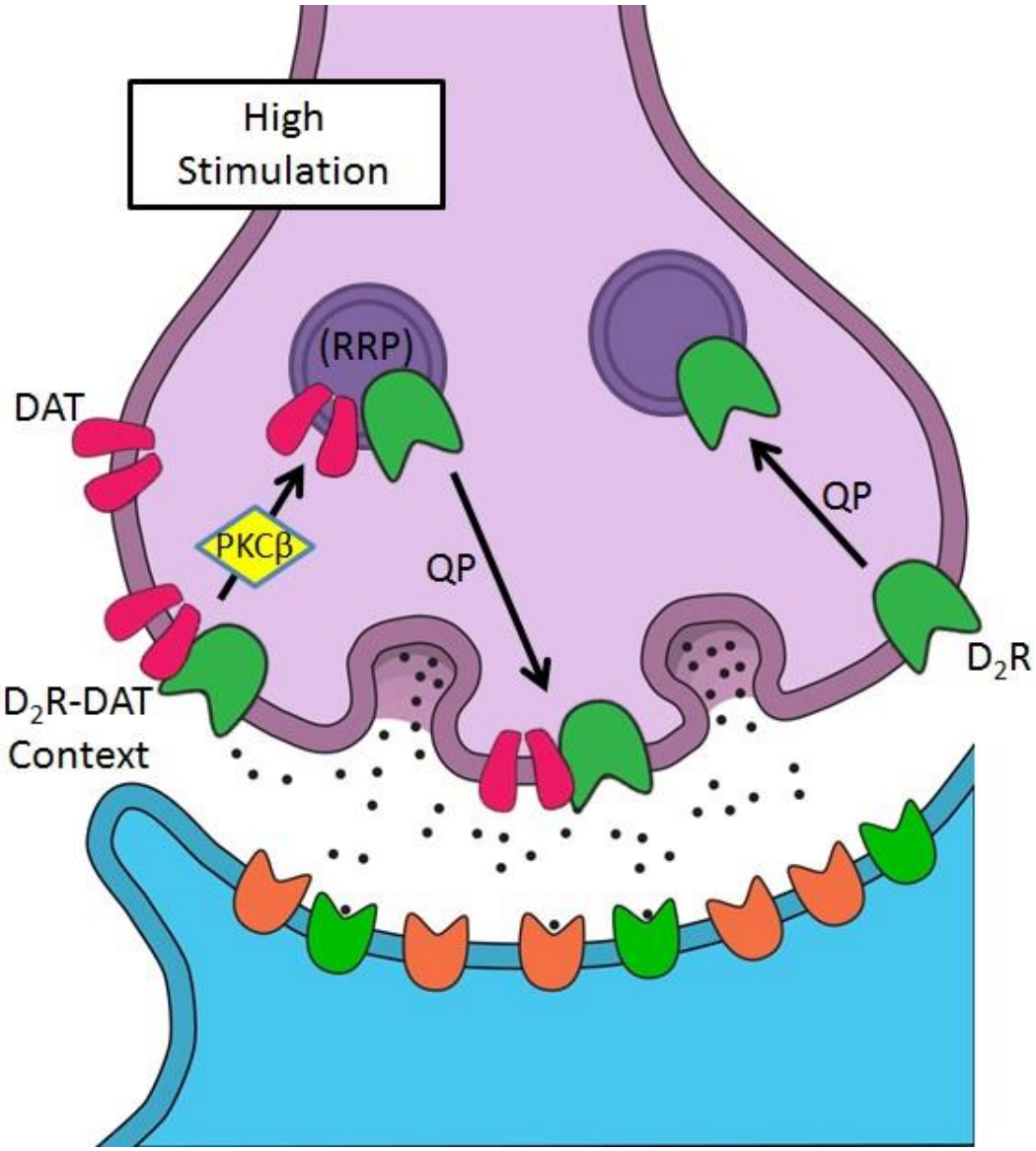

Figure 4-2: D_2 autoreceptor regulation of extracellular dopamine during high frequency stimulation. RRP, readily releasable pool; QP, quinpirole.

to quickly decrease extracellular dopamine concentrations following periods of burst firing. This increased neuron firing occurs in response to cues for natural rewards, such as food, but is increased by many abused drugs, including amphetamine (Daberkow et al., 2013).

D_2 Autoreceptor and Amphetamine

The highly abused DAT substrate amphetamine increases extracellular dopamine by inducing the reverse transport of dopamine through DAT (Fleckenstein et al., 2007) and by increasing burst firing and exocytotic release of dopamine (Daberkow et al., 2013). Additionally, amphetamine changes the surface localization of the transporter. Exposing the transporter to amphetamine for short periods increases surface DAT localization (Furman et al., 2009; Johnson et al., 2005a); longer exposures drives internalization of DAT (Chi and Reith, 2003; Saunders et al., 2000). It is interesting to speculate how amphetamine treatment would affect the D_2 autoreceptor-DAT interaction. I hypothesize that amphetamine would stabilize the D_2 autoreceptor-DAT interaction through its activation of PKC (Giambalvo, 2004). With the D_2 autoreceptor and DAT interacting, I predict that D_2 autoreceptor surface localization would follow the same pattern as DAT, with short amphetamine treatments increasing surface localization and longer treatments triggering internalization. As I demonstrated in Chapter 2, PKCβ inhibition increases the D_2 autoreceptor control of dopamine release. Thus, the amphetamine-mediated activation of PKC would also inhibit the D_2 autoreceptor decrease in dopamine release, perhaps mediating the amphetamine stimulated increase in phasic dopamine release (Daberkow et al., 2013). The D_2 autoreceptor-DAT interaction could serve as a mechanism to rapidly clear dopamine from the extracellular space following amphetamine treatment: dopamine activation of the D_2 autoreceptor would increase ERK signaling, DAT surface localization, and dopamine reuptake. Based on the reduction in amphetamine-stimulated behaviors and dopamine release, the Gnegy

lab has proposed that a PKCβ inhibitor would block the reinforcing effects of amphetamine. My data suggest that such a treatment would block the formation of the D_2 autoreceptor-DAT interaction. However, a PKCβ inhibitor would increase the D_2 autoreceptor suppression of dopamine release, which may block the increase in phasic dopamine release observed following treatment with amphetamine and other abused drugs (Daberkow et al., 2013).

D_2R and Disease

D_2R has been implicated in several psychiatric diseases, including drug addiction and schizophrenia. Human cocaine abusers have less available postsynaptic D_2R in their basal ganglia than normal controls and this effect persists for several months after cocaine taking (Volkow et al., 1993; Volkow et al., 1990). Few studies have determined changes in the D_2 autoreceptor during or following drug abuse. In humans, novelty-seeking is a strong predictor for susceptibility to drug abuse (Piazza et al., 1989). Novelty seeking and D_2-like autoreceptor availability are inversely proportional as measured by PET imaging studies in healthy humans using the D_2-like antagonist [^{18}F]-fallypride (Zald et al., 2008). Additionally, higher impulsivity was correlated with decreased D_2-like autoreceptor binding in healthy human volunteers. The decreased autoreceptor binding also resulted in greater amphetamine-stimulated dopamine release in the striatum (Buckholtz et al., 2010). A mutation in human D_2R (Ser311Cys) correlates with increased incidents of schizophrenia and persecution type delusional disorder (Arinami et al., 1994; Morimoto et al., 2002). This mutation is in the third intracellular loop of D_2R and interferes with G protein activation in cell culture models (Chen and Zhuang, 2003). Lack of D_2 autoreceptor control clearly impairs dopamine signaling, in both drug abuse, schizophrenia, and other psychiatric diseases. I would propose that these subjects lose the

additional D_2 autoreceptor-DAT complex and the ability to correct high extracellular levels of dopamine. This could contribute to the dopamine dysfunction and disease development.

Future Directions

D_2-like receptors involved in D_2 autoreceptor-DAT complex

The D_2-like receptor family is comprised of three receptor types: D_2R, D_3R, and D_4R. After determining the distribution of these receptors, it was decided that D_2R and D_3R but not D_4R could function as autoreceptors (Gingrich and Caron, 1993; Sibley et al., 1993). Experiments with D_2R knockout mice concluded that D_2R and not D_3R functioned as the autoreceptor (L'Hirondel et al., 1998). Using co-staining experiments and D_{2L} knockout mice, it was postulated that the short splice variant of D_2R, D_{2S}, and not the long variant of D_2R was located presynaptically and functioned as the autoreceptor (Khan et al., 1998; Lindgren et al., 2003; Usiello et al., 2000). However, both D_2R isoforms and D_3R are expressed in dopaminergic neurons isolated from rat substantia nigra, often in the same cell (Jang et al., 2011). All three receptor types decreased neuron firing following agonist treatment, suggesting that all can function as autoreceptors. Many of the reports measuring the D_2 autoreceptor coordination of surface DAT localization used the D_{2S} variant of D_2R (Bolan et al., 2007; Chen et al., 2013; Lee et al., 2007). Stimulation of D_3R increases surface DAT localization similarly to D_{2S} (Zapata et al., 2007). Interestingly, co-expression of D_{2L} with DAT did not increase DAT surface localization or dopamine uptake (Lee et al., 2007). Further work is needed to determine if D_{2L} or D_3R can undergo the same D_2 autoreceptor-DAT context regulation I found for D_{2S}. These experiments could easily be done *in vitro* using a heterologous cell system similar to the one described in Chapter 3. However, measurement of this D_2 autoreceptor-DAT context *ex vivo* or *in vivo* and determining the D_2-like receptor involved would be more difficult and would require

the use of peptides against the D_2 autoreceptor-DAT interaction (Lee et al., 2007) and/or siRNA knockdown of various D_2-like receptors.

G protein coupling and the D_2 autoreceptor-DAT context of regulation

Within the D_2 autoreceptor-DAT context, I found that D_2 autoreceptor-mediated signaling was shifted towards ERK signaling (Chapter 3). In cells co-expressing DAT and D_2R, G protein activation is right-shifted following quinpirole treatment. I have done preliminary studies to investigate the involvement of G protein signaling in the regulation of surface D_2R regulation within the D_2 autoreceptor-DAT context. For these experiments, I transfected cells with FLAG-D_{2S} $\pm$ HA-DAT and treated them overnight with vehicle or pertussis toxin to inhibit $G_{i/o}$ signaling. Following pertussis toxin treatment, cells were treated with 1 μM quinpirole for five minutes. Surface FLAG-D_{2S} and HA-DAT were determined using the immunofluorescence technique described in Chapter 3. In cells expressing FLAG-D_{2S} only, the short treatment with quinpirole did not significantly decrease surface FLAG-D_{2S} localization. However, pertussis toxin significantly decreased surface localization of FLAG-D_{2S} (Figure 4-3A, two-way ANOVA, interaction $F(1, 781) = 3.537$, $p = 0.0607$, pertussis toxin $F(1, 781) = 83.58$, $p < 0.0001$, quinpirole $F(1, 781) = 2.888$, $p = 0.0897$). Pertussis toxin treatment gave a very different result in cells co-expressing FLAG-D_{2S} and HA-DAT. In these cells, surface levels of FLAG-D_{2S} were significantly increased following pertussis toxin treatment. Five minutes of quinpirole treatment significantly increased surface levels of FLAG-D_{2S} in vehicle-treated FLAG-D_{2S}/HA-DAT cells but had no effect following pertussis treatment (Figure 4-3B). A two-way ANOVA revealed a significant effect of pertussis toxin, $F(1, 783) = 93.83$, $p < 0.0001$, and quinpirole, $F(1, 783) = 24.23$, $p < 0.001$, and a significant interaction $F(1, 783) = 35.37$, $p < 0.0001$. Pertussis toxin had no effect on surface HA-DAT in cells co-expressing HA-DAT with FLAG-D_{2S} (Figure 4-3C).

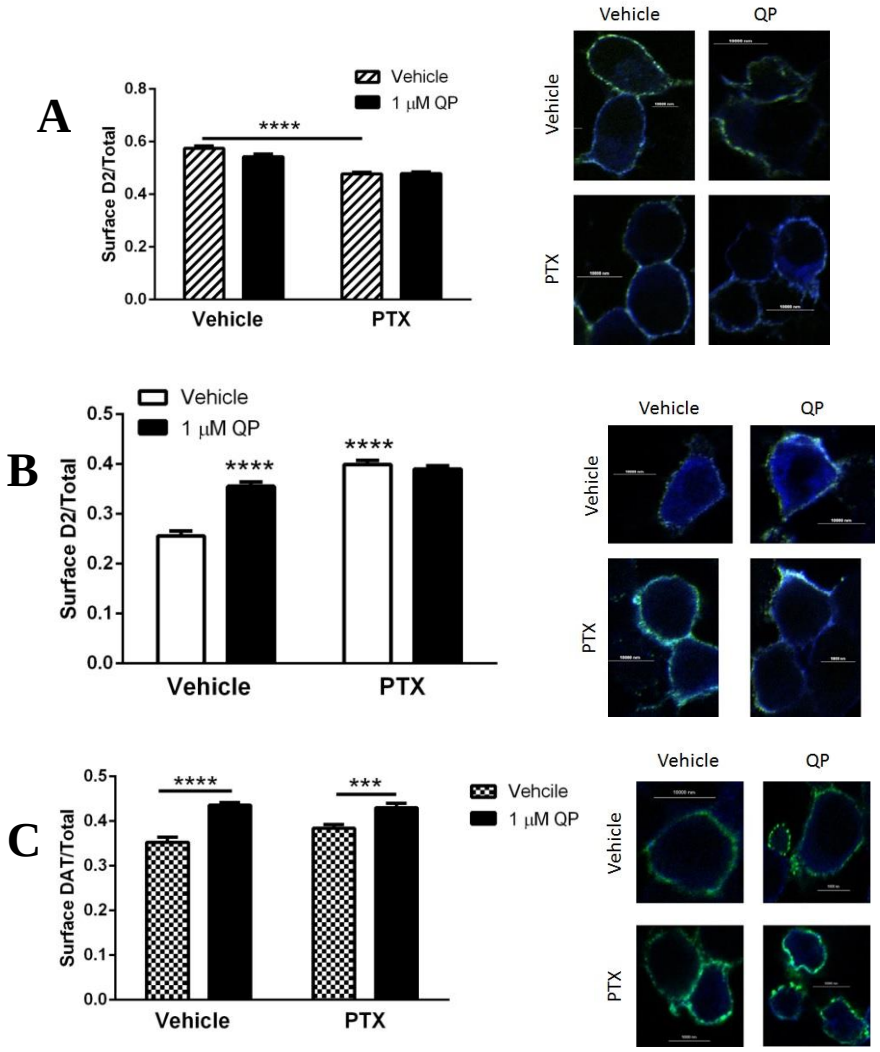

Figure 4-3: Surface D_2R localization but not surface DAT localization is changed following pertussis toxin (PTX) treatment. N2A neuroblastoma cells were transfected with FLAG-D_{2S} ± HA-DAT and treated overnight with vehicle or 100 pg/mL PTX. On experiment day, cells were treated for 5 minutes with vehicle or 1 μM quinpirole (QP). Surface FLAG-D_{2S} and HA-DAT were labeled using the immunofluorescence protocol outlined in Chapter 3. Representative images accompany the quantification for each graph with FLAG-D_{2S} or HA-DAT surface labeled in green and intracellular labeled in blue. (A) Surface FLAG-D_{2S} in cells expressing FLAG-D_{2S} and vector control, N = 83-317, **** $p < 0.0001$ by two-way ANOVA with Tukey's post-hoc analysis. (B) Surface FLAG-D_{2S} in cells expressing FLAG-D_{2S} and HA-DAT, N = 100-288 cells, **** $p < 0.0001$ vs. Vehicle/Vehicle control by two-way ANOVA with Tukey's post-hoc analysis. (C) Surface HA-DAT in cells co-expressing FLAG-D_{2S} and HA-DAT, N = 81-256, *** $p < 0.001$, **** $p < 0.0001$ by two-way ANOVA with Tukey's post-hoc analysis.

Surprisingly, pertussis toxin did not completely block the quinpirole-stimulated increase in surface HA-DAT localization, as was previously reported (Bolan et al., 2007). By two-way ANOVA, there was a significant effect of quinpirole, $F(1, 683) = 33.06$, $p < 0.0001$, but neither a significant effect of pertussis toxin, $F(1, 683) = 1.335$, $p = 0.2484$, nor an interaction, $F(1, 683) = 2.680$, $p = 0.1021$. These results suggest that $G_{i/o}$ protein signaling is involved in the D_2 autoreceptor-DAT interaction. Because inhibiting $G_{i/o}$ with pertussis toxin increased basal surface FLAG-D_{2S} signaling but had no effect on surface HA-DAT localization, G protein signaling may be involved in stabilizing the D_2 autoreceptor-DAT interaction. This further suggests that the D_2 autoreceptor can activate ERK signaling to increase surface DAT localization because pertussis toxin treatment did not block this effect in the FLAG-D_{2S}/HA-DAT cells. Further work is needed to understand how G protein signaling is involved in the D_2 autoreceptor-DAT complex.

Identification of D_2 autoreceptor-DAT context in vivo

I have measured changes in D_2R regulation in the presence and absence of DAT using a homologous cell system. It is yet to be determined if this D_2R-DAT context exists *in vivo*. Proving this effect of the D_2 autoreceptor-DAT context would be technically challenging. First, D_2R is expressed both pre- and post-synaptically, so care would be needed to separate these two pools of receptor, such using Percoll-purified synaptosomes (Dunkley et al., 1988). Lack of specific antibodies for D_2R limits the ability to measure localization changes of the native receptor. However, changes in D_2 autoreceptor-ERK signaling could be measured. I found that cells expressing FLAG-D_{2S} and HA-DAT had greater quinpirole-stimulated ERK activation than that in cells expressing FLAG-D_{2S} alone. In order to measure D_2 autoreceptor signaling in the presence and absence of DAT, I propose using interfering peptides to disrupt the D_2

autoreceptor-DAT complex (Lee et al., 2007). A peptide against the N-terminus of DAT increased locomotor activity in mice and decreased dopamine reuptake, which the authors concluded resulted from prevention of the D_2 autoreceptor-DAT physical interaction. I predict that this interfering peptide would also increase the D_2 autoreceptor-mediated suppression of dopamine release, as disruption of the D_2 autoreceptor-DAT complex allows greater D_2 autoreceptor control of exocytosis such as in the PKC$\beta^{-/-}$ mice. A seemingly simple solution to measure D_2 autoreceptor activity in the presence and absence of DAT would be to use the DAT knockout mice (Giros et al., 1996). However, these mice have an approximate 50% decrease in D_2R expression in both the midbrain and basal ganglia.

Conclusions

The D_2 autoreceptor can be regulated differently depending on its context. I determined that PKCβ suppresses D_2 autoreceptor activity, as loss of PKCβ activity increased D_2 autoreceptor control of dopamine exocytosis. This resulted in increased locomotor suppression following treatment with the D_2R agonist quinpirole, indicating that the PKCβ regulation of D_2R is physiologically relevant. Using a heterologous cell system, I found that DAT changes the regulation of D_2R. This D_{2S}-DAT context suppresses D_{2S} basal surface localization, likely through increased PKCβ-mediated internalization. Agonist treatment increases surface localization of D_{2S} similarly to DAT. The D_{2S}-DAT context of regulation can be disrupted by removing three PKC phosphorylation sites from D_2R (T225A/S228G/S229G) or the DAT N-terminus. This D_{2S}-DAT regulation context extends to signaling. I found that cells co-expressing D_{2S} and DAT have increased ERK activation following quinpirole treatment but a decrease in the quinpirole stimulated activation of G proteins, suggesting a switch to an arrestin signaling pathway. My results further our understanding of how the D_2 autoreceptor is regulated

and has identified a novel, DAT-mediated mechanism for D_2 autoreceptor regulation. These findings, along with the future directions I proposed, add to our knowledge of how the D_2 autoreceptor is regulated and will be useful for future studies regarding other GPCRs, dopamine regulation, and diseases of the dopaminergic system.

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