Title
Investigating the consequences of compartmentalized dopaminergic signaling

Author
Puri, Natasha Mukherjee

Publication Date
2022

Peer reviewed|Thesis/dissertation

Investigating the consequences of compartmentalized dopaminergic signaling

by
Natasha Mukherjee Puri

DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

DocuSigned by:
David Raleigh
7A381335D7C9402...

David Raleigh
Chair

DocuSigned by:
Roshanak Irannejad
DocuSigned by:
David Pearce
DocuSigned by:
Orion Weiner
DC0B9413C4A0416...

Roshanak Irannejad
David Pearce
Orion Weiner

Committee Members

Acknowledgments

Getting a PhD truly takes a village, and I have so many people to thank. First and foremost, I must thank my thesis advisor, Dr. Roshanak Irannejad. I met her before I even started my PhD, when I was working as a lab manager at UCSF. When I got into the Tetrad program, she was already hinting that I should rotate in her lab, in the very sweet and polite way that she does. At the time, I wasn't sure if I wanted to join her lab, mainly because I was intent on joining a cancer research lab. Nevertheless, I decided to rotate with her during my third and final rotation, thinking that even if I didn't join her lab, she was the type of PI whose support I would love to have in the future. A little into my first week there, I knew I wanted to be in her lab. She is one of the most caring and encouraging mentors I have had. She always seemed to sense whenever I was feeling unsure and made a point to tell me what I was doing well, and what I could work on. I looked forward to our weekly meetings because I knew I'd leave feeling better than when I had entered. Not only because of how supportive she is, but because of our scientific discussions, especially when we'd excitedly jump up to the whiteboard to get our ideas across. From early on, I was stunned by the beauty of her science. Even though I wanted to study cancer, her passion for her work completely drew me in. Thanks to her, I am a GPCR cell biologist all the way.

I'd also like to thank all the lab members along the way. Being a part of such a new lab was an incredible experience. We got to build up the lab together, and all our successes and failures were felt together. Gia Romano, the first member of the lab, was welcoming, hard-working, and silly all in one, and she absolutely defined our lab culture. Working with the post docs of the lab felt like working with brilliant superheros. Quynh Mai constantly supported all of us scientifically, emotionally, and mentally. Ting-Yu Lin would drop everything to help if I ever asked for it, which happened quite often. Federica Liccardo is so generous and helpful, she always listened to any complaints I had at face-value without any judgement. I could not have gone through this without the graduate students in the lab either. CJ Sarabia is one of the sweetest people I have met and is an incredible scientist. Evelyn Hernandez knows how much she means to me, but what I'll say here is that from early on in our friendship I was blown away by her intelligence, her energy, and her passion for the things she cares about. I am so incredibly grateful that we have gotten to go through nearly our entire PhDs together. Thanks so much to my committee as well. Dr. David Raleigh, my thesis chair and my very first rotation. His enthusiasm is truly infectious; I had so much fun talking science with him. He has supported me since pretty much day one of grad school, and always seemed happy to hear from me and hear about my science. I thank him for his spirited energy during all of our meetings, as well as his extremely prompt email responses. I thank Dr. David Pearce, who joined my committee to advise on my kidney project.

From our very first meeting when I asked him to join my committee, he was so kind and helpful. Him and his lab never hesitated to help with that side of my thesis work, and even when I moved more towards the brain, his support never faltered. Finally, I must thank Dr. Orion Weiner. He too has supported me since the first day of graduate school and even beyond that. I worked as a lab manager in his lab for two years before starting my PhD. I was shocked when he had complete faith in me to start my own project in his lab. But I think he knew that even if I had no idea what I was doing, I would get support from his lab. So many members of his lab helped me when they really didn't have to, and I learned exponentially when I was in there. Orion has been such a positive part of my scientific career and has helped me gain so much confidence as a scientists. He is a huge reason why I even got into grad school, and I will be forever grateful to him.

I would not have gotten through the first two years of graduate school without my classmates. We really supported and looked out for each other, and thankfully had similar ideas of what a good time was. Helping each other with problem sets, dressing up for Halloween, and planning friendsgivings and holiday parties were some of my favorite memories from graduate school. I feel so lucky to have such supportive and fun classmates, and while I wish we had gotten to spend more time together towards the end, I will be lifelong friends with many of them.

I'd like to thank my sister, Priya (aka Didi), who has been my advocate since the day I was born. I was a highly sensitive child, and while she gave me what I used to call tough love while we were growing up, her fierce love and loyalty is stronger than anything I've encountered in life. She is an incredibly strong-willed and confident person. She can always laugh at herself and is unbothered when people try to put her down. I always wanted to be more like her when we were growing up. Because of her, I have learned to accept criticism, keep trying if I fail at something, to advocate for myself, and to go after the things I want.

Thank you to my husband and best friend, Troy Trevino. He has been there for me through so much, from roommate drama to grad school applications to qualifying exam practices over zoom. We went from living and working together to being over two-thousand miles apart while we both got our PhDs, planning our wedding during the pandemic while not seeing each other for months. While these five years apart might have been the hardest thing we've ever had to do, the reward of being together afterwards is the sweetest I could imagine. His love and support have been constants in my life for so long that I honestly do not know what I would do without them.

Finally, I must acknowledge the massive role my parents played in my success as a graduate student. Dr. Pinku Mukherjee and Dr. Rahul Puri, who got their PhDs before I was even born. My dad wanted to study in the US, and he ended up leaving India in 1985 to get his PhD in combustion engineering. Having to send money to his family

back home combined with paying for international calls to my mom, he had to drive a bus and give private tutoring lessons on the side while he earned his degree. My mom got her PhD in London, in Biology. She didn't really know what she wanted to do before she ended up there, but her father valued education and consistently pushed her towards it. Interestingly, academic research was her true calling, while my dad left academia, and engineering, altogether. My dad saw how most of his professors didn't have time for their families, and wanted to make sure he could provide for us, so he joined the corporate world. But ultimately, all he really wanted was to answer to no one. Once he ensured my sister and I were stable, he ended up starting his own business with my mom, patenting one of the antibodies she had developed in her lab. My mom, on the other hand, worked consistently without thinking much about what she wanted to do. She would tell you that she was never the star student, never overly-ambitious, but now she is the Dean of the graduate division at University of North Carolina, Charlotte. My parents had extremely divergent trajectories after their PhDs, but they both taught me to work hard, but even more importantly to do what makes me happy. They both have been inspirational to me. When my sister and I were young, my dad would plan Science Sundays, using things around the house to conduct little experiments, like using straws to understand the properties of water. What is truly amazing about him is that he has been able to use all the knowledge and lessons he's learned from all his different careers and apply them to anything in his life. My mom

inspired us in a different way. Every day after middle school, we would trek over to her office and lab at the Mayo Clinic. We watched her work and run a lab, occasionally stealing a serological pipet or two to pretend to conduct our own research. She is absolutely the reason I got interested in and pursued a career in biology. Many women of color do not get to see themselves in professional careers, and I do not take lightly the privilege I have by growing up with a role model like that. As I start my own career, I hope I can emulate both of them.

Abstract

Investigating the consequences of compartmentalized dopaminergic signaling

Natasha Mukherjee Puri

Compartmentalized G protein-coupled receptor (GPCR) signaling has been increasingly proven to play a large role in regulating distinct cellular responses. The idea that GPCRs could signal from endosomes was already a relatively novel concept a mere two decades ago. However, early evidence showed that internalization of some GPCRs led to sustained activity rather than an abolishment of signaling. Still, this secondary phase of GPCR signaling was implicated in activation sourced from the cell surface. The concept of distinct signaling hubs originating from other membrane-bound organelles was truly novel. The discovery that the beta 1 adrenergic receptor, an important GPCR in regulating heart contractility and relaxation responses, could signal from the Golgi apparatus begged the question of whether other similar GPCRs could signal from subcellular organelles. Here, I uncover that D1 dopamine receptor (D1DR) can signal from multiple signaling hubs, identify the mechanism in which dopamine accesses intracellular membrane compartments, and speculate on the potential implications of this novel work. First, I use a biosensor tool to visualize compartmentalized D1DR signaling using live-cell confocal fluorescence microscopy. I

identified that organic cation transporter 2 (OCT2), a low-affinity high-capacity transporter of several neurotransmitters such as dopamine, facilitated dopamine transport intracellularly to access the Golgi pool of receptor. I also observed compartmentalized dopaminergic signaling in physiologically relevant cell types, including in key dopaminergic neurons and in kidney cells. Finally, I show that GPCR signals originating from distinct subcellular signaling hubs leads to distinct modulations of the downstream signaling cascades, indicating that receptor signaling from the Golgi could be regulating transcriptional responses. Put together, this work identifies a novel pathway of dopaminergic signaling and suggests that discrete signaling hubs within the cell distinctly regulate vital cellular responses.

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Chapter 1: Introduction and overview of G protein-coupled receptors

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1.1 Overview

G protein-coupled receptors (GPCRs) are responsible for maintaining an overwhelming variety of cellular processes, ensuring proper function of nearly all organs in our body. While the field of GPCR biology is at a critical turning point, with the revelation of novel and non-canonical activation pathways, the study of this incredibly prolific protein family spans decades. This chapter will be able to cover only a fraction of the information known about GPCRs and their signaling pathways.

This thesis will focus on a specific receptor, the D1 Dopamine receptor. Here, I uncover how these receptors can signal from intracellular compartments like the Golgi Apparatus, and in what cell types this signaling occurs. It will also explore cAMP/PKA signaling dynamics downstream of compartmentalized signaling. Perhaps like many dissertations, this work began with a rather simple question, revealed some unexpected findings, and ended with even more questions being asked than answered. Although I could not answer all these questions during my PhD, the results laid out in this thesis are just the tip of the iceberg of incredibly novel findings in relation to dopaminergic signaling.

1.2 Introduction to G protein-coupled receptors:

The cells that make up tissues and organs must respond to minute changes in their environment to maintain proper function. Cells accomplish this in part through G protein-coupled receptors (GPCRs), the largest and most diverse protein family in the mammalian genome. It's estimated that over 800 GPCRs exist in the human genome alone (Fredriksson et al., 2003; Pierce et al., 2002). Not only that, GPCRs are also incredibly conserved on an evolutionary scale, indicating their necessity for all life (Strotmann et al., 2011). GPCRs enable our cells to respond to their environment, even minute changes in their environment, by translating external stimuli into intracellular signals. They allow us to see and smell, to learn and remember and move (Pierce et al., 2002). It is due to GPCRs that our hearts beat, that our immune systems work. Because they are so critical to life, their dysfunction can lead to a number of diseases. It is unsurprising that nearly 30% of all pharmacological drugs in the market target GPCRs.

GPCRs are characterized by a seven transmembrane alpha-helical domain, a cytoplasmic C-terminal tail, and 3 loops spanning each helix on either side of the lipid membrane (Kroeze et al., 2003). Receptors are activated by a ligand that directly interacts with a binding pocket, often within the transmembrane helix bundle. Agonists that target GPCRs are incredibly diverse in themselves, ranging from lipids, peptides, amino acids, neurotransmitters, hormones, even odorants and light. GPCRs are split

into classes based on their function. Class A or rhodopsin-like GPCRs are the most common class of receptors in humans.

GPCRs are aptly named for their ability to couple to heterotrimeric G proteins, which act as molecular switches in GPCR-mediated signaling cascades. The G protein complex, consisting of a nucleotide-bound Gα subunit and a Gβγ dimer, is directly activated by the receptor it couples to. Once a ligand binds its receptor, a conformational change in the receptor facilitates the Gα protein to exchange the GDP its bound to in the inactive state for GTP. This promotes uncoupling of the Gα in its active, GTP bound state as well as the Gβγ dimer, both of which are free to interact with their downstream targets (Nygaard et al., 2013; Weis & Kobilka 2018).

The heterotrimeric G proteins are functionally divided into a few subtypes based on the Gα subunit, including $G\alpha_{s/olf}$, $G\alpha_{i}$, $G\alpha_{q}$, $G\alpha_{12/13}$. All Gα proteins have a conserved GTPase domain and a helical domain. The determination of these subtypes is based on which effectors they regulate, from adenylyl cyclases to phospholipases to ion channels. $G\alpha_{s}$ proteins in their active, GTP-bound conformation will directly interact with adenylyl cyclase to stimulate production of cyclic adenosine '3,5'-monophosphate (cAMP), a secondary messenger that regulates several cellular processes. $G\alpha_{i}$, on the other hand, counteracts $G\alpha_{s}$ activation by inhibiting adenylyl cyclase. Gβγ dimers also

have a few isoforms and mediate a diverse array of cellular processes, mainly through regulation of ion channels (Syrovatkina et al., 2016; Yang et al., 2021).

The signaling molecule cAMP, produced downstream of $G\alpha_s$ protein activation, was the first secondary messenger to ever be identified. Although cAMP is a highly ubiquitous messenger produced by a diverse array of stimuli, based on its location either within a cell, tissue, or organ, can accomplish several distinct cellular tasks. This is mediated in part by modulating intracellular levels of cAMP. Concentration of cAMP within a cell is regulated by PDEs, which degrade cAMP (Agarwal, Clancy, & Harvey, 2016; Bock et al., 2020; Saucerman, Greenwald, & Polanowska-Grabowska, 2014). One of the downstream targets of cAMP, protein kinase A (PKA), is a holoenzyme consisting of a regulatory dimer and two catalytic subunits. Upon binding the regulatory subunits by cAMP, the catalytic subunits are released, free to phosphorylate their downstream targets. Recent evidence showing a higher ratio of free regulatory subunits to catalytic subunits of PKA within a cell suggest that cAMP could be further sequestered by PKA regulatory subunits. The recent studies of cAMP dynamics has shown that its activity to be on the nanometer scale and its diffusion is quite limited (Saucerman et al., 2014) Agarwal et al., 2016; Zaccolo, Zerio, & Lobo, 2021).

1.3 Compartmentalized signaling of GPCRs

Due to the notion that GPCRs are important for transducing extracellular cues into intracellular signaling cascades, it was thought that these receptors were only functional on the cell surface. In fact, many ligand types that activate GPCRs, such as catecholamines like dopamine, epinephrine, and norepinephrine, are rather hydrophilic and cannot easily diffuse across the plasma membrane. Further, once activated by a ligand, many receptors are phosphorylated by GPCR kinases (GRK) that promote recruitment of arrestin proteins and can block G protein coupling, a process called receptor desensitization. Beta arrestin also promotes activation of the clathrin-mediated endocytic pathway and subsequent removal of GPCRs from the plasma membrane through direct interaction with adaptor protein 2-clathrin complex (Sorkin & Von Zastrow, 2009; Von Zastrow & Sorkin, 2021). Internalized receptors are then either trafficked to lysosomes for degradation or recycled back to the plasma membrane, or re-sensitized. Thus, receptor endocytosis was previously thought to be a mechanism of attenuating the GPCR-mediated signaling cascade (Ariano et al., 1997; Bloch, Bernard, & Dumartin, 2003; Vickery & von Zastrow, 1999).

However, evidence in the last decade has shown that rather than abolishing the signal, a number of GPCRs that internalize contribute to a distinct phase of cell signaling. The earliest evidence of compartmentalized GPCR signaling showed persistent cAMP

signaling following robust internalization of the parathyroid hormone receptor and the thyroid stimulating hormone receptor (Calebiro et al., 2009; Ferrandon et al., 2009), and several other GPCRs have been shown to function at this intracellular compartment. For example, beta 2 adrenergic receptor (B1AR), vasopressin 2 receptor (V2R), and the D1 dopamine receptor (D1DR) have all been shown to signal from endosomes (Kotowski et al., 2011; Lazar et al., 2020; Tsvetanova & von Zastrow, 2014; Feinstein et al., 2013). As these are $G\alpha_s$-coupled receptors, the role of endosomal signaling on cAMP production was measured in these studies. By blocking clathrin-mediated endocytosis, endosomal GPCR signaling was identified as a distinct signaling phase that contributed to cAMP production. Importantly, endosome-localized signaling has been shown to regulate distinct transcriptional responses, indicating its role in signaling cascades is functionally distinct from the plasma membrane pool of receptor. While there are more compartmentalized $G\alpha_s$ -coupled receptors that have been identified, there are a growing number of receptors coupled to $G\alpha_i$ that have been shown to signal at distinct membrane compartments, such as neurokinin receptor, and mu and delta opioid receptor (Jensen et al., 2017; Yarwood et al., 2017; English et al., 2018; Stoeber et al., 2018).

Not only have many GPCRs been shown to signal from endosomes after internalization from the plasma membrane, but recent evidence has shown that some GPCRs exist as

a stable pool at the Golgi Apparatus and can signal from there (Stoeber et al., 2018; Lin et al., 2023; Radoux-Mergault et al., 2023; Zhang et al., 2013). For example, the beta 1 adrenergic receptor (B1AR) has been shown to signal from both the plasma membrane and Golgi membrane. The B1AR is important in regulating heart contraction and relaxation responses by regulating cAMP/PKA modulation in cardiomyocytes. The Golgi pool of B1AR, and subsequent subcellular cAMP/PKA at this distinct location, has been implicated in regulating relaxation rates in cardiomyocytes. Given that dysfunction of the relaxation rate has been implicated in heart failure, identifying Golgi-B1AR as the primary regulator of this response is key to developing more targeted therapies. B1AR at the Golgi has also been identified as the primary mediator of the phospholipase C epsilon (PLCe) signaling pathway, which has been shown to regulate cardiac hypertrophy in cardiomyocytes (Nash et al., 2019). Interestingly, PLC is anchored to the outer nuclear envelope, revealing a role for Golgi-localized signaling molecules in regulation of nuclearly-localized effectors and suggesting the importance of effector proteins being in the local vicinity of signaling cascades.

It was previously thought that cAMP, the signaling molecule downstream of Ga_s-GPCR activation, was readily diffusible from the plasma membrane as it's such a small molecule. However, recent evidence has shown that cAMP is highly compartmentalized and as such is not readily diffusible (Agarwal et al., 2016; Zaccolo, Zerio, & Lobo, 2021).

This is due in part to the fact that PDEs that degrade cAMP are anchored to several membrane compartments by A kinase anchoring proteins (AKAPs). Further, as PKA is also present in several subcellular compartments through interaction with AKAPs, it has been proposed that the regulatory subunit of PKA can act as a cAMP sink in several locations within the cell (Saucerman et al., 2014). The knowledge that cAMP is microdomains are limited to the vicinity of their production further emphasizes the importance of distinct pools of GPCR-$G\alpha_s$ complexes.

1.4 Dopamine and Dopamine receptors

For the first half of the 20th century, dopamine was thought to be nothing more than a precursor to norepinephrine. Once dopamine was identified as a true neurotransmitter that was produced in the brain, it quickly became implicated in the regulation of a wide array of neurological processes ranging from motor function, learning, memory, and reward-mediated behavior. Dysfunction of dopamine neurotransmission was identified in almost all neurological diseases, namely Parkinson's Disease, schizophrenia, and substance abuse disorders (Berke, 2018; Costa & Schoenbaum, 2022; Girault & Greengard, 2004).

L-dihydroxyphenylalanine (L-DOPA), the precursor to dopamine, is synthesized from the amino acid tyrosine via the enzyme tyrosine hydroxylase. Amino acid decarboxylase (AADC) is responsible for converting L-DOPA into dopamine, which is then turned into norepinephrine by dopamine hydroxylase (Nishijima & Tomiyama, 2016; Stansley & Yamamoto, 2013). Dopamine neurotransmission is in part modulated by the enzyme monoamine oxidase (MAO) that degrade dopamine, catechol-O-methyltransferase that inactivate dopamine, and dopamine transporter (DAT) on presynaptic neurons that clear dopamine from synaptic clefts (Mercuri et al., 1997; Sader-Mazbar et al., 2013; Youdim et al., 2006). Dopamine transported intracellularly

by DAT is transferred into vesicles through vesicle transporters (VMAT) to be recycled and released later.

The dopaminergic neurons that produce and release dopamine, along with the neurons that respond to this release, make up the midbrain dopamine system. The substrantia nigra (SN) and ventral tegmental area (VTA) are the source for dopamine signaling that project into three distinct regions of the midbrain: the striatum, nucleus accumbens, and cortex (Berke, 2018; Costa et al., 2022). The pattern of dopamine release, or firing pattern, not only differs between the dopaminergic neurons of the SN and VTA but is very distinctive itself (Dreyer et al., 2016). There are three modes of firing patterns that help to modulate the distinctive rates of dopamine release. They range from a constant, low level of dopamine release called tonic firing, to either short bursts or pauses of dopamine which are thought to be a phasic response. Tonic firing interspersed with phasic dopamine release in the striatum play an important role in behavioral regulation (Dreyer et al., 2010).

Like many hormones and neurotransmitters, dopamine acts through a family of GPCRs to carry out its functions. Dopamine interacts with 5 GPCR types: D1, D2, D3, D4, and D5 dopamine receptors, though D1 and D2 are the most predominant and well-studied receptors in this family. This group is further split into D1-like (D1 and D5) and

D2-like (D2-D4), based on their G protein selectivity. D1-like receptors are Ga_s coupled and contribute to increases in cAMP after activation by dopamine. D2-like receptors counteract D1-like activity as they are coupled to Ga_i proteins that inhibit adenylyl cyclase and block cAMP production. In the striatum, neurons predominantly express either D1 or D2 dopamine receptors (D1DR or D2DR) that mediate the direct and indirect dopaminergic pathways, respectively (Yapo et al., 2017; Pan et al., 2021). The indirect pathway is activated by tonic dopamine release. These lower levels of dopamine preferentially bind to D2DR, which has almost 100-fold higher affinity for dopamine compared to D1DR. Therefore, D1DR requires a much higher concentration, in the micromolar range, of extracellular dopamine in the synaptic cleft to be activated (Berke, 2018; Costa et al., 2022). Thus, D1DR activates the direct pathway through phasic bursts of dopamine (Berke, 2018; Costa et al., 2022; Dreyer et al., 2010).

Dopaminergic signaling is not limited to neural circuits. There exists an independent dopaminergic system in the kidney. L-DOPA is produced in adrenal glands and filtered through the glomerulus, the initial gateway into the kidney (Hansel et al Catecholamines Dopamine, 1996). Once filtered into the nephron, a functional unit of the kidney, L-DOPA diffuses into proximal tubule cells, where it is converted to dopamine by AADC and is transported out to act on dopamine receptors on the luminal membrane. D1DR are predominantly expressed in the proximal convoluted

tubule and regulate sodium excretion. Activation by dopamine causes D1DR to inhibit the apically-localized sodium-hydrogen exchanger 3 (NHE3) and basolateral sodium-potassium ATPase, thus preventing translocation of sodium from the lumen of the nephron to the bloodstream. D1DR regulation of sodium reabsorption helps maintain proper blood pressure in instances of high salt intake (De Donato et al., 2022; Harris & Zhang, 2012; Olivares-Hernández et al., 2021).

1.5 Organic Cation Transporters

Transporters are another family of membrane proteins that connect the extracellular environment to the inside of the cell. Uptake transporters help clear hormones and neurotransmitters from extracellular spaces to regulate several cellular processes such as homeostasis, metabolism, and cell growth. This family of transporters are further divided into uptake 1 and uptake 2 transporters. Uptake 1 transporters are dopamine, norepinephrine, and serotonin transporters (DAT, NET, and SERT) exclusively expressed on dopaminergic, noradrenergic, and serotonergic neurons, respectively (Barrett et al., 2017). These are high-affinity but low-capacity Na+/Cl- dependent monoamine transporters, allowing them to continue transporting neurotransmitters against their electrogenic gradient from extracellular environment despite their high intracellular concentration (Barrett et al., 2017). Their primary role is rapid uptake of neurotransmitters, making them essential for the modulation and maintenance of synapses. Thus, uptake 1 transporters play an essential role in regulating neurotransmission and facilitating crosstalk between neurons (Voutsinos-Porche et al., 2003). As such, these transporters have been a major target of drugs developed to treat psychological and behavioral disorders.

The uptake 2 family are low-affinity, high-capacity transporters. Uptake 2 transporters are made up by the organic cation transporters (OCT) and plasma membrane

transporter (PMAT) (Koepsell et al., 2007). Unlike uptake 1 transporters, they are bidirectional and electrogenic, following a concentration gradient and negative membrane potential on the intracellular side of the plasma membrane. They play an important role in the transport of nutrients, toxins, and hormones in a variety of epithelial tissues that make up the kidney, liver, and intestine. For instance, in the kidney OCT2 is expressed on surface of cells of the proximal convoluted tubule, the first segment of the nephron. OCT2 is responsible for the secretion of amines, cationic drugs, and toxins into the kidney to be excreted as waste (Gomes et al., 1997; Gründemann et al., 1997; Müller et al., 2013).

While they were originally thought to function only in epithelia of non-CNS organs, the past two decades have revealed their importance in regulating brain function. In the brain, OCTs have been thought to be responsible for aiding uptake 1 transporters in clearance of neurotransmitters from the interstitial space between synapses (Gasser et al., 2006; Gasser & Lowry, 2018; Matsui et al., 2016; Wang et al., 2014). They are mainly expressed in postsynaptic neurons and other brain cell types whereas uptake 1 transporters are expressed solely in presynaptic neurons, allowing for dynamic neuromodulation. Their role becomes especially key when neurotransmitter concentrations are high, as well as when uptake 1 transporter function is chemically or genetically impaired (Gasser et al., 2017).

OCTs and PMAT have a distinct expression pattern across different brain areas. OCT2 and OCT3 have been shown to express in areas of the brain important for behavioral regulation (Amphoux et al., 2006; Hayer-Zillgen et al., 2002). They are expressed not only in neurons but in the astrocytes and microglia of these brain regions as well (Gasser et al., 2017). Their role in these cell types has been thought to aid in removal of catecholamines that have diffused out of the synaptic cleft. All the OCTs and PMAT are also responsible for transport of drugs and toxins across the blood brain barrier. OCTs also have been more recently shown to be localized not only to the plasma membrane but intracellular membranes. Specifically, OCT3 has been shown to express on the outer nuclear membrane as well as mitochondrial membrane, suggesting they play a role in the regulation of intracellular concentrations of biogenic amines (Gasser et al., 2017). This provides another example of a transmembrane protein that regulate cellular function from distinct membrane compartments.

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Chapter 2: The organic cation transporter 2 regulates dopamine D1 receptor signaling at the Golgi apparatus

Natasha M. Puri, Gia R. Romano, Quynh N. Mai, Ting-Yu Lin, and Roshanak Irannejad

Adapted from eLife publication: https://doi.org/10.7554/eLife.75468

2.1 Abstract:

Dopamine is a key catecholamine in the brain and the kidney, where it is involved in a number of physiological functions such as locomotion, cognition, emotion, endocrine regulation and renal function. As a membrane impermeant hormone and neurotransmitter, dopamine is thought to signal by binding and activating dopamine receptors, members of the G protein couple receptor (GPCR) family, only on the plasma membrane. Here, using novel nanobody-based biosensors, we demonstrate for the first time that the dopamine D1 receptor (D1DR), the primary mediator of dopaminergic signaling in the brain and kidney, not only functions on the plasma membrane but becomes activated at the Golgi apparatus in the presence of its ligand. We present evidence that activation of the Golgi pool of D1DR is dependent on Organic Cation Transporter 2 (OCT2), a dopamine transporter, providing an explanation for how the membrane impermeant dopamine accesses subcellular pools of D1DR. We further demonstrate that dopamine activates Golgi-D1DR in the striatal medium spiny neurons (MSN) and this activity depends on OCT2 function. We also introduce a new approach to selectively interrogate compartmentalized D1DR signaling by inhibiting Gαs coupling, using a nanobody-based chemical recruitment system. Using this strategy, we show that Golgi-localized D1DRs regulate cAMP production and mediate local protein kinase A activation. Together, our data suggest that spatially compartmentalized signaling hubs are previously unappreciated

regulatory aspects of D1DR signaling. Our data provide further evidence for the role of transporters in regulating subcellular GPCR activity.

2.2 Introduction

Dopamine (DA) is a major hormone and neurotransmitter that regulates a wide range of physiological responses including reward-motivated behavior, aversion, cognition, and motor control in the central nervous system, CNS (Di Chiara & Imperato, 1988; Salery, Trifilieff, Caboche, & Vanhoutte, 2020; Sulzer, 2011). DA also regulates physiological responses in non-CNS tissues such as sodium secretion in the kidney (Missale, Nash, Robinson, Jaber, & Caron, 1998). All known cellular actions of DA are mediated by Dopamine receptors, members of the G protein-coupled receptor (GPCR) superfamily. GPCRs are the largest and most diverse family of membrane receptors in the mammalian genome and are responsible for regulating an enormous variety of cellular responses.

In both the CNS and the kidney, DA is produced locally. There are five subtypes of Dopamine receptors, D1, D2, D3, D4 and D5, that are classified as D1-class receptors (D1 and D5) or D2-class receptors (D2, D3 and D4) (Kebabian, 1978; Spano, Govoni, & Trabucchi, 1978). The D1-class receptors are primarily coupled to Gαs/olf proteins and stimulate the activity of adenylyl cyclase, AC, leading to the production of the second messenger cyclic AMP, cAMP (Beaulieu, Espinoza, & Gainetdinov, 2015). In contrast, the D2 class are associated with Gαi/o proteins and inhibit cAMP production (Beaulieu et al., 2015). D1 Dopamine Receptors (D1DRs) are highly expressed in the CNS where

they underlie major brain functions such as locomotion, learning and memory, attention, impulse control, and sleep (Missale et al., 1998). D1DRs in the kidney regulate trafficking of sodium ATPase and transporters, thereby affecting renal function (Honegger et al., 2006; Wiederkehr et al., 2001).

Impermeable agonists such as DA have long been thought to activate D1DRs only at the plasma membrane. Like many GPCRs, removal of D1DRs from the cell surface by endocytosis has been described as a mechanism that attenuates cellular signaling (Ariano et al., 1997; Bloch, Bernard, & Dumartin, 2003; Vickery & von Zastrow, 1999). As such, efforts at modulating DA signaling as a therapeutic strategy for various pathophysiological conditions have only taken into consideration the consequences of signaling by plasma membrane localized DA receptors(Jin, Goswami, Cai, Zhen, & Friedman, 2003; Panchalingam & Undie, 2001; Undie, Weinstock, Sarau, & Friedman, 1994). However, evidence from the past decade suggests that for some GPCRs endocytosis might in fact activate a second phase of acute or prolonged Gαs-mediated cAMP response from the endosomes (Calebiro & Koszegi, 2019; Calebiro, Nikolaev, & Lohse, 2010; Feinstein et al., 2013; Ferrandon et al., 2009; Irannejad et al., 2013; Irannejad, Tsvetanova, Lobingier, & von Zastrow, 2015; Irannejad & von Zastrow, 2014; Kotowski, Hopf, Seif, Bonci, & von Zastrow, 2011; Lobingier & von Zastrow, 2019; Stoeber et al., 2018; Thomsen, Jensen, Hicks, & Bunnett, 2018). Recent studies further

support this notion by providing evidence that cAMP generation by activated receptors at the endosome are necessary in regulating transcriptional responses that are distinct from those elicited by activation of the plasma membrane receptor pool (Bowman, Shiwarski, & Puthenveedu, 2016; Godbole, Lyga, Lohse, & Calebiro, 2017; Jean-Alphonse et al., 2014; Jensen et al., 2017; Peng, Pessino, Huang, & von Zastrow, 2021; Tsvetanova & von Zastrow, 2014).

Most of the receptors that have been shown to exhibit a second phase of signaling from internal compartments are primarily coupled to Gαs protein. cAMP diffusion is within the nanometer scale around phosphodiesterases at physiological conditions (Agarwal, Clancy, & Harvey, 2016; Bock et al., 2020; Saucerman, Greenwald, & Polanowska-Grabowska, 2014). Given this narrow range of diffusion, it has been difficult to explain how receptor activation solely on the plasma membrane results in the activation of downstream effectors at distant subcellular locations such as the endoplasmic reticulum, Golgi and the nucleus (Agarwal et al., 2016; Richards et al., 2016; Saucerman et al., 2014). As one explanation for this observation, we recently showed that activation of the Golgi-localized beta1 adrenergic receptors (β1AR) cause local production of cAMP by Golgi localized Gαs protein. Importantly, we demonstrated that a catecholamine transporter facilitates the transport of epinephrine, a membrane impermeant endogenous β1AR agonist, to the lumen of the Golgi to

activate the Golgi pool of β1AR (Irannejad et al., 2017). The importance of generation of a local pool of cAMP by Golgi-localized β1AR was further supported by the finding that activated Golgi-β1ARs, but not activated plasma membrane-β1ARs , cause PLCε activation at the perinuclear/Golgi membrane, which mediates hypertrophic responses in cardiomyocytes (Irannejad et al., 2017; Nash, Wei, Irannejad, & Smrcka, 2019).

Whether the need for local cAMP generation is unique to cell types or specific GPCRs is not well understood. The lack of cAMP mobility in cells becomes prominent in larger cells with higher membrane compartmentation that present physical barriers to cAMP diffusion. Considering the high degree of membrane compartmentation of neurons and proximal tubules of the kidney, the two main cell types that express D1DRs, we wondered whether D1DR signaling is also compartmentalized. Here, using a conformational sensitive nanobody that recognizes activated D1DR, we show that the pre-existing pool of D1DR that is localized to the Golgi membrane is activated upon stimulation with extracellular DA. Furthermore, we demonstrate that OCT2 facilitates the transport of DA to the Golgi-localized D1DR and regulates its local activity at the Golgi. We further show that OCT2 has a distinct expression pattern in the kidney and specific regions of the brain including the MSNs, where D1DRs are endogenously expressed. Thus, our findings reveal that DA can activate D1DR signaling at the Golgi

and point to a novel role for OCT2 as a factor that determines which cell types exhibit DA-mediated subcellular signaling.

2.3 Results

Nanobody-based conformational sensitive biosensors detect active D1DR and Gs protein at subcellular membranes

We have previously shown that a single-domain camelid antibody, nanobody 80 (Nb80), originally developed to stabilize an active conformation of beta 2 adrenergic receptor (β2AR) for crystallography purposes (Rasmussen et al., 2011), can be repurposed as a conformational biosensor to detect activated β2AR and β1AR in living cells (Irannejad et al., 2017; Irannejad et al., 2013). Through directed evolution on Nb80, a high-affinity nanobody (Nb6B9) was generated that stabilizes the active conformation of epinephrine bound β2AR (Ring et al., 2013). Given that β2AR/Nb6B9 binding sites are highly conserved among other aminergic receptors such as β1AR and D1DR (Supplementary Figure 1a)(Rasmussen et al., 2011), we reasoned that this nanobody could also be used as a conformational sensitive biosensor to detect activated D1DR in real time and in living cells (Figure 1a). In HeLa cells expressing Snap-tagged D1DR, Nb6B9 fused to GFP (Nb6B9-GFP) was diffuse throughout the cytoplasm (Figure 1b, 0 min). Upon stimulation of these cells with 10μM DA, Nb6B9-GFP was rapidly recruited first to the plasma membrane and shortly after to the Golgi apparatus (Figure 1b, 2 min). Nb6B9-GFP recruitment to the plasma membrane and the Golgi was dose dependent starting at 10nM and 100nM DA stimulations, respectively (Figure 1c and Supplementary Figure 1b). Similar Nb6B9-GFP dose

dependent recruitments were observed upon activation of β1AR at the plasma membrane and the Golgi (Supplementary Figure 1c and d). Importantly, no Nb6B9 recruitment to any membrane was detected when delta opioid GPCRs, which lack sequence homology to Nb6B9 biding sites, were activated (Supplementary Figure 1e), suggesting the specificity of this conformational biosensor. Together these data suggest that the D1DR Golgi pool is activated in response to extracellular DA addition. Similar to HeLa cells, treatment of D1DR-expressing HEK293 cells with 10μM DA resulted in the recruitment of NB6B9-GFP to only the plasma membrane (Figure 1b, lower panel, Figure 1d, 2 min). By contrast, SKF81297, a selective membrane permeant D1DR agonist, activated both the plama membrane and the Golgi receptor pools in both HeLa and HEK293 cells (Supplementary Figure 2). In addition to the Golgi recruitment and consistent with a previous report (Kotowski et al., 2011), Nb6B9 was also found to colocalize with D1DR at the endosomes, at a later time after agonist addition, indicating an active pool of D1DR at endosomes. We further used mini-Gs protein, a more general biosensor for Gs-coupled GPCRs (Wan et al., 2018), to show that the active pool of D1DR at the plasma membrane, endosomes, and the Golgi can also be detected by mini-Gs recruitment to these locations. Consistent with the Nb6B9 findings, this biosensor allowed detection of activated D1DR at the Golgi starting at 10nM DA addition (Supplementary Figure 3a and b).

To investigate whether activated D1DRs couple to G proteins to elicit a G-protein mediated response at the Golgi, we took advantage of another nanobody-based biosensor, Nb37-GFP. We previously used Nb37-GFP to detect transiently active β1AR/Gs and β2AR/Gs complexes at the Golgi and endosomes, respectively (Irannejad et al., 2017; Irannejad et al., 2013). Nb37 was recruited to the plasma membrane and the Golgi upon stimulation with DA, suggesting that the D1DR Golgi pool couples to G protein and activates it (Supplementary Figure 4b and c). Together, these findings suggest a distinct spatiotemporal regulation of D1DR signaling at the plasma membrane and the Golgi membranes.

OCT2 facilitates the transport of Dopamine to the Golgi-localized D1DR

These observations raised the key question of how DA, a hydrophilic/membrane impermeant monoamine, can access the Golgi pool of D1DR. The first clue came from the observation that DA activates Golgi-D1DR in HeLa cells but not HEK293 cells (Figure 1b) whereas SKF81297, a hydrophobic/membrane permeant agonist, activates the Golgi pool of D1DR in both cell types (Supplementary Figure 2). These distinct effects of DA and SKF81297 are not based on their differential potency for activating D1DR, as they have comparative EC50 values in inducing cAMP responses (Supplementary Figure 5a). Moreover, D1DR activation at the Golgi is not dependent on receptor internalization, as inhibiting endocytosis by blocking dynamin did not

block D1DR activation at the Golgi (Supplementary Figure 4a). We previously found that a membrane transporter, Organic Cation Transporters 3 (OCT3), facilitates epinephrine transport resulting in activation of the Golgi-localized β1AR. OCT3 is a member of the solute carrier (SLC) family 22, uptake 2 transporters that are electrogenic and transport catecholamines in a bidirectional manner. Importantly, OCT transporters are localized on the plasma membrane and intracellular compartments including nuclear envelope, thus they can transport catecholamines across the plasma membrane and across internal membrane compartments (Gasser, Hurley, Chan, & Pickel, 2017; Irannejad et al., 2017). Therefore, we hypothesized that another OCT family transporter can similarly function in DA transport to allow for its delivery to the Golgi and for the activation of Golgi-localized D1DR pools.

There are three main OCTs that have largely overlapping distribution but distinct substrates (Nies, Koepsell, Damme, & Schwab, 2011; Roth, Obaidat, & Hagenbuch, 2012; Schomig, Lazar, & Grundemann, 2006; Taubert, Grimberg, Stenzel, & Schomig, 2007). OCT3 facilitates the transport of epinephrine and norepinephrine (Nies et al., 2011). DA has been identified as a key endogenous substrate for another member of the SLC22A family, OCT2 (SLC22A2)(Amphoux et al., 2006; Bednarczyk, Ekins, Wikel, & Wright, 2003; Busch et al., 1998; Grundemann et al., 1998; Schomig et al., 2006; Taubert et al., 2007). Therefore, we asked whether OCT2 has a role in transporting DA

to the Golgi membranes. We found robust OCT2 protein expression in HeLa cells as measured by western blotting, whereas expression in HEK293 cells was significantly lower (Supplementary Figure 5b). In immunostaining experiments, we found OCT2 localization on both the plasma membrane and the Golgi in HeLa cells by using an OCT2 specific antibody. This immunostaining was abrogated in HeLa cells expressing OCT2 specific shRNAs but not those expressing the control, scrambled shRNA (Supplementary Figure 5d). To test the role of OCT2 in DA transport, we first used corticosterone, an inhibitor that has been shown to broadly inhibit OCTs and found that it did not inhibit DA-mediated D1DR activation at the Golgi in HeLa cells (Figure 2b and Supplementary Figure 6a). We then used an OCT2 selective inhibitor (10μM and 100μM imipramine) and found that DA-mediated D1DR activation at the Golgi is inhibited in HeLa cells. By contrast, SKF81297, a membrane permeant D1DR agonist that can diffuse across membranes and does not require facilitated transport, could still access and activate Golgi-D1DR in imipramine-treated cells (Figure 2a-c). Next, we overexpressed OCT2-mApple in HEK293 cells and used Nb6B9-GFP to assess D1DR activation in live cells. By expressing OCT2-mApple in HEK293 cells, we found that Nb6B9-GFP could now be recruited to activated D1DR at the Golgi membranes. (Figure 1c and Supplementary Figure 5c). To further confirm the role of OCT2 in DA transport, we used two different OCT2 shRNAs to genetically decreases OCT2 expression (Figure 2e). We found that DA-mediated, but not that of SKF81297, D1DR

activation at the Golgi was inhibited in HeLa cells expressing OCT2 shRNA (Figure 2d-g). Together, these results suggest that OCT2 facilitates the transport of DA to the Golgi lumen where it then activates D1DR at the Golgi membranes.

Golgi and plasma membrane-localized D1DR both contribute to the cAMP response

Our data suggested that the plasma membrane and Golgi pools of D1DR both couple to the Gs protein. In addition to its presence at the plasma membrane, adenylyl cyclase has been reported to localize at the Golgi/perinuclear membranes **(Boivin et al., 2006; Cancino et al., 2014)**. We therefore asked whether D1DR/Gs complexes at both the plasma membrane and the Golgi activate Gs-mediated cAMP responses. To address this question, we utilized a rapamycin dimerization system composed of FK506-binding protein (FKBP) and FKBP-rapamycin binding domain of FRAP (FRB), to rapidly induce recruitment of Nb6B9 to specific membrane compartments. This makes it possible to specifically block D1DR/Gs coupling at each distinctly localized pool. We have previously shown that βARs nanobody, Nb80, which binds to the same region as G protein (Chung et al., 2011; Rasmussen et al., 2011), blocks either the plasma membrane or the Golgi-β1AR mediated cAMP responses when it is recruited locally to these compartments at high concentrations(Irannejad et al., 2017). This inhibition is likely due to steric occlusion of the Gαs protein. Using HEK293 cells expressing either FKBP at the plasma membrane or the Golgi with FRB fused to Nb6B9 (FRB-Nb6B9), we

demonstrated that treatment with rapalog, a rapamycin analog, specifically targets Nb6B9 to either membrane (Figure 4 a-c). Upon stimulation with membrane permeant agonist SKF81297, Nb6B9 targeted to the plasma membrane disrupts plasma membrane-D1DR/G proteins coupling, while Golgi-D1DR is still able to elicit a cAMP response (Figure 4d). In turn, treatment with rapalog in cells expressing Golgi-targeted FKBP and FRB-Nb6B9 and subsequent stimulation with SKF81297 resulted in inhibition of the Golgi-D1DR pool (Figure 4e). Importantly, rapalog treatment alone had no effect on the overall cAMP production elicited by Forskolin, a direct activator of adenylyl cyclase (Figure 4f). These data indicate that Golgi-localized D1DR is able to promote cAMP response.

Dopamine uncaging triggers rapid activation of Golgi localized-D1DR and local PKA. To further investigate the role of Golgi-localized D1DR in activating PKA locally, we utilized a photo-sensitive caged dopamine that becomes uncaged upon blue light exposure (Figure 6a). Unlike DA, caged-DA is hydrophobic and thus membrane permeant (Castro et al., 2013; Yapo et al., 2017). To ensure that caged-DA accumulates inside the cell and reaches the Golgi-localized D1DR, we incubated HEK293T PKAcat-GFP knock in cells with 1 μM caged-DA for 10 min in a dark incubator. Addition of caged-DA to HEK293T PKAcat-GFP cells did not activate D1DR, as indicated by cytoplasmic localization of Nb6B9-mApple, confirming that DA is

inactive in its caged form (Figure 6b top panel). Upon stimulation of cells with blue light for 10 seconds, we observed D1DR activation at the Golgi, as detected by rapid Nb6B9-mApple recruitment to the Golgi membranes within seconds after blue light exposure (Figure 6b and c). This was then followed by PKAcat-GFP dissociation from the perinuclear/Golgi regions, as a result of cAMP production and PKA activation (Figure 6b and c). These data further support the notion that Golgi-localized D1DR activates PKA locally.

2.4 Discussion

Our findings demonstrate for the first time that dopaminergic receptors can signal from the Golgi apparatus. We present evidence that dopamine, a hydrophilic catecholamine, can be transported to the Golgi membrane to reach the pre-existing Golgi pool of D1DRs. This transport is facilitated by OCT2. The Golgi-D1DR comprises a functional signaling pool as it can activate Gαs and stimulate cAMP production. Moreover, we introduced a new approach to selectively interrogate compartmentalized D1DR signaling by inhibiting Gαs coupling, using a nanobody-based chemical recruitment system. Finally, utilizing caged-dopamine, we showed that photo-release of dopamine at the Golgi upon rapid blue light exposure triggers D1DR-mediated cAMP production and local PKA activation.

As the signaling activities of D1DR have thus far been thought to be limited to the plasma membrane, substantial efforts have been focused on designing small molecules agonists of D1DR to bias signaling towards a particular signaling pathway, without the consideration of spatial D1DR signaling **(Jin et al., 2003; Kuroiwa et al., 2008; Panchalingam & Undie, 2001; Undie et al., 1994)**. Our findings on D1DR signaling from the Golgi membrane suggest location-bias as an overlooked aspect of signaling specificity. The present study demonstrates the important role of local generation of cAMP by GPCRs in controlling local PKA activation at specific subcellular

compartments. It is well established that cAMP mediated signaling specificity depends on the function of compartment specific phosphodiesterases, enzymes that degrade cAMP, limiting the diffusion of this second messenger (Agarwal et al., 2011; Buxton & Brunton, 1983; Gold, Gonen, & Scott, 2013; Musheshe, Schmidt, & Zaccolo, 2018; Steinberg & Brunton, 2001; Warrier et al., 2007). Recent measurements of cAMP mobility suggest a nanometer scale diffusion domain (Bock et al., 2020; Saucerman et al., 2014). The model where cAMP generation by plasma membrane localized receptors propagates in a linear fashion to then control intracellular effectors of cAMP is inconsistent with the nanometer scale of cAMP diffusion range within the cell (Agarwal et al., 2016; Saucerman et al., 2014; Zaccolo, Zerio, & Lobo, 2021). Thus, our data further provide evidence that PKA activation at a specific compartment requires GPCR activation locally in the vicinity of that compartment. Given that each subcellular membrane compartment has a distinct lipid environment (Balla, 2013), it is likely that PKA activation at each location will recruit a unique set of effectors and proteins and regulates distinct signaling and physiological outcomes. The importance of local generation of cAMP by Golgi-localized GPCR has been demonstrated for β1AR. Nash et al have demonstrated that activated Golgi-β1ARs only, but not the plasma membrane pool, lead to PLCε activation at the perinuclear/Golgi membrane, which mediates hypertrophic responses in cardiomyocytes (Irannejad et al., 2017; Nash et al.,

2019). Whether distinct signaling pathways are regulated by plasma membrane or Golgi-localized D1DR is not yet clear but strongly suggested by our findings. Published studies suggest that OCT2 is expressed in a number of tissues and cell types that also express D1DRs (Arnsten et al., 1995; Busch et al., 1998; Double & Crocker, 1995). Interestingly, however, there are D1DR expressing cell types that do not express OCT2. For instance, we found that within the brain, OCT2 is highly expressed in the striatum and moderately in the cortex. In contrast, OCT2 has little to no expression in the hippocampus or substantia nigra (Supplementary Figure 7a) (Amphoux et al., 2006; Busch et al., 1998). Therefore, we speculate that the expression pattern of OCT2 may be a determinant of which cell types are able to activate their internal pool of D1DR.

There are two major DA uptake transport mechanisms: i) uptake 1 transporters that have high affinity for DA and are mostly localized in presynaptic neurons, and ii) uptake 2 transporters that have low affinity but high capacity for DA and are expressed in various brain regions as well as different organs in the body (Grundemann et al., 1998; Lin et al., 2011; Nies et al., 2011; Reith, Zhen, & Chen, 2006; Torres, Gainetdinov, & Caron, 2003). OCT2 belongs to the uptake 2 transporter family and has been previously thought to mainly function as an uptake transporter, helping with the clearance of extracellular DA and terminating DA-mediated signaling pathways

(Amphoux et al., 2006; Bednarczyk et al., 2003; Busch et al., 1998; Taubert et al., 2007). Unlike uptake 1 transporters, uptake 2 transporters can transport catecholamines, including DA, across the membrane, in a bidirectional and electrogenic manner, and independent of Na^+ and Cl^- transport (Nies et al., 2011; Schomig et al., 2006). Previous reports have demonstrated that OCTs, in particular OCT3, are localized on both the plasma membrane and subcellular membranes including the outer nuclear membranes near the Golgi (Gasser, 2021; Gasser et al., 2017). We showed that OCT2 is expressed on both the plasma membrane and the Golgi membranes in HeLa cells (Supplementary Figure 5d). As OCT2 is a member of an electrogenic and bidirectional transporter, we speculate that the plasma membrane OCT2 facilitates the transport of DA from the extracellular environment to the cytoplasm and the intracellular-localized OCT2 might facilitate DA transport into the Golgi. Given that the resting membrane potential of inner nuclear membrane (~ -100mV) (Burdakov, Petersen, & Verkhratsky, 2005; Matamala, Castillo, Vivar, Rojas, & Brauchi, 2021; Sanchez et al., 2018) has been reported to be more negative relative to that of the cytoplasmic side of the plasma membrane (~ -40 to -70 mV), it is plausible that, just as the transport of DA from the extracellular space into the cytoplasm by OCT2 takes advantage of the electrogenic gradient, a similar gradient allows for transport of DA from the cytoplasm across the nuclear envelope which is connected to the lumen of the Golgi membrane.

With DA as the substrate, Km measurements ranging from 2 to 46 μM have been reported for OCT2 transporters (Amphoux et al., 2006; Gasser, 2021; Grundemann et al., 1998; Schomig et al., 2006). Thus, as a low affinity but high-capacity transporter, subcellular OCT2 is likely to encounter high concentrations of cytoplasmic DA under physiological conditions (Wiencke, Horstmann, Mathar, Villringer, & Neumann, 2020). Based on the calculated rate constant for OCT2 *in vivo* and the known water space of average cells, cytoplasmic concentrations of DA at equilibrium are thought to be ~10 fold higher compared to the extracellular concentrations (Grundemann et al., 1998). For instance, addition of 100nM DA in the extracellular environment of OCT2 expressing cells results in the accumulation of 1μM cytoplasmic DA over 10 minutes (Grundemann et al., 1998). We found that the requirement for OCT2 in activating Golgi-localized D1DRs is seen even at low concentrations of exogenously added DA (10nM) (Supplementary Figure 3). Notably, knock down or inhibition of OCT2 abrogated Golgi-localized D1DR signaling (Figure 2 and 3), highlighting the specificity of OCT2 in this signaling regulation.

The present results expand the concept of GPCR compartmentalized signaling and open additional interesting questions for further studies regarding mechanisms that regulate subcellular activity of other monoamine receptors such as 5-HT (serotonin) and histamine receptors by other monoamine transporters (Lin et al., 2011; Torres et al.,

2003). Establishing GPCR signaling from subcellular compartments is the first step in unraveling the physiological consequences of compartmentalized signaling for each GPCR family member.

2.5 Figures

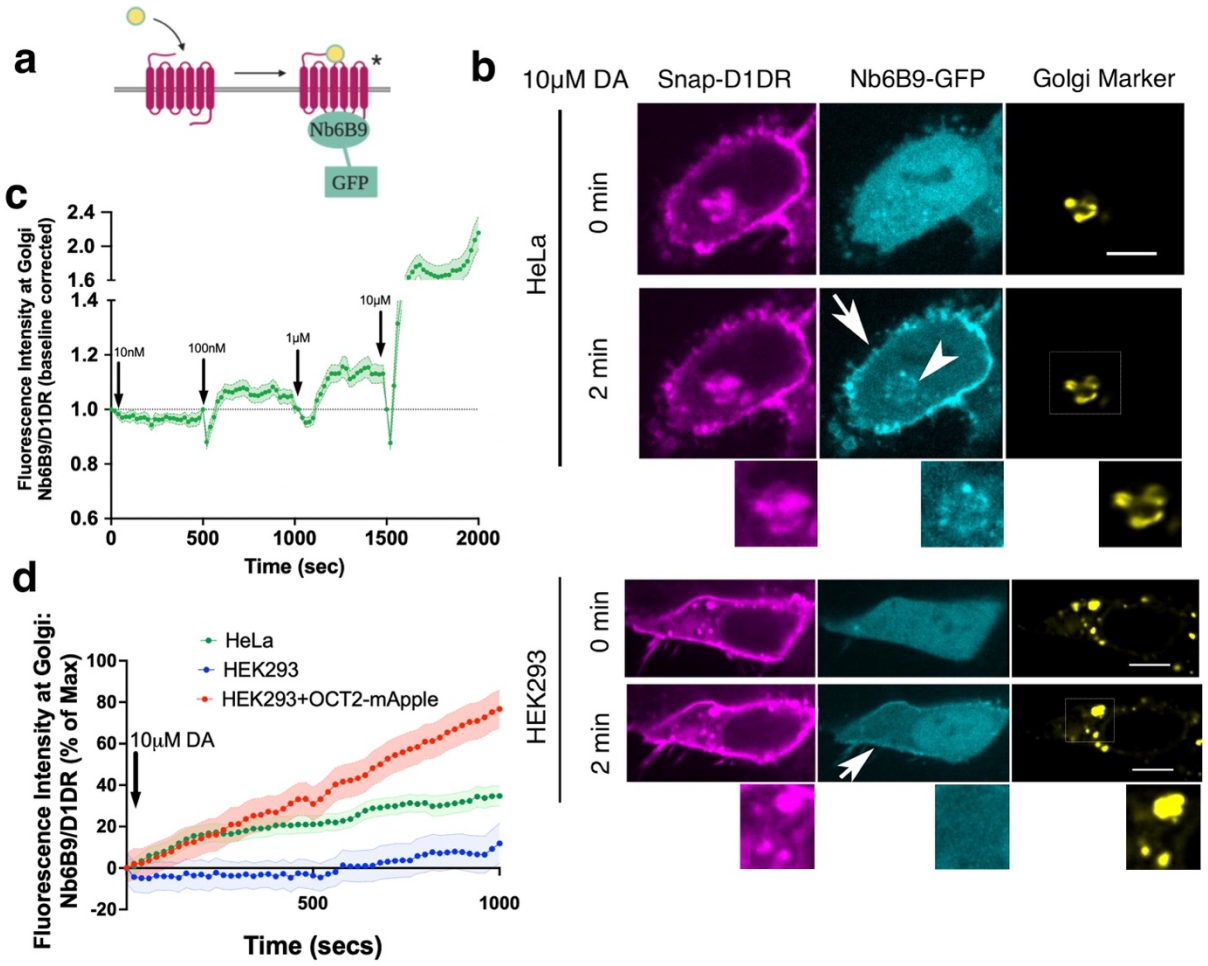
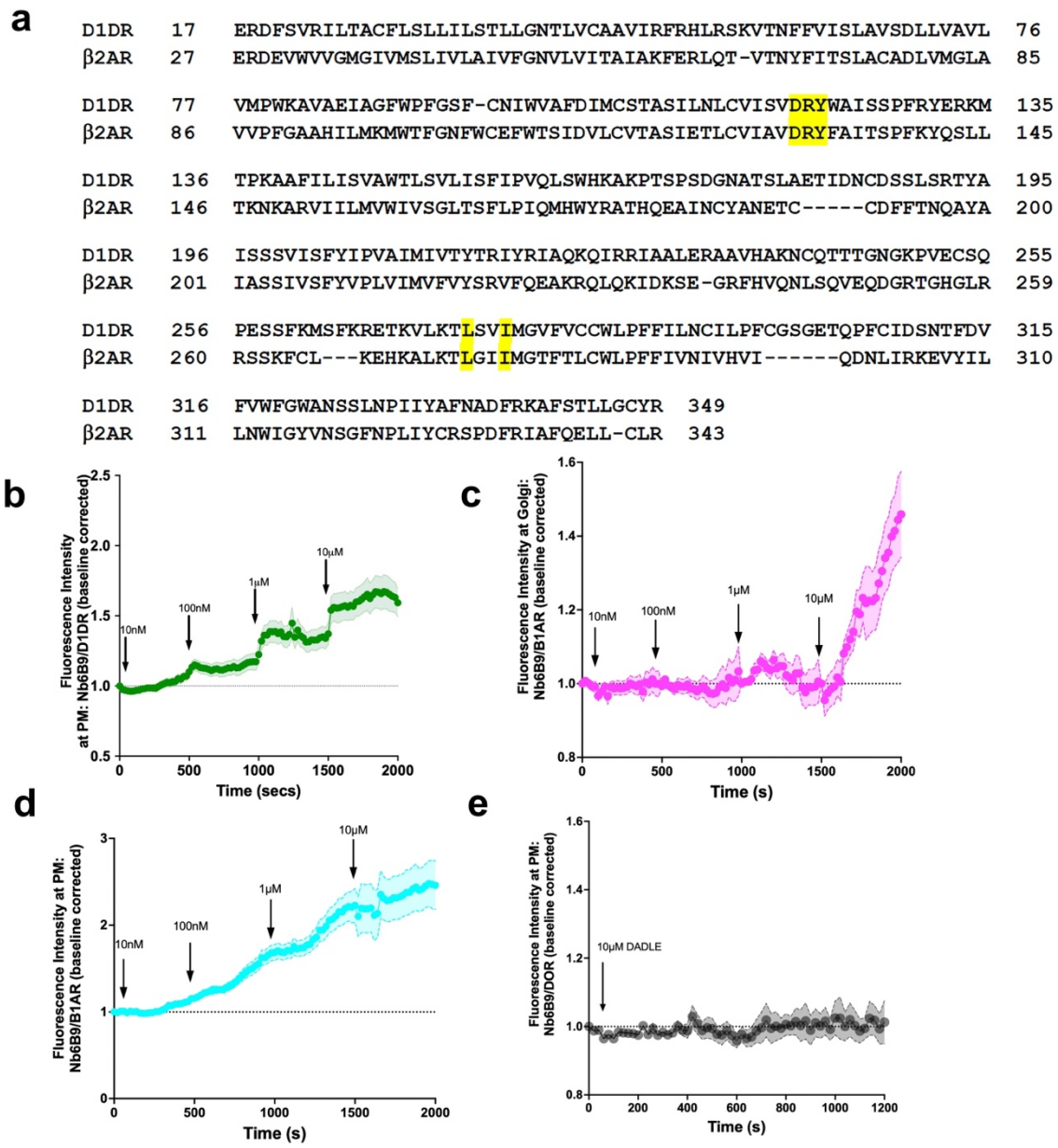
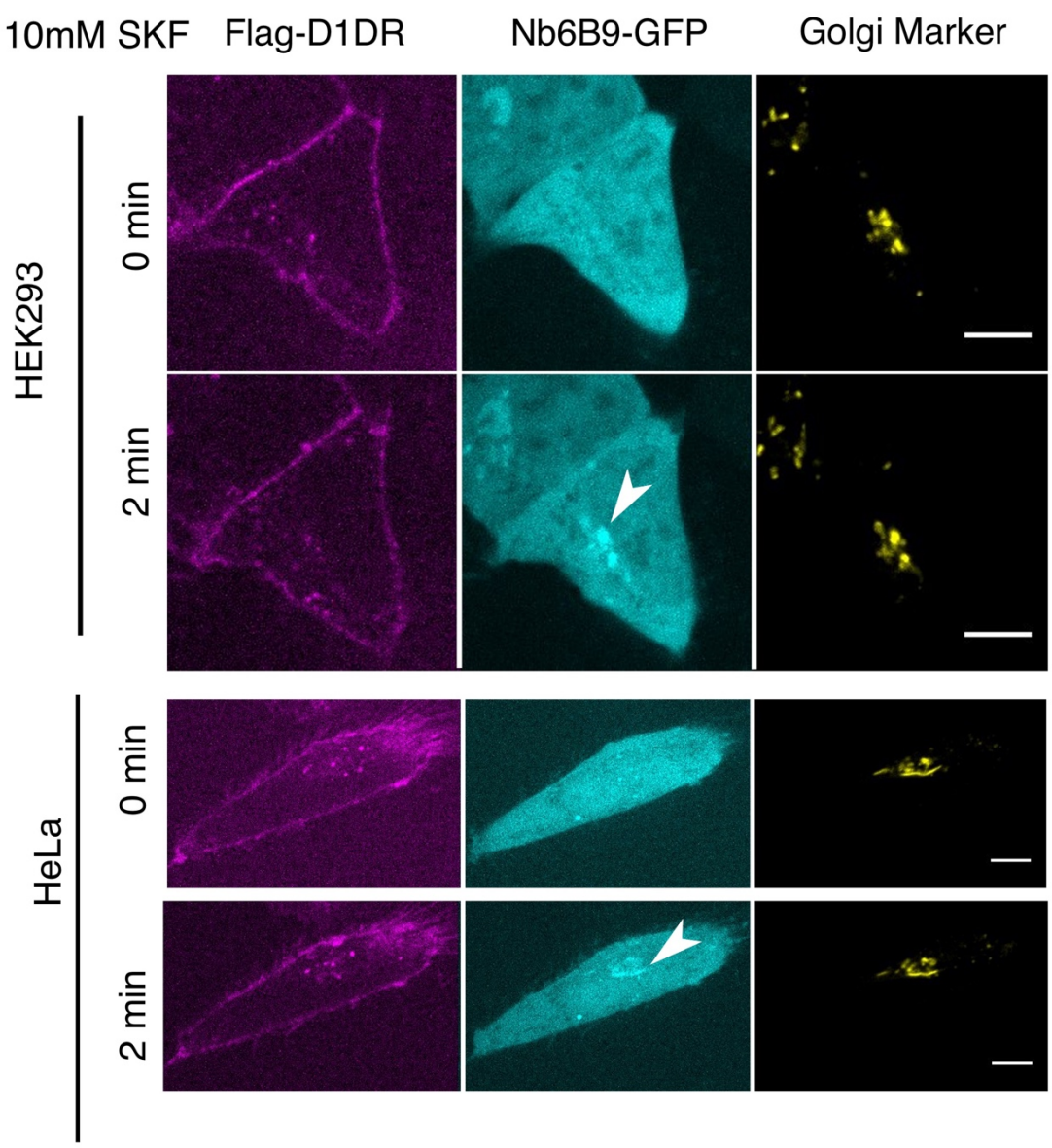


Figure 2.1 Conformational biosensor of D1DR activation shows signaling at plasma membrane and Golgi. a. Nanobody6B9 (Nb6B9) binds to the receptor exclusively in its active conformation. We have tagged Nb6B9 to fluorophore GFP to act as a conformational biosensor for D1 Dopamine receptor (D1DR) activity. **b.** Confocal images of representative D1DR-expressing HeLa and HEK293 cells with Nb6B9-GFP and GalT-mRFP expression before and after 10μM dopamine addition. Stimulation with 10uM dopamine results in recruitment of Nb6B9 to active D1DR at plasma membrane and Golgi in HeLa (*n* = 15 cells, Pearson's coefficient = 0.63, 5 experiments); 10μM DA treatment only activates plasma membrane localized D1DR in HEK 293 cells (*n* = 17 cells, Pearson's coefficient = 0.15, 5 experiments). Arrow indicates active D1DR at plasma membrane; Arrowhead indicates active D1DR at Golgi membrane; Scale bar = 10μm. **c.** Quantification of D1DR activity at Golgi in HeLa and HEK293 cells; fluorescence intensity of Nb6B9 at Golgi relative to D1DR at Golgi from confocal imaging of live cells labeled Snap-tagged D1DR and expressing Nb6B9-GFP and GalT-mRFP.

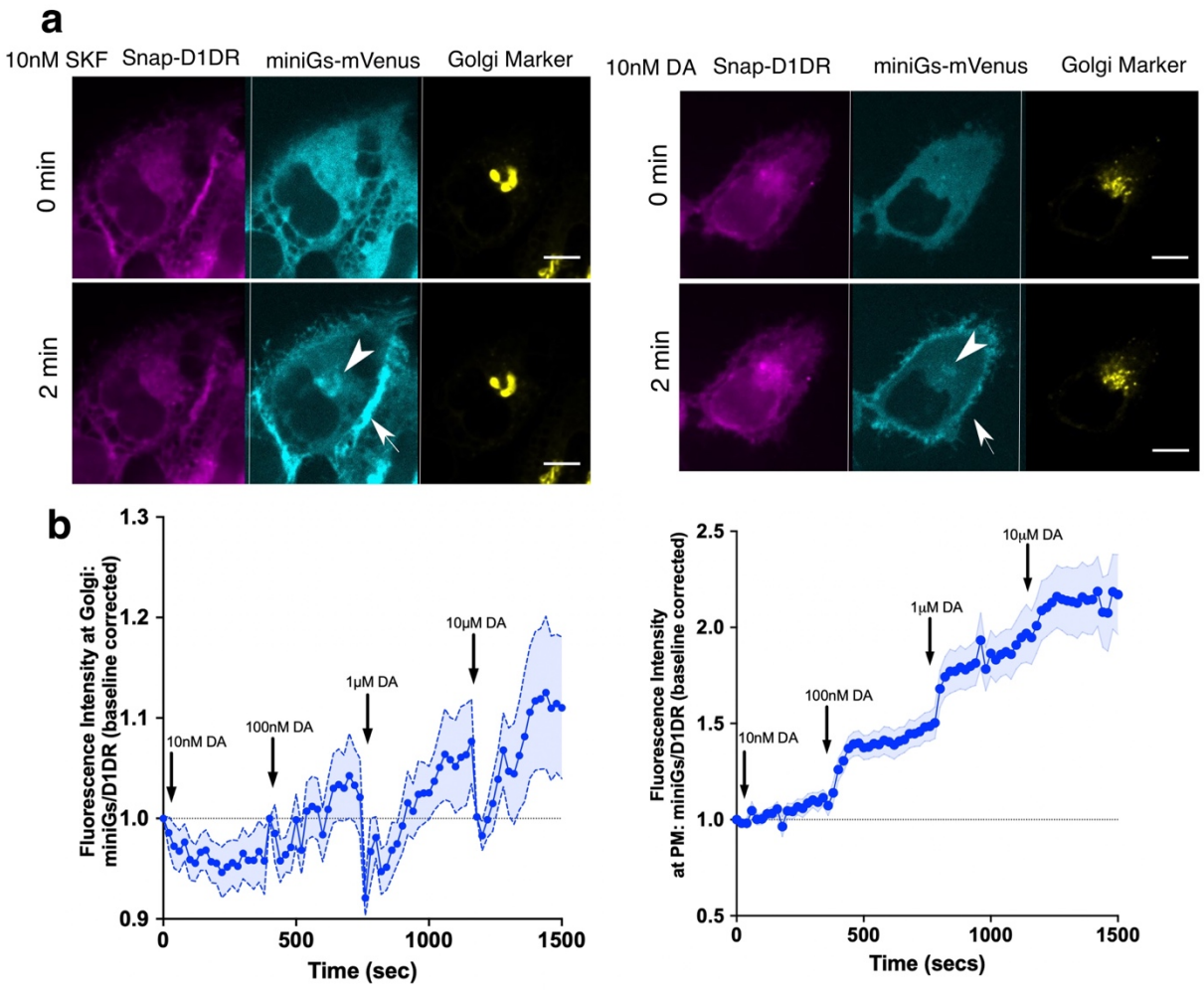


Supplementary Figure 2.1 legend on page 55

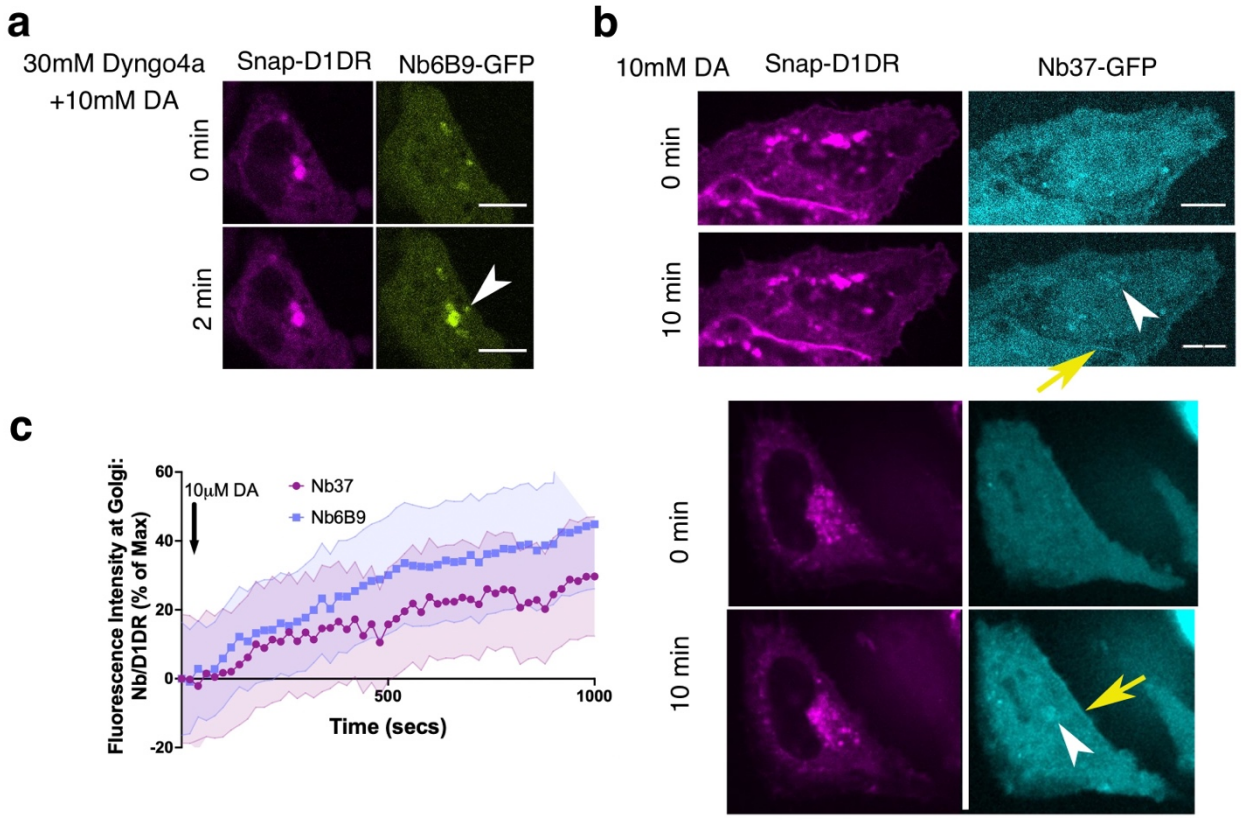
Supplementary Figure 2.1 a. Motifs of nanobody binding are conserved between beta2 adrenergic receptor (B2AR) and D1 dopamine receptor (D1DR). Nanobodies developed for B2AR can therefore bind D1DR in the active conformation. b. Quantification of D1DR activation at the PM in HeLa cells upon addition of increasing DA concentrations; Normalized fluorescence intensity of Nb6B9 at plasma membrane relative to Snap-tagged-D1DR at plasma membrane (*n*=26, 3 biological replicates). c. Quantification of β1AR activation at the Golgi in HeLa cells upon addition of increasing epinephrine concentrations; Normalized fluorescence intensity of Nb6B9 at Golgi relative to Snap-tagged- β1AR at Golgi (*n*=27, 3 biological replicates). d. Quantification of β1AR activation at the plasma membrane in HeLa cells upon addition of increasing epinephrine concentrations; Normalized fluorescence intensity of Nb6B9 at plasma membrane relative to Snap-tagged- β1AR at plasma membrane (*n*=33, 3 biological replicates). Quantifications were baseline corrected after addition of each dose. e. Quantification of Delta Opioid receptor (DOR) activation at the PM in HeLa cells upon addition of 10μM DADALE. Normalized fluorescence intensity of Nb6B9 at plasma membrane relative to GFP-DOR at the plasma membrane (*n*=14, 2 biological replicates).



Supplementary Figure 2.2 Representative D1DR-expressing HEK293 and HeLa cells with Nb6B9-GFP and GalT-mRFP localization at indicated times after 10uM SKF-81297 addition. Stimulation with 10uM SKF-81297 results in recruitment of Nb6B9 to active D1DR at plasma membrane and Golgi in both HEK 293 and HeLa cells.



Supplementary Figure 2.3 a. Representative D1DR-expressing HeLa cell with miniGs-mVenus and GalT-mRFP localizations at indicated times after SKF-81297 addition. The miniGs-construct is the GTPase domain only of the protein Gs, and was engineered as an alternative to using the nanobody. **b.** Quantification of D1DR activation at the Golgi in HeLa cells upon addition of increasing DA concentrations; Normalized fluorescence intensity of miniGs-mVenus relative to Snap-tagged-D1DR at Golgi (left) or the plasma membrane (right). Quantifications were baseline corrected after addition of each dose (n=30, 3 biological replicates).



Supplementary Figure 2.4 a. Representative HeLa cell expressing D1DR and Nb6B9GFP. Cells were incubated with 30μM Dyngo, a dynamin inhibitor that blocks endocytosis, then imaged and stimulated with dopamine at indicated times. Golgi-localized D1DR is still activated when endocytosis is blocked, suggesting this pool is distinct from plasma membrane-localized D1DR. **b.** Representative HeLa cell expressing D1DR and Nb37-GFP. Nb37 binds to the GPCR-Gs protein complex in the nucleotide free state, before the Gs protein binds GTP and dissociates in its active form. **c.** Quantification of Nb37 and Nb6B9 intensity at Golgi, normalized to Golgi-D1DR. Both nanobodies show similar kinetics of Golgi-localized D1DR activation after addition of dopamine.

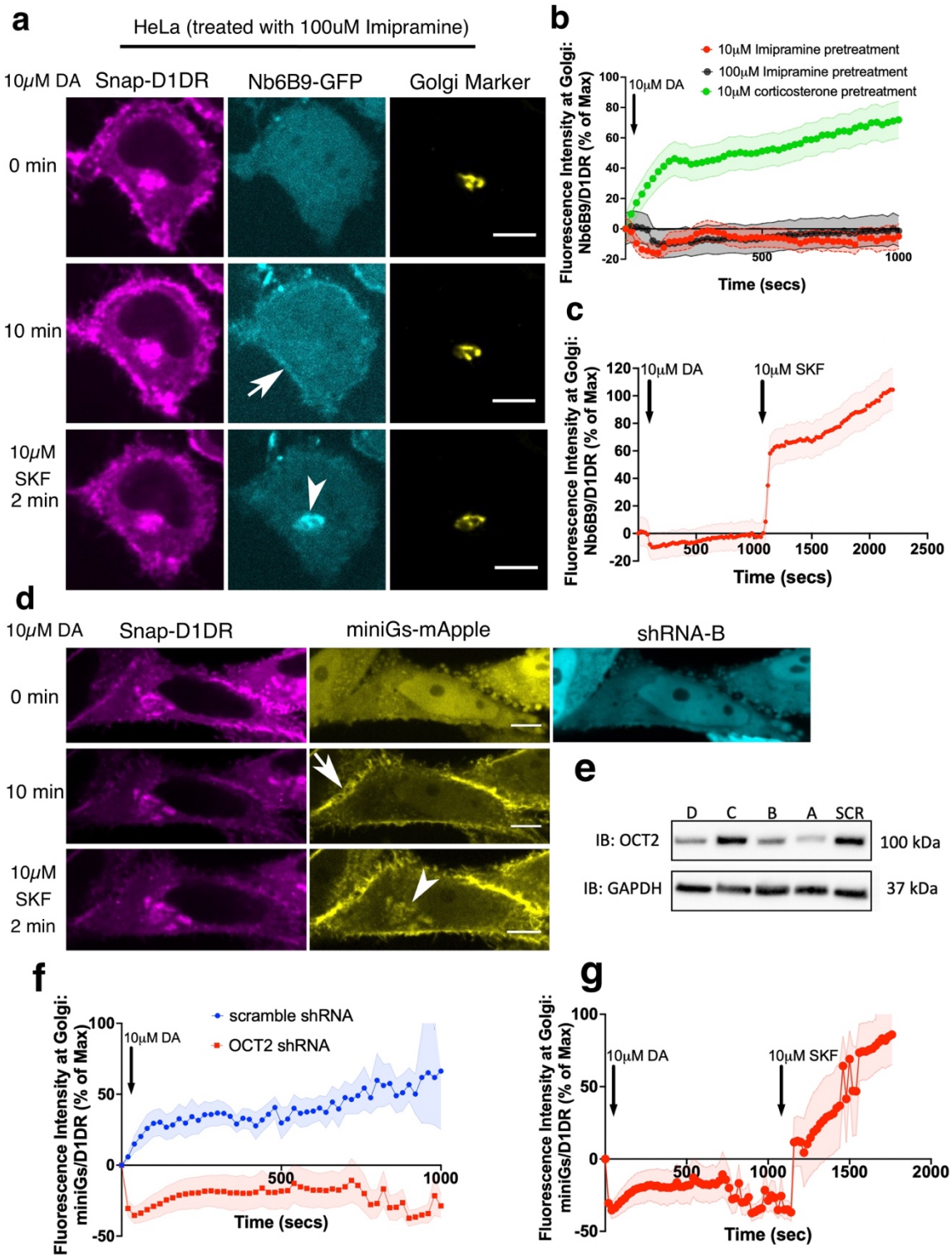


Figure 2.2 legend on page 60

Figure 2.2 OCT2 facilitated DA transport to the Golgi-Localized D1DR. a. Representative HeLa cell expressing Snap-D1DR, Nb6B9-GFP, and GalT-mRFP expression pretreated with 100μM imipramine for 15 min, before and after 10μM dopamine addition. Inhibition of OCT2 blocks Golgi-localized D1DR activation but SKF81297 can still reach the Golgi membranes and activate D1DR Golgi pool (*n* = 30 cells, Pearson's coefficient = 0.2 and 0.68 after DA and SKF addition respectively, 3 biological replicates). Arrow indicates active D1DR at plasma membrane; Arrowhead indicates active D1DR at Golgi membrane; Scale bar = 10μm. **b.** Quantification of Nb6B9-GFP recruitment at Golgi upon 10μM DA stimulation in HeLa cells pretreated with 10μM and 100μM imipramine, 10μM corticosterone and **c**. after 10μM SKF81297 addition; Normalized fluorescence intensity of Nb6B9-GFP relative to Snap D1DR at Golgi (*n*= 30, 3 biological replicates). **d.** Representative HeLa cell expressing Snap-D1DR, miniGs-mApple and OCT2 shRNA-B-GFP, before and after 10μM dopamine addition. OCT2 shRNA blocks Golgi-localized D1DR activation. 10μM SKF81297 addition activates D1DR at the Golgi (*n* = 16 cells, Pearson's coefficient = -0.05 and 0.74 after DA and SKF addition respectively, 3 biological replicates). **e.** Detection of OCT2 expression in HeLa cells expressing different shRNAs by Western Blot. **f.** Quantification of D1DR activation at the Golgi in HeLa cells expressing scramble or OCT2 shRNAs upon addition of 10μM DA; Normalized fluorescence intensity of Nb6B9 at Golgi relative to Snap-tagged-D1DR at Golgi. **g.** Quantification of D1DR activation at the Golgi in HeLa cells expressing OCT2 shRNA-B and D upon addition of 10μM SKF81297.

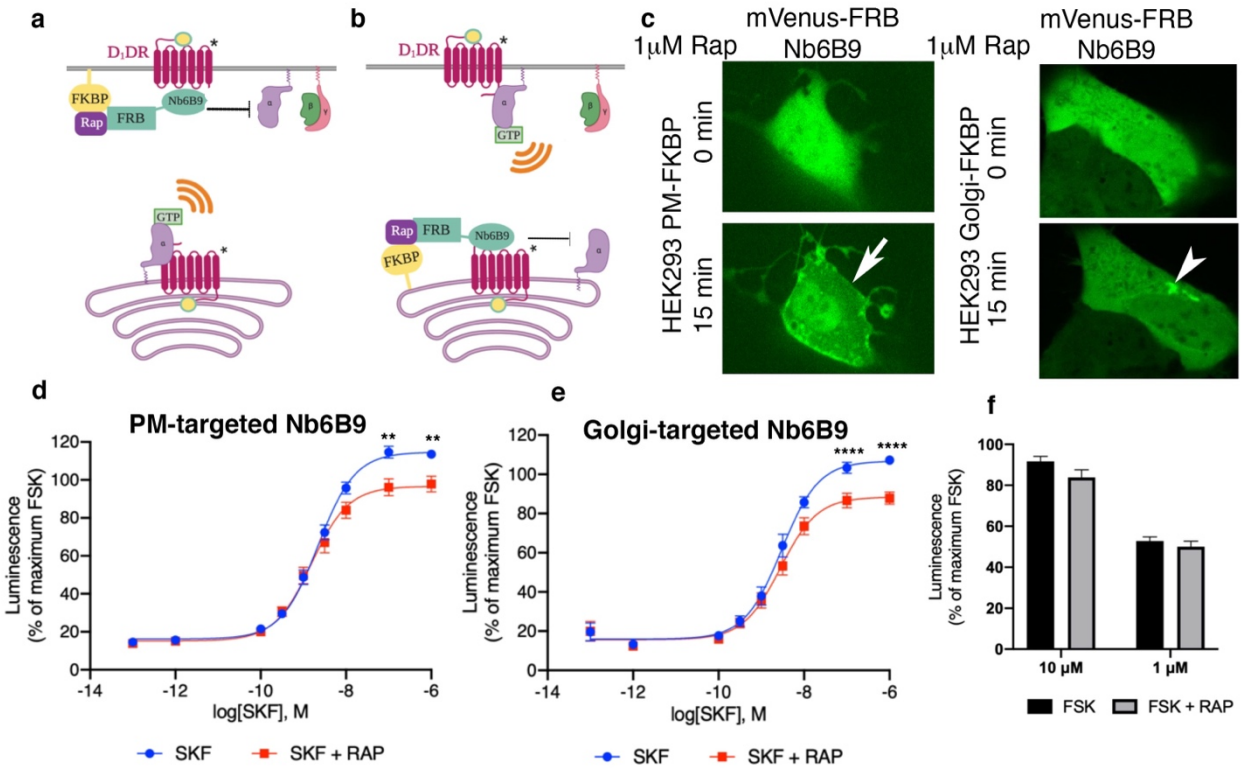


Figure 2.3 Both plasma membrane and Golgi-localized D1DR promote cAMP production. Model of blocking D1DR-Gs coupling at the plasma membrane (**a**) and Golgi membrane (**b**) after recruitment of mVenus-FRB-Nb6B9. FKBP was targeted to either the plasma membrane (**a**) or Golgi membrane (**b**), and its binding partner FRB-mVenus was fused to Nb6B9. Upon addition of rapalog (rapamycin analog), FKBP and FRB heterodimerize and sequester Nb6B9 to either membrane, disrupting G protein coupling to the receptor and thus blocking signaling from each respective location. **c.** Representative confocal images of HEK293 cells expressing either plasma membrane (PM) or Golgi targeted FKBP showing mVenus-FRB Nb6B9 localization at indicated times after rapalog addition. Representative cells confirm inducible sequestration of Nb6B9 to either PM or Golgi. Arrow indicates PM; Arrowhead indicates Golgi. **d.** Forskolin-normalized D1DR-mediated cAMP response with and without rapalog pretreatment (1μM, 15 min) and SKF81297 at indicated concentrations in HEK293 expressing PM-FKBP (mean ± s.e.m., *n* = 6 biological replicates, P values of 0.0021 and 0.0015 at 10^{-7} and 10^{-6}, respectively) **e.** Forskolin-normalized D1DR-mediated cAMP response with and without rapalog pretreatment (1μM, 15 min) and SKF81297 at indicated concentrations in HEK293 expressing Golgi-FKBP (mean ± s.e.m., *n* = 6 biological replicates, P values of <0.0001 at 10^{-7} and 10^{-6}). **f.** Effect of 1μM and 10μM rapalog on forskolin-mediated cAMP response (*n* = 3 biological replicates)

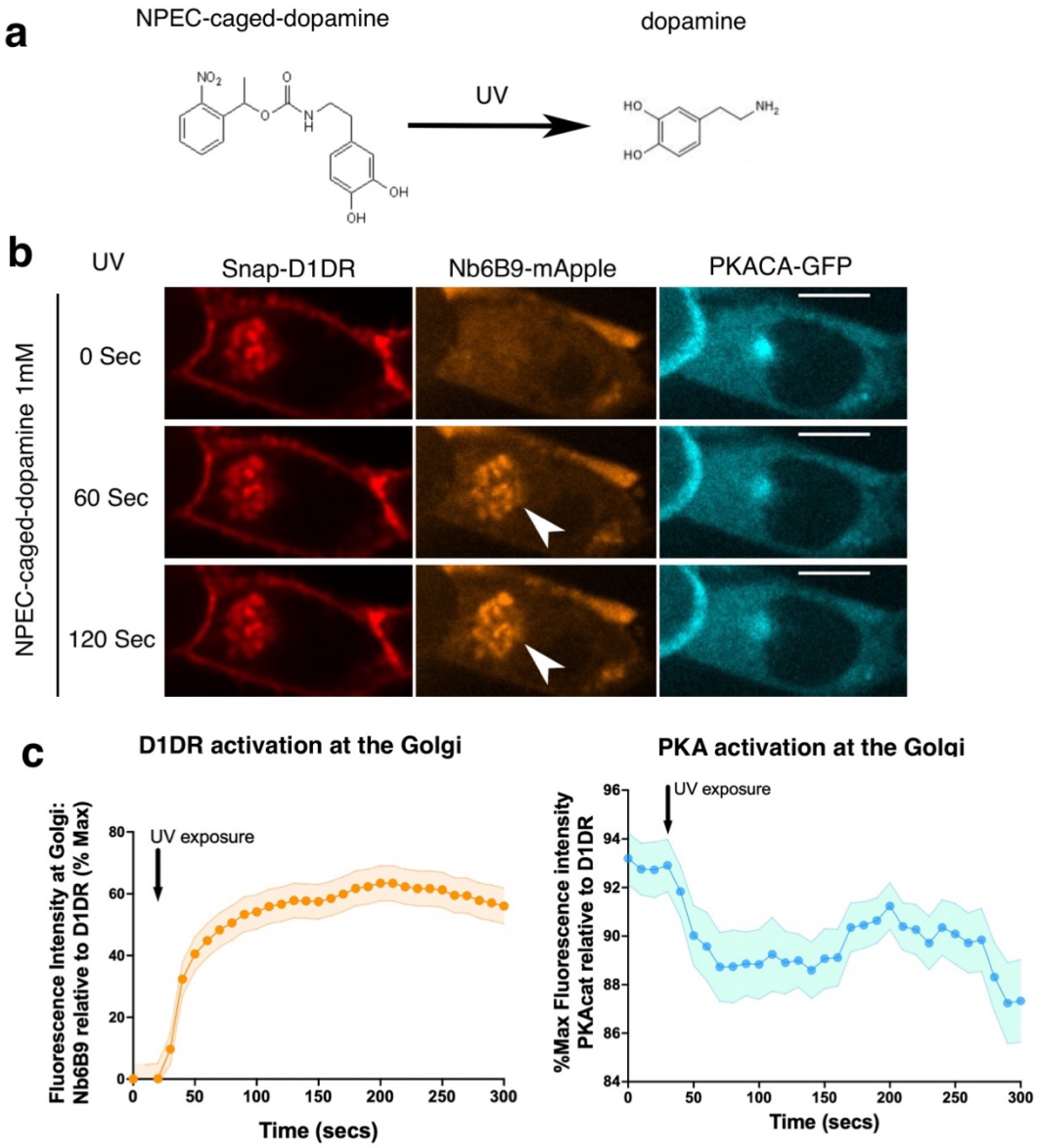
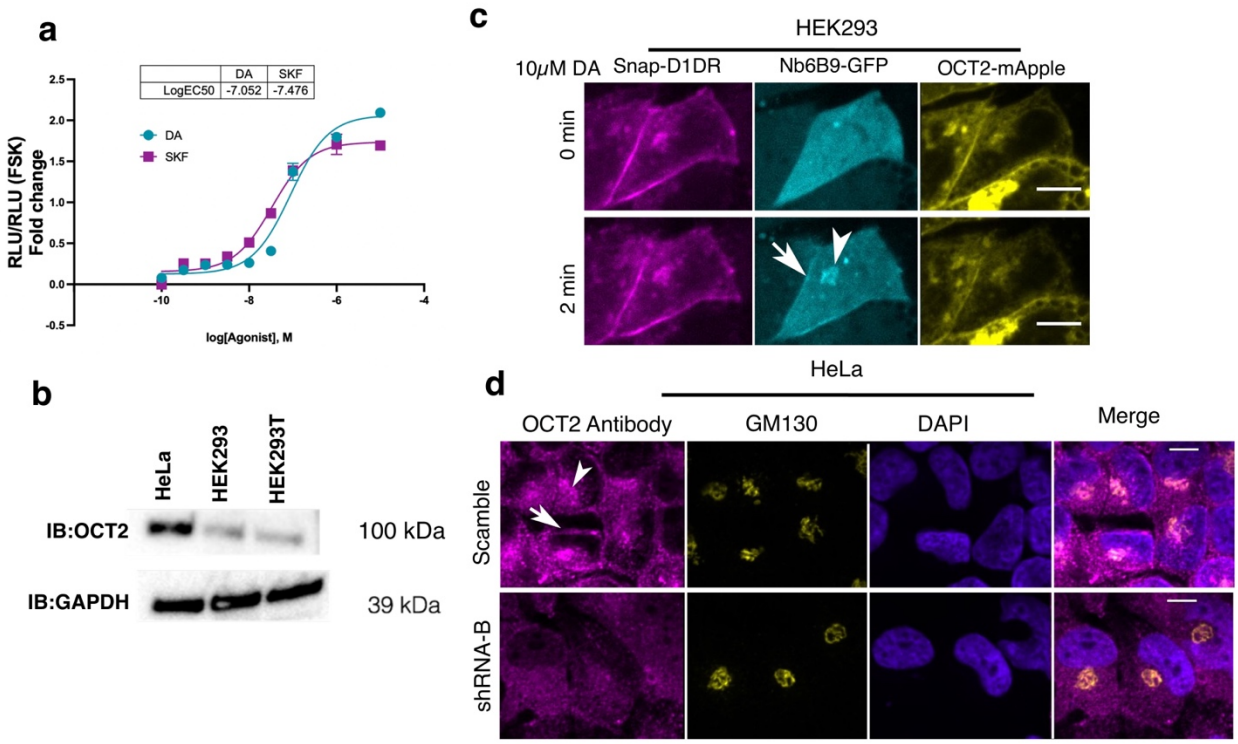
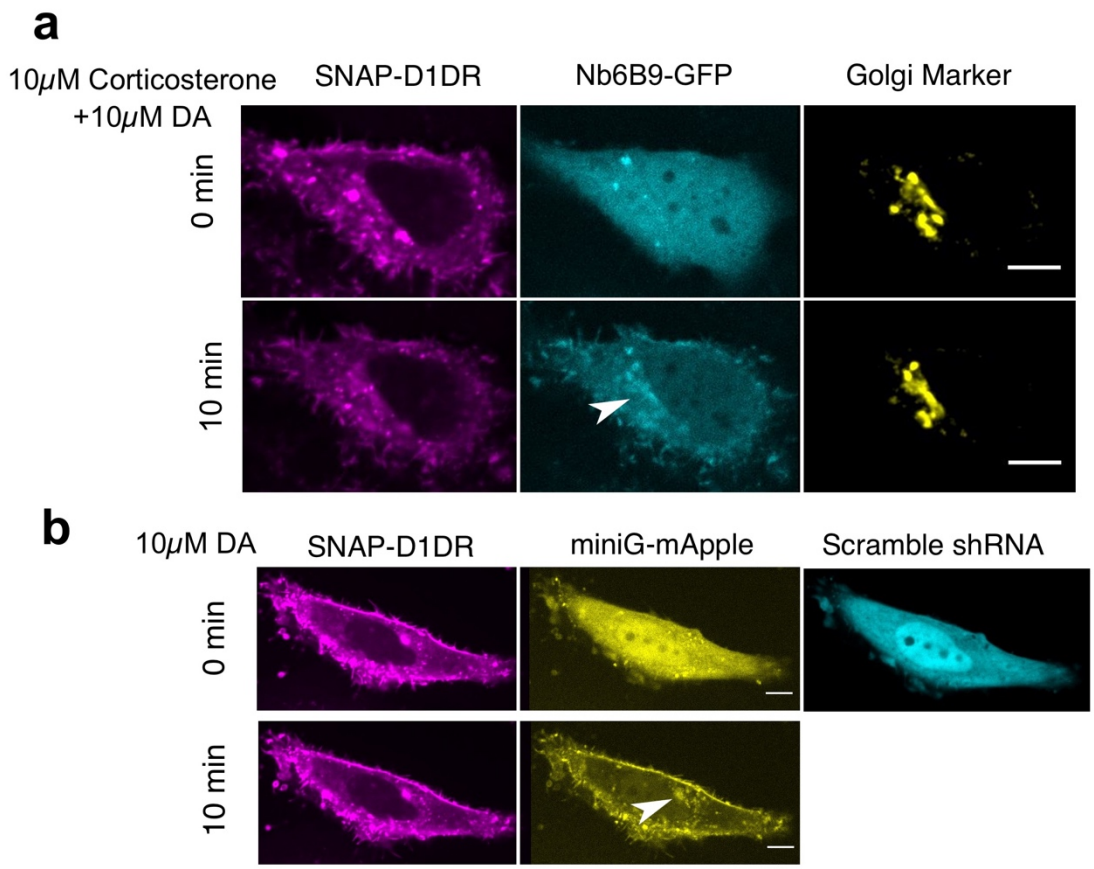


Figure 2.4 Rapid activation of Golgi localized-D1DR and PKA by photo-release of dopamine. a. Dopamine is un-caged from (N)-1-(2 Nitrophenyl) ethylcarboxy-3,4-dihydroxyphenethylamine (NPEC) upon blue light (UV) exposure. b. Confocal images of representative D1DR-expressing HEK293 cells with endogenous PKA-cat-GFP and Nb6B9-mApple expression, incubated with 1 μM NPEC-caged dopamine and at indicated times after blue light exposure (*n* = 46 cells, 4 biological replicates). Arrowhead indicates Nb6B9 recruitment to the Golgi membrane; Scale bar = 10μm. c. Normalized fluorescence intensity of Nb6B9-mApple and PKAcat relative to Golgi-D1DR after blue light exposure.



Supplementary Figure 2.5 a. Dose-response curve of forskolin-normalized D1DR-mediated cAMP response in HEK 293 cells treated with SKF81297 or dopamine; SKF and DA have similar potency and efficacy, thus differences in signaling at Golgi in HEK293 cells are not due to differences in drug potency or efficacy (mean ± s.e.m., *n* = 3 biological replicates). **b.** Detection of OCT2 expression by Western Blot. HeLa cells express OCT2, while HEK 293 cells do not. **c**. Representative HEK293 cell expressing Snap-D1DR, Nb6B9-GFP, and OCT2-mApple at indicated times after 10μM dopamine addition. Overexpression of OCT2 in HEK293 cells rescues Golgi-localized D1DR activation (*n* = 16 cells, Pearson's coefficient = 0.67, 6 biological replicates). Arrow indicates active D1DR at plasma membrane; Arrowhead indicates active D1DR at Golgi membrane; Scale bar = 10μm. **d.** Endogenous localization of OCT2 at the plasma membrane and the Golgi membranes. HeLa cells were labeled with OCT2 specific antibody and the Golgi antibody (GM130) (top panels). Antibody staining of OCT2 is not detectable in OCT2 shRNA expressing cells; *n* = 25 cells, Pearson's coefficient = 0.64, 3 biological replicates). Arrow indicates active OCT2 at plasma membrane; Arrowhead indicates active OCT2 at Golgi membrane; Scale bar = 10μm.



Supplementary Figure 2.6 a. Representative HeLa cell expressing Snap-D1DR, Nb6B9-GFP, and GalT-mRFP pretreated with 10μM corticosterone, an OCT3 selective inhibitor, for 15 min, before and after 10μM dopamine addition. Inhibition of OCT3 does not block Golgi-localized D1DR activation (*n* = 18 cells, Pearson's coefficient = 0.72, 2 biological replicates). **b.** Representative HeLa cell expressing Snap-D1DR, miniGs-mApple and scramble shRNA-GFP, before and after 10μM dopamine addition. Scramble shRNA does not block Golgi-localized D1DR activation (*n* = 10 cells, Pearson's coefficient = 0.6, 4 biological replicates)

2.6 Materials and Methods

Table 2.1 Key resources

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Cell line	HEK293	ATCC	CRL-1573.3	Mycoplasma Tested negative
Cell line	HEK293T	ATCC	ACS-4500	Mycoplasma Tested negative
Cell line	HeLa	ATCC	CRM-CCL-2	Mycoplasma Tested negative
Antibody	Rabbit Anti-Dopamine Receptor D1	Abcam	ab216644	1:100
Antibody	Mouse Anti-GM130	BD biosciences	610822	1:1000
Antibody	Rabbit Anti-SLC22A2	Abcam	ab170871	1:1000
Antibody	Rabbit Anti-SLC22A2	ABClonal	A14061	1:100
Antibody	GAPDH	Proteintech		1:10000
Antibody	HRP-conjugated rabbit IgG	GE Healthcare		1:10000
Antibody	Sheep anti-mouse IgG	GE Healthcare		1:10000
Antibody	Sheep anti-rabbit IgG	GE Healthcare		1:10000
Antibody	Rabbit Anti-Dopamine Receptor D1	Proteintech	17934-1-AP	1:100
Peptide	D1DR blocking peptide	Proteintech	Ag12366	1:20
Conjugated dye	Snap-Cell 647	NEB		
Chemical compound, drug	Dopamine hydrochloride	Sigma		
Chemical compound, drug	SKF81297 hydrobromide	Tocris		
Chemical compound, drug	NPEC-caged-dopamine	Tocris		
Chemical compound, drug	A/C heterodimerizer	Takara	635056	

Table 2.1 continued Key Resources

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Chemical compound, drug	Dyngo	Abcam	ab120689	
Chemical compound, drug	Forskolin	Sigma	F6886-10MG	
Chemical compound, drug	Imipramine	Sigma	113-52-0	
Chemical compound, drug	Corticosterone	Sigma		
Software, algorithm	Prism	GraphPad		
Software, algorithm	ImageJ	Imagej.net/contributors		
Software, algorithm	MatLab	MatLab		
Recombinant DNA reagent	pGloSensor-20F	Promega		
Recombinant DNA reagent	signal sequence Snap-D1DR	This study		pCDNA3 backbone; Snap vector
Recombinant DNA reagent	pVenus-FRB-Nb6B9	This study		pVenus-C1 vector
Recombinant DNA reagent	FKBP-GalT-mApple	This study		pm-Apple-M1 vector
Recombinant DNA reagent	Lyn-2xFKBP-CFP		Plasmid # 20149	
Recombinant DNA reagent	pCAG-Snap-D1DR	This study		pCAG vector
Recombinant DNA reagent	pCAG-Nb6B9-GFP pCAG-Nb37-GFP	This study		pCAG vector

Cell Culture, cDNA constructs and transfection

HeLa and HEK293 cells (purchased from ATCC as authenticated lines CCL-2, CRL-1573 and CRL 1446 respectively) were grown in Dulbecco's minimal essential medium supplemented with 10% Fetal Bovine Serum (FBS) without antibiotics. Cell cultures were free of mycoplasma contamination. Signal Sequence-Snap-tagged D1DR was created by amplifying D1DR from Flag-D1DR using 5'- GCCTGGGCTGGGTCTTGGATCCGATGACGCCATGGACG -3'; 5'-ATAGGGCCCTCTAGAGCCTCAGGTTGGGTGCTG -3' primers, and inserted into the Snap vector using BamHI and XbaI. pVenus-FRB-Nb6B9 was created by amplifying Nb6B9 and FRB from Nb6B9-GFP (Irannejad et al., 2017), and pC_4-R_HE plasmid (ARIAD Pharmaceuticals), using 5'-TGGTGGACAGGTGCAGCT -3'; 5'-GGATCCTCATGAGGAGACGGTGACCTGGGT -3' and 5'-GCTTCGAATTCAATCCTCTGGCAT -3'; 5'- TGCACCTGTCCACCAGCACTA-3 primers respectively, such that it contained the linker sequence GATAGTGCTGGTAGTGCTGGTGGAC, and inserted into the pVenus-C1 vector using EcoRI and BamH1. FKBP-GalT-mApple was created by amplifying FKBP and GalT from KDELr-FKBP and GalT-mCherry plasmids (a generous gift from Dr.Farese lab), using 5'-CATGCTAGCGCCGCCACCATGGGAGTGCAGGTGGAAACCAT-3', 5'-GAGCTCGAGACCAGCACTACCAGCACTATCCTCCAGCTTCAGCAGCTCCACG3' and 5'- GCTCAAAGCTTGCCGCCACCGGAAGGCTTCGGGAGCCG-3',

5'- ACCGGATCCTTAGGCCCCTCCGGTCCGGAGCTCCCCG-3' primers, respectively and inserted into the pmApple-N1 vector using NheI, XhoI for FKBP and HindIII and BamHI for GalT(Irannejad et al., 2017). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Snap tagged human D1DR constructs were labelled with Snap-cell 647 SiR (New England Biolabs, S9102S) as described previously (Lukinavicius et al., 2013).

Live-cell confocal imaging

Live cell imaging was carried out using Nikon spinning disk confocal microscope with a 60 ×, 1.4 numerical aperture, oil objective and a CO_2 and 37 °C temperature-controlled incubator. A 488, 568 nm and 640 Voltran was used as light sources for imaging GFP, mRFP/mApple, and Snap-647 signals, respectively. Cells expressing both Snap-tagged receptor (2µg) and the indicated nanobody–GFP (200ng) were plated onto glass coverslips. Receptors were surface labelled by addition of Snap-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Live cell images where endocytosis was inhibited were carried out by incubating the cells in 30µM Dyngo 4a (ab120689) at 37 °C for 30 minutes before indicated agonist was added. HEK293 Indicated agonists (dopamine hydrochloride-Sigma, SKF81297 hydrobromide-Tocris) were added and cells were imaged every 20 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. NPEC-caged-

dopamine (Tocris) was incubated for 10 min before cells were stimulated with 3.2 W/cm^2 blue light. Time-lapse images were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS elements).

Fixed-cell confocal imaging

Cells were permeabilized with saponin to reduce the cytoplasmic background, as described previously(Lobert & Stenmark, 2012). Briefly, HeLa cells were permeabilized with 0.05% saponin (Sigma) in PEM buffer (80mM K-Pipes, pH 6.8, 5 mM EGTA, 1mM MgCl2) for 5 min on ice. Cells were then fixed with 3% paraformaldehyde in PBS for 10 min and then quenched with 50mM Nh4Cl in PBS for 15 min. Primary antibodies D1DR antibody (ab216644) (1:100), D1DR (Proteintech 17934-1AP) (1:100) with or without D1DR blocking peptide (Proteintech Ag12366) (1:25), GM130 (BD biosciences 610822) (1:1000), and SLC22A2/OCT2 antibody (ab170871) or SLC22A2/OCT2 (ABClonal-A14061) (1:100), were diluted in PBS supplemented with 0.05% saponin. D1DR and GM130 antibodies were diluted in TBS with 5% donkey serum and 0.1% Triton X-100. Confocal images were taken using Nikon spinning disk confocal microscope with a 60x 1.4 numerical aperture, oil objective.

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (http://rsb.info.nih.gov/ij). For measuring

kinetics of Nb6B9–GFP and miniGs recruitment at the Golgi membrane over time in confocal images and kinetics of PKA-Cat GFP dissociation from the Golgi, analyses were performed on unprocessed TIFF images using custom scripts written in MATLAB (Jullie et al., 2020). Values were normalized by calculating the percent relative to the maximum value, then baseline corrected using Prism 6.0 software so that the first value of each condition was set to 0. The same MATLAB script was used to analyze the dose-response kinetics of both Nb6B9-GFP and miniGs-mApple recruitment to the Golgi membrane in response to increasing concentrations of agonists. In this case, to better visualize the increase in fluorescence at the Golgi after addition of agonist, values were normalized to the baseline following each addition of agonist. This was done in Microsoft Excel, and each baseline value was set to 1 to measure the fold change in fluorescence. Co-localization analysis at the Golgi was estimated by calculating the Pearson's coefficient between the indicated image channels with the Golgi marker channel, using the co-localization plug-in for ImageJ (Coloc2). *P* values are from two-tailed unpaired Student's *t*-tests calculated using Prism 6.0 software (GraphPad Software).

Luminescence-based cAMP assay

HEK 293 cells stably expressing D1DR were transfected with a plasmid encoding a cyclic-permuted luciferase reporter construct, (pGloSensor-20F, Promega) and

luminescence values were measured, as described previously (Irannejad et al., 2013). Briefly, cells were plated in 96-well dishes (~100,000 cells per well) in 500µl DMEM without phenol red/no serum and equilibrated to 37 °C in the SpectraMax plate reader and luminescence was measured every 1.5 min. Software was used to calculate integrated luminescence intensity and background subtraction. In rapamycin heterodimerization experiments, cells were pre-incubated with 1µM A/C heterodimerizer, a rapamycin analog (Takara 635056) for 15 min. 5µM forskolin was used as a reference value in each multi-well plate and for each experimental condition. The average luminescence value (measured across duplicate wells) was normalized to the maximum luminescence value measured in the presence of 5µM forskolin. For rapamycin treated cells, the average luminescence value was normalized to the maximum luminescence value measured in the presence of 5µM forskolin and 1µM Rapamycin.

Western blotting

Cells from HEK293, HEK293T and HeLa were lysed in extraction buffer (0.2% Triton X-100, 50mM NaCl, 5mM EDTA, 50mM Tris at pH 7.4 and complete EDTA-free protease inhibitor cocktail (Roche). Kidney and neural tissues from B6 adult mice were disrupted in RIPA buffer (50mM Tris at pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate 0.1% SDS and complete EDTA-free protease inhibitor cocktail).

After agitation at 4°C for 30 min, supernatants of samples were collected after centrifuging at 15000 x rpm for 10 min at 4°C. Supernatants were mixed with SDS sample buffer for the protein denaturation. The proteins were resolved by SDS-PAGE and transferred to PVDF membrane and blotted for anti-SLC22A2 (ab170871-1:1000) or GAPDH (1:10,000) antibodies to detect OCT2 and GAPDH expression by horseradish-peroxidase-conjugated rabbit IgG, sheep anti-mouse and rabbit IgG (1:10,000 Amersham Biosciences) and SuperSignal extended duration detection reagent (Pierce).

Data availability

Our research resources, including methods, cells and protocols are available upon request. All reagents developed, such as FRB and FKBP constructs, as well as detailed methods will be available upon request. The corresponding author adheres to the NIH Grants Policy and Sharing of Unique Research Resources.

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2.8 Acknowledgments

We thank D. Jullie, K. Silm, A. B. Lobingier, A. Manglik, E. Hernandez and D. Larsen for assistance, advice and valuable discussion. These studies were supported by the National Institute on General Medicine (GM133521) to R.I and The American Heart Association (908933) to N.P.

2.9 Author Contributions

Natasha Puri designed experimental strategy, carried out most of the experiments and analysis, contributed to interpreting the results and writing the paper. Gia Romano designed some experimental strategy, carried out initial imaging and signaling experiments and analysis. Quynh Mai contributed to the cAMP experimental design and analysis. Roshanak Irannejad designed the experimental strategy, contributed to interpreting the results and writing the paper.

Chapter 3: Investigating the physiological relevance of compartmentalized D1 dopamine receptor signaling

Natasha M. Puri, Ting-Yu Lin, and Roshanak Irannejad

Partially adapted from eLife publication: https://doi.org/10.7554/eLife.75468

3.1 Abstract

In the last decade, studies have established that many G protein-coupled receptors (GPCRs) can signal not only from the plasma membrane but also at other distinct membrane compartments, such as the endosomal and Golgi membranes.

More recently, the D1 dopamine receptor (D1DR) has been identified as another GPCR that is able to signal from subcellular compartments. Organic Cation Transporter 2 (OCT2) mediates Golgi-localized D1DR activation by dopamine (DA). Although OCT2 was mainly thought to function primarily in the kidney, we found OCT2 has a distinct expression pattern in D1DR-expressing tissues across different regions of the brain. Namely, we found OCT2 to be highly expressed in striatal brain tissue, a region that is essential for dopaminergic signaling transmission. This begged the question of whether OCT2 mediates Golgi-D1DR activity in highly differentiated cell types. Here, we show D1DR signaling patterns in two kidney cell lines, primary medium spiny neurons of the striatum, and primary hippocampal neurons. We find that Golgi-D1DR activation is OCT2-mediated in physiologically relevant cell types. We also find that activation of D1DR at distinct locations leads to differential cAMP/PKA signaling dynamics, providing evidence for the first time that intracellular D1DR signaling is functionally and physiologically relevant. The present study highlights the importance of compartmentalized GPCR signaling in maintaining the delicate balance of cellular responses required for proper tissue function.

3.2 Introduction

The consequences of highly localized GPCR signaling hubs are only just starting to be understood. Given the high-degree of compartmentalization of cAMP, the downstream target of GPCR-Gαs activation, these discrete activation nodes would explain how highly localized signaling cascades have far-reaching effects in large, differentiated cells. Moreover, recent evidence has shown that other compartmentalized receptors, like the beta 1 adrenergic receptor (β1AR), are functionally distinct at different locations. β1AR localized at the Golgi Apparatus has been shown to regulate phospholipase C-ε (PLCε) activity and control heart contractility responses in cardiomyocytes (Lin et al., 2023; Nash et al., 2019)). GPCRs localized to endosomes, such as beta 2 adrenergic receptor (β2AR) and opioid receptor, have been well established in regulating distinct transcriptional responses (Irannejad et al., 2017; Stoeber et al., 2018; Thomsen et al., 2018; Tsvetanova & von Zastrow, 2014).

Experts in the field speculate that the distinct lipid environments at different membrane compartments also contributes to distinct signaling responses. A kinase anchoring proteins (AKAP) tether phosphodiesterases (PDE), protein kinase A (PKA), and phosphatases to distinct membrane locations within the cell. Their membrane distribution and binding partners vary depending on the isoform. AKAPs have been shown to play an important role in limiting cAMP activity to highly localized

nanodomains (Agarwal, Clancy, & Harvey, 2016; Bock et al., 2020; Saucerman, Greenwald, & Polanowska-Grabowska, 2014). This is through PDEs that degrade cAMP, limiting their diffusion at the lipid membrane from which they are generated. PKA, a target of cAMP activation, is a holoenzyme made up of two regulatory subunits that cAMP directly binds to and two catalytic subunits that are released to phosphorylate downstream effectors. It has also been proposed that free regulatory subunits of PKA can act as a sink to sequester cAMP (Saucerman et al., 2014). The distribution of cAMP regulatory mechanisms at distinct membrane compartments needs to be further elucidated.

Dopamine receptors are members of the GPCR family and mainly function in the brain and kidney. These receptors are split into D1-like (D1, D5) and D2-like (D2, D3, D4) that couple to Gαs and Gαi, respectively. Neural dopaminergic signaling regulates locomotion, reward-motivated behavior, and learning. The D1DR-expressing medium spiny neurons (MSNs) of the striatum are well established to play significant roles in motivation, aversion and reward (Alburges, Hunt, McQuade, & Wamsley, 1992; Di Chiara & Imperato, 1988; Kim, Alberico, Emmons, & Narayanan, 2015; Nestler & Luscher, 2019; Romach et al., 1999). Dysfunctional dopaminergic signaling in the brain is associated with several neurodegenerative diseases such as Parkinson's Disease, schizophrenia, depression, and addiction. The renal dopaminergic system helps

maintain blood pressure by regulation of sodium excretion, and defects in this signaling have been found in patients with essential hypertension (Harris et al., 2012; Olivares-Hernandez et al., 2021; Donato et al., 2022). Recently, we showed the D1 dopamine receptor (D1DR) can signal from the plasma membrane (PM), Golgi, and endosomes (Puri et al., 2022). Whether D1DR activity at different membrane locations regulates distinct cellular processes in the brain and kidney is not known.

Results from our previous study show that Golgi-D1DR activation is mediated by organic cation transporter 2 (OCT2). A low-affinity, high-capacity, bidirectional transporter, OCT2 is thought to primarily play a role in removing drugs, hormones, and neurotransmitters from extracellular spaces (Koepsell et al., 2007). Although OCT2 was previously thought to function primarily in the kidney, it has more recently been identified in distinct regions of the brain that regulate behavioral responses. Its primary role in the brain has been thought to aid high-affinity uptake 1 transporters for dopamine, norepinephrine, and serotonin (DAT, NET, and SERT) in clearing excess neurotransmitters from synapses to attenuate signaling and neural crosstalk (Barrett et al., 2017). In polarized human kidney cells, OCT2 has been shown to be targeted to the basolateral membrane, but there are different isoforms of OCT2 in some mammals that are localized to the apical membrane (Gasser et al., 2017; Gründemann et al., 1997). Its

primary function in the kidney is thought to aid in the removal of cations, drugs, and toxins from the bloodstream into the kidneys to be excreted.

Highly differentiated cell types, like cells of the kidney and brain, have high degrees of membrane compartmentalization and polarization. Kidney cells that make up the nephron, or the functional unit of the kidney, are highly polarized. Forming apical and basolateral membranes that are separated by tight junctions allow this epithelial cell layer to act as a barrier for tissues (Zihni et al., 2016). The polarized membranes then become the molecular gateway between different compartments of the body. Trafficking of proteins to apical and basolateral membranes is very tightly regulated to ensure the proper functions of these membranes (Hua et al., 2006; Stoops & Caplan, 2014; Torres et al., 2011). The apical membrane faces the lumen of the nephron, interacting with everything that gets filtered through the glomerulus to be transported to the bladder. The kidney is innervated with blood vessels that face the basolateral membrane of the nephron. Through these membranes, necessary solutes and water can be reabsorbed back into the bloodstream or secreted into the nephron to be expelled. The distinct transport mechanisms at each membrane location are essential for proper kidney function. D1DR is the predominant dopamine receptor type in the proximal convoluted tubule (PCT) of the nephron, the functional unit of the kidney (Jose et al., 2002; Olivares-Hernández et al., 2021; Soares-da-Silva & Fernandes, 1990;

Wiederkehr et al., 2001). Sodium reabsorption occurs via sodium hydrogen exchanger 3 (NHE3) localized to the luminal side of PCT cells and a sodium potassium ATPase (NKA) on the basolateral membrane (Honegger et al., 2006; Wiederkehr et al., 2001). D1DR activity regulates both NHE3 and NKA by promoting their endocytosis in a cAMP/PKA dependent manner (Ekengren et al., 2004; Satoh et al., 1993). D1DR localization has been debated to exist either on both apical and basolateral membranes or limited to the apical membrane (Fiorentini et al., 2003; Kong et al., 2006; Olivares-Hernández et al., 2021). Given the contested localization of D1DR and the high-degree of cAMP compartmentation, the mechanism of basolateral-NKA regulation is unclear.

Neurons of the brain also have high degrees of membrane compartmentation. While not technically polarized, they have a very distinctive shape and size that allow them to communicate to regions of the brain both near and far. In fact, dopaminergic neurons are the most unique and diverse neurons in the mammalian brain in terms of morphology and physiology. Dopaminergic neurons of the substantia nigra can reach nearly a meter in length in order to project into other brain regions and display distinct arborizations depending on which area of the brain they are projecting into. Dopaminergic neurons in different regions of the brain have been shown to have distinct kinetics and mechanisms of activation. Moreover, the plasma membrane of

neurons is further compartmentalized into distinct functional segments that are essential for propagation of neuronal signaling. The lipid and phospholipid distribution at the PM helps to target and confine proteins to their functional segment.

The significance of Golgi-D1DR signaling in regulating cellular responses in both the brain and kidney is not known. Given the limited diffusion of cAMP from its microdomain and the high degree of compartmentation in both these cell types, we hypothesize that Golgi-localized D1DR activation does occur. Further, because of our findings on the role of OCT2 in mediating this intracellular signaling, we speculate that the relevant D1DR-expressing cells also require OCT2 to facilitate Golgi-D1DR activation. In addition to several kidney cell lines, here we demonstrate that Golgi-localized D1DR signaling is also a feature of primary striatal MSNs. We also show that these localized signaling cascades lead to differential cAMP/PKA signaling dynamics.

3.3 Results

Regulation of dopamine-mediated activation of the Golgi-localized D1DR in primary murine neurons by OCT2

To investigate the role of OCT2 in D1DR signaling in physiologically relevant cell types, we measured OCT2 expression patterns in cell types derived from the kidney and the brain, the two main organs where D1DRs are known to have function (Figure 1a). Previous reports, some of which depended on RNA measurements, suggested that OCT2 is robustly expressed in the striatum and cortex, where D1DR is known to express and have function (Castro et al., 2013; Hallett, Spoelgen, Hyman, Standaert, & Dunah, 2006; Tang & Bezprozvanny, 2004), but at low levels in the hippocampus and substantia nigra, regions in which D1DR also has known functions (Alburges et al., 1992; Arnsten, Cai, Steere, & Goldman-Rakic, 1995; Busch et al., 1998; Double & Crocker, 1995). By western blotting using a validated antibody (Supplementary Figure 1d), we similarly found significant OCT2 expression in the striatum and cortex, slightly lower expression in the hippocampus, and negligible expression in substantia nigra (Figure 1a).

To determine the role of OCT2 in regulating a distinct pool of D1DR signaling in neurons, we isolated primary murine striatal MSNs, where OCT2 is expressed at high levels (Supplementary Figure 1a) (Bacq et al., 2012; Matsui, Nakata, & Kobayashi,

2016). Within the striatum, D1DR-expressing MSNs have been shown to play roles in DA-regulated processes such as motivation, aversion and reward seeking (Alburges et al., 1992; Di Chiara & Imperato, 1988; Kim et al., 2015; Nestler & Luscher, 2019; Romach et al., 1999). We detected endogenous D1DR on both the plasma membrane and the Golgi membranes in MSNs using two different D1DR antibodies (Supplementary Figure 1a, lower panel and Supplementary Figure 1c). D1DR immunostaining was diminished when MSNs were immuno-stained in the presence of D1DR blocking peptide (Supplementary Figure 1c). We also showed that MSNs express OCT2 on both the plasma membrane and the Golgi (Supplementary Figure 1b). To visualize receptor activation in living neurons, we cloned a neural-specific promoter into our Snap-tagged D1DR construct as well as our biosensor for receptor activity Nb6B9-GFP, as described in Chapter 1 and Puri et al. Stimulating D1DR expressing MSNs with DA resulted in the recruitment of Nb6B9-GFP to both the plasma membrane and the perinuclear regions (Figure 1b and c). The perinuclear region in MSNs is indeed colocalized with the Golgi membranes markers (Supplementary Figure 1a, top panel). Importantly, OCT2 inhibition resulted in the inhibition of DA-mediated Golgi-D1DR activation. By contrast, the membrane permeant SKF81297 activated D1DR at the Golgi (Figure 1c and d). To demonstrate that D1DR can form a functional complex with G protein at the Golgi in MSNs, we took advantage of Nb37-GFP to detect transient D1DR/G protein coupling. Similar to what we have observed in HeLa

cells, DA stimulation resulted in the recruitment of Nb37-GFP to the Golgi, suggesting that the D1DR Golgi pool is able to couple to G protein and activate it in MSNs (Supplementary Figure 1d). These data demonstrate that Golgi-localized G protein signaling by D1DRs occurs in a physiologically relevant cell type and that this signaling requires OCT2. Moreover, as there are cell types that express D1DR but not OCT2, our findings suggest that OCT2 expression could determine which cell types exhibit both plasma membrane and Golgi-localized D1DR signaling under physiological conditions.

To further determine whether this observation was true, we isolated primary murine hippocampal neurons, where OCT2 is expressed at lower levels than in MSNs of the striatum. We predicted that, given the low expression of OCT2, Golgi-D1DR signaling would not occur in hippocampal neurons. We did in fact observe modest recruitment of Nb6B9-GFP to Golgi-localized D1DR upon stimulation with DA in isolated hippocampal neurons (Supplementary Figure 1e and f). This was not consistent across different isolations. We have reason to believe that OCT2 expression changes from early stages in development to adult mice, suggesting that neonatal mice may have higher levels of OCT2 in the hippocampus compared to adult mice (data not shown). Nevertheless, these results confirm that the level of OCT2 expression can determine the scope of D1DR activation at the Golgi.

Compartmentalized D1DR activation in different kidney cell lines

To test whether we could observe compartmentalized D1DR activation in physiologically relevant dopaminergic system functionally distinct from the brain, we used Mardin Derby canine kidney (MDCK) cells, a kidney-derived immortalized cell line often used to represent the proximal tubule epithelium. First, we confirmed that this cell line does in fact endogenously express OCT2, making it a suitable candidate to study OCT2 mediated Golgi-D1DR activity (Figure 2a). Although this cell line also endogenously expresses D1DR, we transfected Snap-tagged D1DR, Nb6B9-GFP, and a Golgi marker in order to regulate the ratio of receptor to biosensor (Figure 2b, Figure 2c top panel). Upon stimulation with 10μM DA, Nb6B9-GFP was rapidly recruited to active D1DR at the PM and Golgi membrane (Figure 2c). To test whether OCT2 is still important in regulating Golgi-D1DR activation, we used murine principal kidney cortical collecting duct (mpkCCD), a representative cell type of the collecting duct of the nephron which is known to not express OCT2. Stimulating mpkCCD cells with 10μM DA resulted in recruitment of Nb6B9-GFP to active D1DR at the PM, but not at the Golgi (Figure 2d). To ensure that Golgi-D1DR was functional, we stimulated mpkCCD cells with 10μM SKF-81297, a membrane permeant D1DR agonist. We found that SKF was able to activate D1DR localized to the Golgi membrane, as shown by the quantification of Nb6B9-GFP recruitment to D1DR at the Golgi region (Figure 2e, f).

It's important to note that these studies were done in unpolarized kidney cell lines. Next, we tested whether PM-D1DR is further compartmentalized in polarized cells. We grew MDCK cells on transwell filters, a well-established method to produce three-dimensional (3D) cultures by promoting formation of apical and basolateral membranes. We transiently expressed Snap-labeled D1DR and either Flag-tagged NHE3 or Myc-tagged NKA in polarized MDCK cells grown on transwell filters for seven days (Supplementary Figure 3a). After immunostaining for Snap and either Flag or Myc, we analyzed the localization of D1DR as well as NHE3 or NKA. We found that both D1DR and NHE3 do in fact colocalize on the apical membrane of 3D MDCK cells, while NKA was localized to the basolateral membrane (Supplemental Figure 3b, 3c). This confirms the preliminary evidence in the literature that D1DR is limited to the apical membrane in kidney cells. It is unlikely that D1DR at the apical membrane can regulate NKA on the basolateral membrane, further strengthening the hypothesis that the internal Golgi-D1DR is regulating basolateral NKA, while apically-localized PM-D1DR regulates the apical NHE3. However, OCT2 has been very well-established to localize to the basolateral membrane, although some contesting evidence suggests that certain OCT2 isoforms can localize to apical membranes in some mammalian tissues (Cite). While kidney cells do express OCT2 (Figure 1a), they also express aromatic amino acid decarboxylase (AADC) that converts L-DOPA to dopamine. L-DOPA is normally produced in the adrenal gland, where it travels to the lumen of the nephron, crosses

the cell membrane and is converted into DA in the cytoplasm of proximal convoluted tubule cells of the nephron. DA is then secreted out of these cells to act on apical D1DR later in the proximal tubule as well as other dopamine receptors that are localized to the luminal membrane of the distal convoluted tubule. However, the fact that L-DOPA is converted to DA within proximal convoluted tubule cells suggests that there exists a cytoplasmic pool of DA within these cells independent of OCT2. This led us to hypothesize that presence of L-DOPA allows for an internal pool of DA in proximal tubule cells that can activate Golgi-D1DR. To test this, we incubated MDCK cells expressing Snap-D1DR as well as Nb6B9-GFP with 10mM L-DOPA and measured intracellular receptor activation. Interestingly, we found that treatment of cells with L-DOPA was able to activate Golgi-D1DR (Supplementary Figure 3d). This surprising finding suggests that in proximal tubules of the kidney, L-DOPA could be more important than OCT2 for Golgi-D1DR activation (Supplementary Figure 3e-model).

Golgi-D1DR activity leads to sustained intracellular cAMP signaling

We have previously established that D1DR localized to the Golgi can be activated and produces a local pool of cAMP, and that Golgi-D1DR activation is mediated by OCT2 in physiologically relevant cell types (Chapter 2, Puri et al). Next, we wondered what cAMP activity looked like downstream of distinct pools of D1DR activity. D1DR is coupled to the Gαs protein that, upon activation by the receptor, is released to

stimulate adenylyl cyclase to produce cAMP. This secondary messenger is a ubiquitous small molecule, however its diffusion rate is low due to buffering by its direct effectors phosphodiesterases that degrade cAMP, all of which are anchored to several lipid membranes by a kinase anchoring proteins (Figure 3a). To measure cAMP signaling dynamics downstream of D1DR-activation at distinct membrane compartments, we utilized the biosensor Pink Flamindo, comprising of a cAMP binding domain flanked by fluorophore mApple, that increases in fluorescence intensity upon cAMP binding. We targeted Pink Flamindo to the trans Golgi Network (PF-TGN) to develop a biosensor able to detect cAMP diffusion to the Golgi region. We expressed our biosensor in HEK293 cells, which importantly do not express OCT2 (Chapter 2, Puri et al) (Figure 3b). Using this cell line, we took advantage of the two distinct agonists of D1DR; one being DA, the membrane permeant endogenous agonist that cannot diffuse across the lipid membrane and thus activates PM-D1DR exclusively, the second being SKF 81297, the hydrophobic synthetic agonist that can cross the PM to activate Golgi-D1DR. Given the high-degree of cAMP compartmentation in cells, we hypothesized that cAMP generated solely from the PM would be unable to diffuse to the perinuclear/Golgi region. Surprisingly, we found at early time points, stimulating HEK293 cells with 10nM DA led to a modest increase in PF-TGN fluorescence (Figure 3c). Stimulating cells with 10nM SKF resulted in a more robust and sustained increase in PF-TGN, suggesting that

Golgi-D1DR activation is necessary for prolonged cAMP signaling in the perinuclear region.

Local activation of PKA at the Golgi depends on selective activation of Golgi-D1DR

A key downstream effector sensed by cAMP is protein kinase A (PKA). PKA is a holoenzyme, consisting of two catalytic and two regulatory subunits (Figure 3a). There are two PKA types (type I and II) that are anchored to distinct subcellular locations through interactions with distinct A kinase anchoring proteins (Soberg & Skalhegg, 2018) (Figure 3a). PKA type II has been shown to localize to the perinuclear/Golgi membranes (Nigg, Schafer, Hilz, & Eppenberger, 1985). Binding of cAMP to the PKA regulatory subunit (PKAreg) induces rapid dissociation and activation of the PKA catalytic subunit (Figure 3a) (Tillo et al., 2017; Walker-Gray, Stengel, & Gold, 2017). To test whether cAMP generation by Golgi-localized D1DR/Gs complex results in the activation of PKA at the perinuclear/Golgi, we utilized a previously described HEK293T knock-in cell line expressing a split fluorescent protein, labeling native PKA catalytic subunit gene with GFP (PKAcat-GFP) (Figure 3d) (Feng et al., 2017; Peng et al., 2021). Stimulation of HEK293T PKAcat-GFP knock in cell lines expressing D1DR with 10nM SKF81297, a concentration that activates both pools of D1DR (Chapter 2), resulted in rapid dissociation of PKAcat-GFP from the perinuclear/Golgi membranes (Supplementary Figure 4a, top panel). Quantification of these data shows that

stimulation with SKF81297 results in sustained activation of PKA at the perinuclear/Golgi regions (Figure 3e). We then asked whether PKAcat dissociation is mediated by the activation of D1DR Golgi pool. Given that HEK293T express very low levels of OCT2 transporter and thus DA cannot be sufficiently transported to the Golgi membranes, we used DA to specifically activate the plasma membrane pool of D1DR. Importantly, stimulation of HEK293T PKAcat-GFP knock-in cells with 10nM DA, a concentration with similar potency as SKF81297 (Chapter 2, Puri et al) did promote PKAcat dissociation at early time points, but rapidly reassociated with PKAreg (Figure 3e, Supplementary Figure 4a lower panel). Together, these data indicate that activation of the D1DR at the Golgi, but not the plasma membrane, results in sustained local PKA activation at the perinuclear/Golgi. Interestingly, D1DR activation at the PM does result in perinuclear PKA activation, but only transiently.

To further elucidate D1DR signaling dynamics from the plasma membrane or Golgi membrane, we used a PKA reporter containing a phosphorylation motif that can be detected by a phospho-specific antibody. We targeted this reporter to the Golgi membrane or PM in HEK293 cells and measured PKA activation by probing for phosphorylation of the PKA reporter via western blot (Supplementary Figure 4b, 4c). Preliminary results show that stimulating cells with 10μM DA to activate only PM-D1DR, PKA-dependent phosphorylation at the PM peaks at 5 minutes and decreases at the

later time points (Supplementary Figure 4c, 4d). Golgi phosphorylation of the PKA reporter was not significantly changed by DA stimulation at 5 or 10 minutes. This supports the evidence suggesting signaling cascades downstream of Gαs-coupled receptors are highly compartmentalized, and cAMP generated from the PM does not result in significant PKA activity at the Golgi. It remains to be seen whether Golgi-PKA reporter increases in phosphorylation at earlier time points, in which transient increases in cAMP and PKA have been shown by biosensors. Stimulating cells with synthetic membrane permeant agonist SKF81297 led in a robust increase in Golgi-PKA reporter phosphorylation (Supplementary Figure 4c, 4d).

Our findings led us to question the purpose of sustained cAMP/PKA signaling at the perinuclear region downstream of Golgi-D1DR. Evidence from other compartmentalized GPCRs has shown that signaling from endosomes leads to distinct transcriptional responses. We wondered whether Golgi-localized GPCR activity could also regulate transcription, given the proximity of the Golgi to transcriptional machinery in the nucleus. To determine the local pool of cAMP generated downstream of Golgi-D1DR activation could act on effectors in the nucleus, we measured cAMP diffusion to the nucleus using the cAMP biosensor Pink Flamindo targeted to the nucleus by nuclear localization sequence (PF-NLS) (Figure 4a). Using HEK293 cells expressing Snap-labeled D1DR and PF-NLS, we found that stimulating cells with

membrane impermeant DA resulted in a brief but modest increase in cAMP diffusion to the nucleus (Figure 4b). Though surprising, cAMP levels in the nucleus decreased to baseline levels three minutes after stimulation of PM-D1DR (Figure 4b). However, stimulation of both pools of D1DR with membrane permeant SKF81297 resulted in a robust increase in cAMP in the nucleus that was sustained over a longer time period (Figure b). To directly test the role of distinct pools of cAMP on transcription, we utilized bacterial photoactivatable adenylyl cyclase (bPAC), an AC isoform that is activated by blue light. We targeted bPAC to either the PM or Golgi then measured phosphorylation of CREB by western blot (Supplementary Figure 5a). Preliminary results suggest that cAMP produced at the Golgi has a larger impact on CREB activation (Supplementary Figure 5b, 5c). Altogether, these data show that Golgi-D1DR activity leads to sustained cAMP/PKA levels that reach the nucleus, suggesting that another compartmentalized Golgi pool of GPCR, distinct from the endosomal pool, can also regulate transcriptional responses.

3.4 Discussion

In this present study we have shown that D1DR can signal at intracellular membrane compartments such as the Golgi Apparatus in relevant D1DR-expressing cell types. We present evidence that OCT2 facilitates transport of dopamine intracellularly to access this internal pool of receptor, although our preliminary evidence suggests there may be other methods of creating intracellular DA. We also demonstrate unique biosensor tools targeted to distinct membrane compartments to further elucidate the role of compartmentalized D1DR activation on cAMP/PKA signaling dynamics. Our work suggests that distinct pools of D1DR contribute to dopaminergic signaling cascades in both the brain and kidney and speculate that subsequent differences in cAMP and PKA activity mediate distinct cellular responses.

We showed that OCT2 is expressed in MSNs in the striatum (Supplementary Figure). Consistent with this, we have further shown that DA activates Golgi pool of D1DR in MSNs (Figure 1). Moreover, we showed that activation of the D1DR at the Golgi, but not the plasma membrane, results in sustained cAMP and PKA activation at the perinuclear/Golgi region (Figure 3). This could potentially explain some of the distinct cAMP/PKA signaling patterns that have been observed in different D1DR expressing neurons. For instance, cAMP-dependent PKA responses have been shown to be sustained in striatal neurons compared to pyramidal cortical neurons (Castro et al.,

2013). Interestingly, we found that stimulating PM-D1DR with DA in non-OCT2 expressing cells resulted in transient, modest cAMP diffusion to both the Golgi and nucleus, whereas stimulation of both PM and Golgi-D1DR led to sustained, robust cAMP at these regions. Given our preliminary evidence that cAMP generated from the Golgi leads to increased activity of transcription factor CREB, it is possible that the sustained cAMP generated by Golgi-D1DR can regulate transcriptional responses.

Our observation that small amounts of cAMP can diffuse from the PM to perinuclear regions was surprising given that cAMP is known to be limited to microdomains on the nanometer scale. We speculate that with high levels of cAMP generated at the PM, small amounts of the signaling molecule can leak into regions spatially far from its membrane compartment. It is possible that the regulatory machinery localized to the perinuclear region, such as AKAP-bound PDE and PKA regulatory subunits, can degrade and sequester the low concentrations of cAMP produced from the PM. The larger concentration of cAMP generated from Golgi-localized receptor activation is likely to overwhelm regulatory machinery, thereby leading to sustained cAMP and subsequent PKA activity. It will be interesting to determine whether the small concentration of PM-cAMP that can diffuse to the nucleus leads to early activation of transcriptional responses that is either functionally distinct from other compartmentalized sources of cAMP or simply compounds transcriptional activity.

Overall, our results suggest that the expression pattern of OCT2 and activation of the Golgi-pool of D1DRs determine which cell types and tissues exhibit sustained cAMP/PKA signaling. This work highlights the need to determine the functional consequences of Golgi-D1DR mediated sustained signaling cascades.

D1DR-mediated cAMP signaling regulates major brain functions. Persistent DA stimulation and sustained receptor activation causes long-term changes in gene expression of neuronal plasticity-related genes, dendritic remodeling and locomotor sensitization (Gerfen et al., 1990; Le Moine & Bloch, 1995; Luscher & Malenka, 2011; J. Zhang et al., 2006). Many drugs of abuse increase the release of DA and elevate the firing rate of midbrain dopaminergic neurons in the striatum, particularly MSNs (Di Chiara & Imperato, 1988; Luscher & Malenka, 2011; Nestler & Luscher, 2019). Whether this sustained receptor-mediated cAMP/PKA activation in MSNs is a consequence of D1DR activation at the Golgi is not clear. Understanding the contribution of D1DR subcellular signaling could potentially help with drug development for disorders where dopaminergic signaling is mis-regulated.

We showed that OCT2 is also expressed in MDCKs, a kidney cell line that resembles the PCT of the nephron, where D1DR signaling regulates sodium reabsorption. We have shown that DA can activate Golgi-D1DR in this cell type. Importantly, in a cortical

collecting duct cell line that does not express OCT2, we could only observe Golgi-D1DR activation by permeant agonist SKF81297, but not DA. We also show that D1DR is targeted to the apical membrane in 3D polarized MDCKs grown on transwell filters, showing that D1DR is further compartmentalized along the PM. Given these results, we speculate that compartmentalized D1DR signaling occurs in the OCT2-expressing PCT of the nephron, and that Golgi-D1DR activation is distinctly regulating effectors at the basolateral membrane of polarized PCT cells.

Accurate measurements of cytoplasmic DA in intact pre- or post-synaptic neurons have been challenging due to lack of sensitivity of most analytical methods and their effects on cell viability (Chang et al., 2021; Olefirowicz & Ewing, 1990; Post & Sulzer, 2021). However, given that DA is present at high millimolar concentrations within the synaptic vesicles (Omiatek et al., 2013; X. W. Zhang, Hatamie, & Ewing, 2020), it is likely that rapid uptake of DA post release will result in high cytoplasmic DA concentrations. In the kidney, dopamine concentrations have been reported to be in the nanomolar range. As discussed in Chapter 2, the Km measurements of OCT2-mediated DA transport suggests that intracellular concentrations of DA would be ten-fold higher than extracellular concentrations. Interestingly, DA in the kidney is sourced from L-DOPA produced in the adrenal glands. L-DOPA is filtered through the glomerulus into the lumen of the nephron, then converted into dopamine by aromatic amino acid

decarboxylase (AADC). Levels of AADC are highest in the PCT, which is known to be the primary source of DA in the kidney. DA is then transported out of PCT cells into the lumen to act on apically localized PM-dopamine receptors throughout all the nephron segments. The role of intracellular DA in the PCT has never been investigated due to functional, intracellular pools of D1DR not being identified until recently. However, this reveals another source of intracellular dopamine independent of OCT2 function that could be facilitating Golgi-D1DR activation. Given our preliminary evidence, we speculate that the membrane permeant precursor L-DOPA could provide another source of intracellular DA that can access Golgi-D1DR.

The results of the present study reveal a novel function of OCT2 in mediating dopaminergic signaling cascades in cells of the brain and kidney. Ever since OCT2 was shown to express in specific brain regions, it has been a subject of study in understanding mechanisms behind behavioral responses and diseases. Drugs of addiction like methamphetamine, originally thought to only interact with the high-affinity uptake 1 dopamine transporter, have now been shown to directly bind OCT2 as well. The role this plays in dysfunctional dopaminergic signaling in the brain is not known but could shed mechanistic insight on how drugs associated with substance abuse impact intracellular dopaminergic signaling. Drugs used to treat Parkinson's Disease (PD) also directly interact with OCT2. Higher levels of interstitial DA in synaptic

cleft due to OCT2 inhibition has provided an explanation for some symptoms of PD treatment. More thought should be given to intracellular dopaminergic mis-signaling in response to drug treatments, both in neurodegenerative diseases like PD and schizophrenia, but drugs of abuse as well. OCT2 in the kidney has been relatively well-characterized in aiding the secretion of hormones, nutrients, and toxins. However, localization of OCT2 in kidneys is contested. OCT2 isolated from pig and rat have shown to localized to apical membrane, but human OCT2 is thought to be targeted to the basolateral membrane. It's possible that OCT2 isoforms exist on the apical membrane of human PCT cells. In fact, Gründemann et al. proposed that OCT2 could be mediating transport of DA out of PCT cells. If true, we propose a model in which OCT2 mediates DA secretion into lumen to act on apical D1DR further down the nephron segment, as well as other dopamine receptors that exist in other regions of the nephron (model).

Overall, the present work reveals that D1DR can signal from distinct membrane compartments in relevant, D1DR-expressing cell types in an OCT2-dependent manner. We also show that D1DR activation at the Golgi results in a distinct cAMP/PKA response, revealing a functional consequence of compartmentalized dopaminergic signaling. This work adds to the growing evidence that compartmentalized GPCR signaling regulates distinct cellular responses. Given the recent evidence for Golgi-

localized B1AR signaling in heart contractility responses through the regulation of specific effectors, we reason that subcellular D1DR signaling in MSNs of the striatum could also be regulating direct downstream effectors. For example, DARPP-32, a master regulator in the MSNs that shuttles between the cytosol and nucleus, could be a candidate for Golgi-D1DR regulation. Whether Golgi-localized signaling and subsequent local cAMP can regulate transcriptional responses remains to be seen. The results of this study are foundational in aiding the development of therapies that target dysfunctional dopaminergic signaling.

3.5 Figures

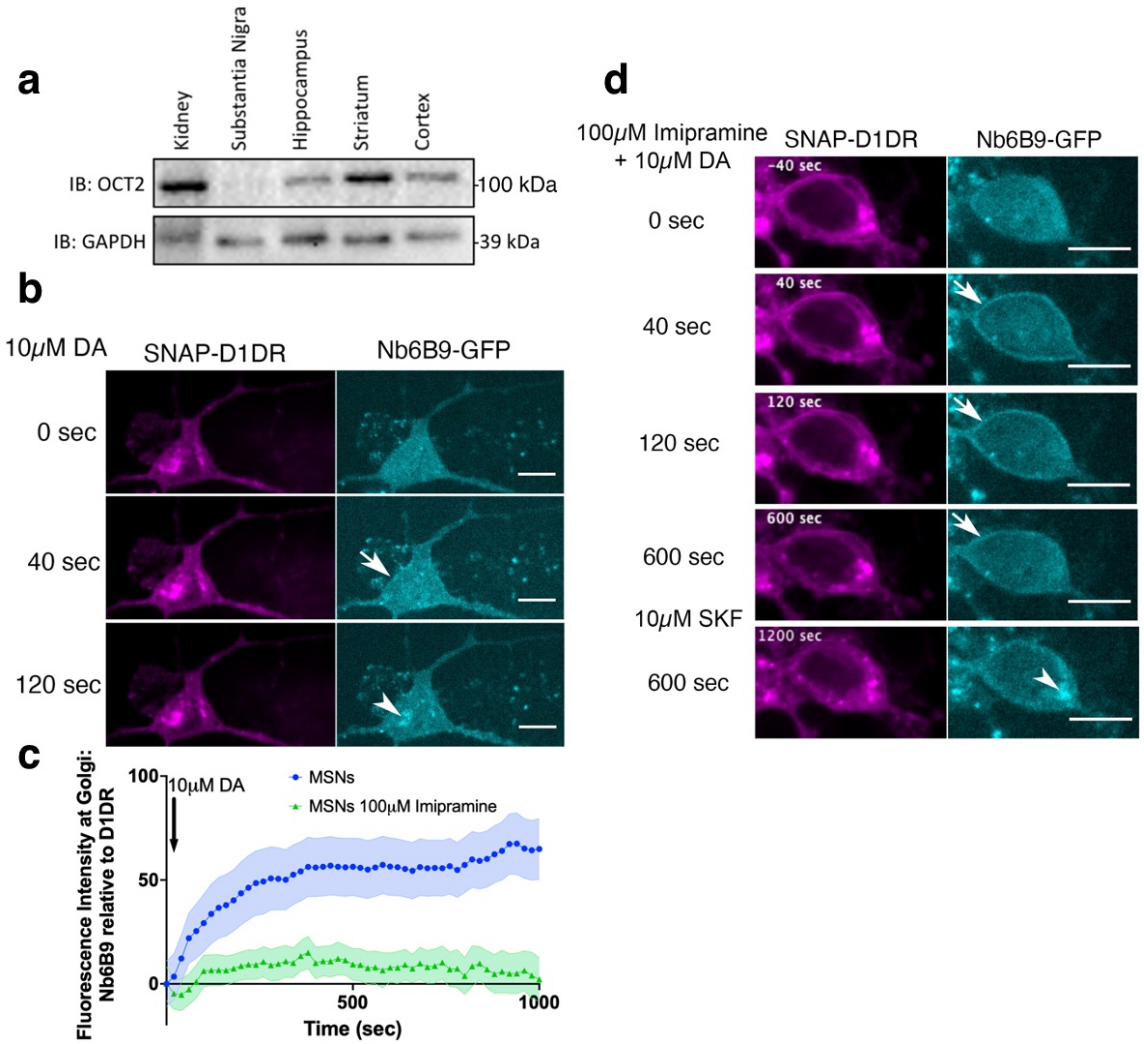


Figure 3.1 Regulation of dopamine-mediated activation of the Golgi-localized D1DR in striatal neurons by OCT2. a. Detection of OCT2 expression in different tissue extracts and brain slices by Western Blot **b.** Representative MSN expressing Snap-D1DR and Nb6B9-GFP at indicated times after 10μM dopamine addition. DA stimulates D1DR activation at the Golgi in MSNs (*n* = 22 cells, Pearson's coefficient = 0.67, 6 biological replicates). Arrow indicates active D1DR at plasma membrane; Arrowhead indicates active D1DR at Golgi membrane; Scale bar = 10μm. **c.** Quantification of Nb6B9-GFP recruitment at Golgi upon 10μM DA stimulation in MSNs cells pretreated with OCT2 inhibitor; Normalized fluorescence intensity of Nb6B9-GFP relative to Snap D1DR at Golgi (*n*= 12 and 7, respectively, 5 biological replicates). **d.** Representative MSN cell expressing Snap-D1DR and Nb6B9-GFP, pretreated with 100μM imipramine for 15 min, before and after 10μM dopamine addition. Inhibition of OCT2 blocks Golgi-localized D1DR activation at MSN (*n* = 18 cells, Pearson's coefficient = 0.38, 6 biological replicates but SKF81297 can still reach the Golgi membranes and activate D1DR Golgi pool (*n* = 6 cells, Pearson's coefficient = 0.75 4 biological replicates). Arrow indicates active D1DR at plasma membrane; Arrowhead indicates active D1DR at Golgi membrane; Scale bar = 10μm.

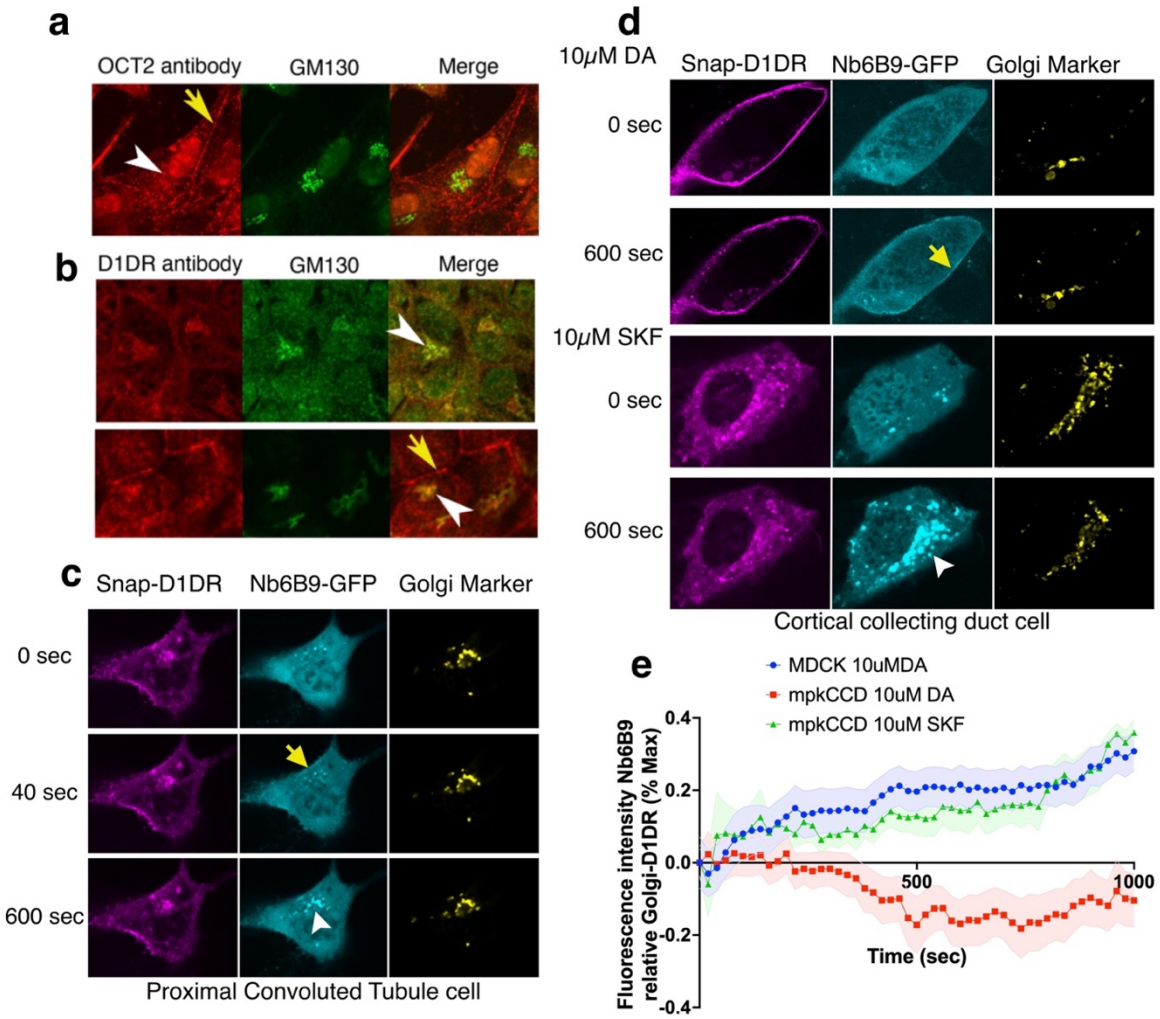


Figure 3.2 Regulation of dopamine-mediated activation of the Golgi-localized D1DR in striatal neurons by OCT2. a. Representative Mardin-Derby Canine Kidney (MDCK) cell, which are often used to depict the proximal convoluted tubule of the kidney. MDCK cell stained for endogenous OCT2. OCT2 is expressed on the plasma membrane and at the Golgi in MDCK cells, **b.** Representative MDCK cell stained for endogenous D1DR, which is endogenously expressed on the plasma membrane and Golgi. **c.** Representative MDCK cell expressing Snap-D1DR and Nb6B9-GFP at indicated times after 10μM dopamine addition. DA stimulates D1DR activation at the Golgi in PCT-like MDCK cells. **d**. Representative mpkCCD cell line, an immortalized murine cell line derived from cortical collecting duct. Cell expressing Snap-D1DR and Nb6B9-GFP at indicated times after 10μM dopamine or SKF addition. Yellow arrow indicates active D1DR at the plasma membrane; arrowhead indicates active D1DR at the Golgi **e.** Quantification of Nb6B9-GFP recruitment at Golgi upon 10μM DA stimulation in MDCK cells, and 10μM DA or SKF stimulation in mpkCCD cells; Normalized fluorescence intensity of Nb6B9-GFP relative to Snap D1DR at Golgi.

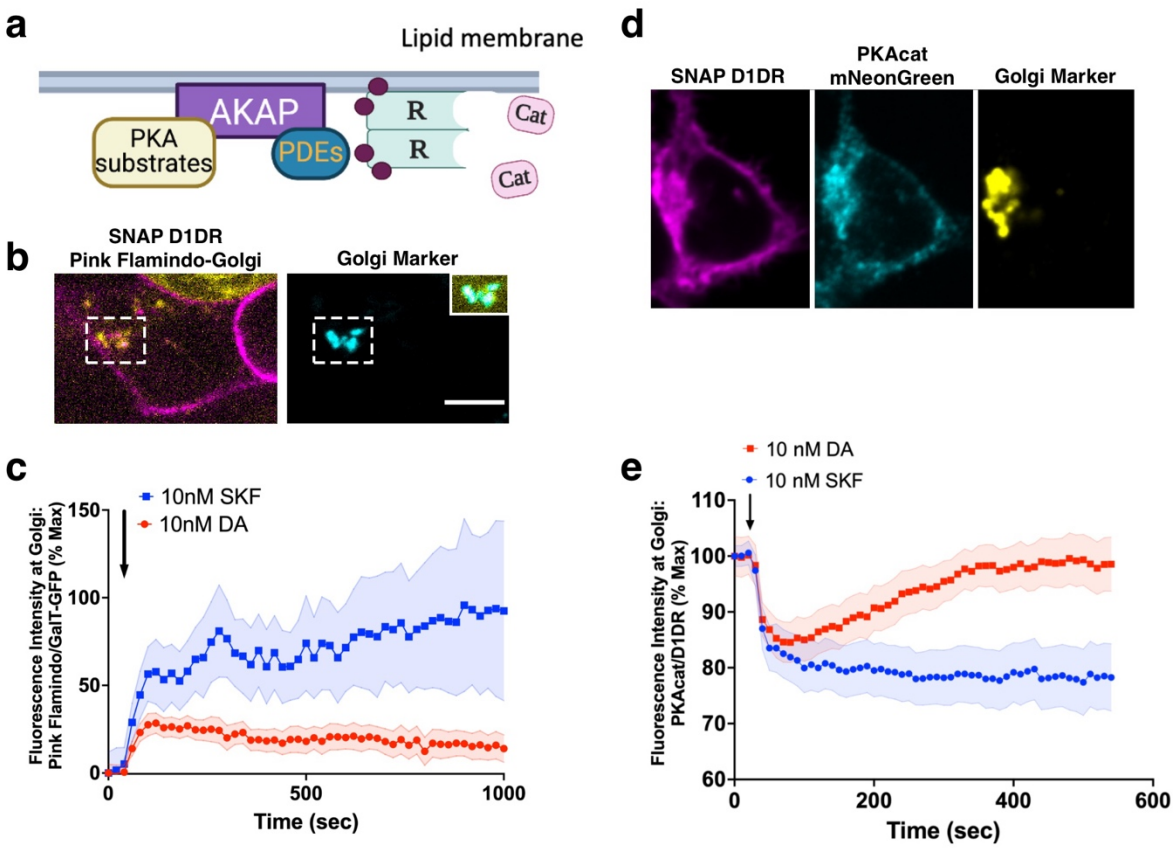


Figure 3.3 Golgi localized cAMP and PKA is activated by D1DR at the Golgi. a. Model of cAMP compartmentalization and PKA activation; phosphodiesterases (PDEs) are scaffolded to lipid membranes by anchoring proteins and degrade cAMP; cAMP binds PKA regulatory subunit (R), rendering PKA catalytic subunit (PKA-cat) dissociation. **b.** Confocal images of representative HEK293 cells expressing Snap-D1DR, Pink Flamindo-TGN, and Golgi marker; Scale bar = 10μm. **c.** Normalized fluorescence intensity of Pink Flamindo-TGN relative to Golgi after treatment with 10nM dopamine or SKF81297. **d.** Confocal images of representative D1DR-expressing HEK293 cells with endogenous PKA-cat-GFP and GalT-mRFP expression at indicated times after 10nM SKF81927or 10nM DA. Arrowhead indicates PKAcat at Golgi membrane. **e.** Normalized fluorescence intensity of PKAcat relative to Golgi-D1DR after treatment with 10nM DA or 10nM SKF81927.

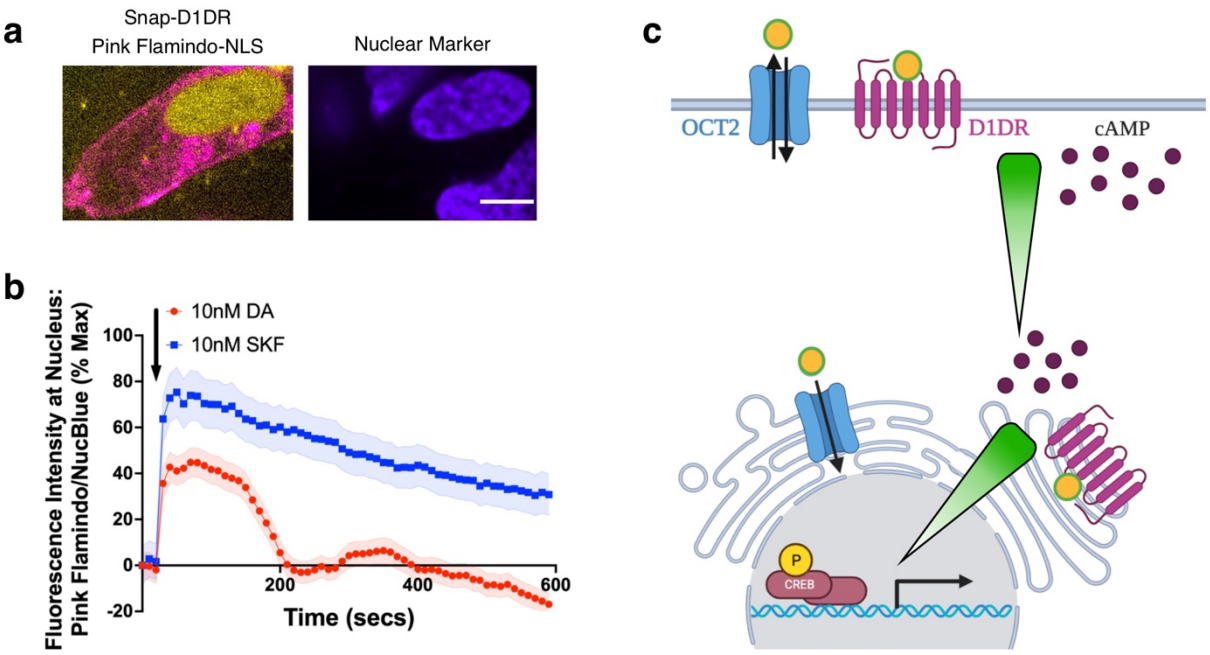
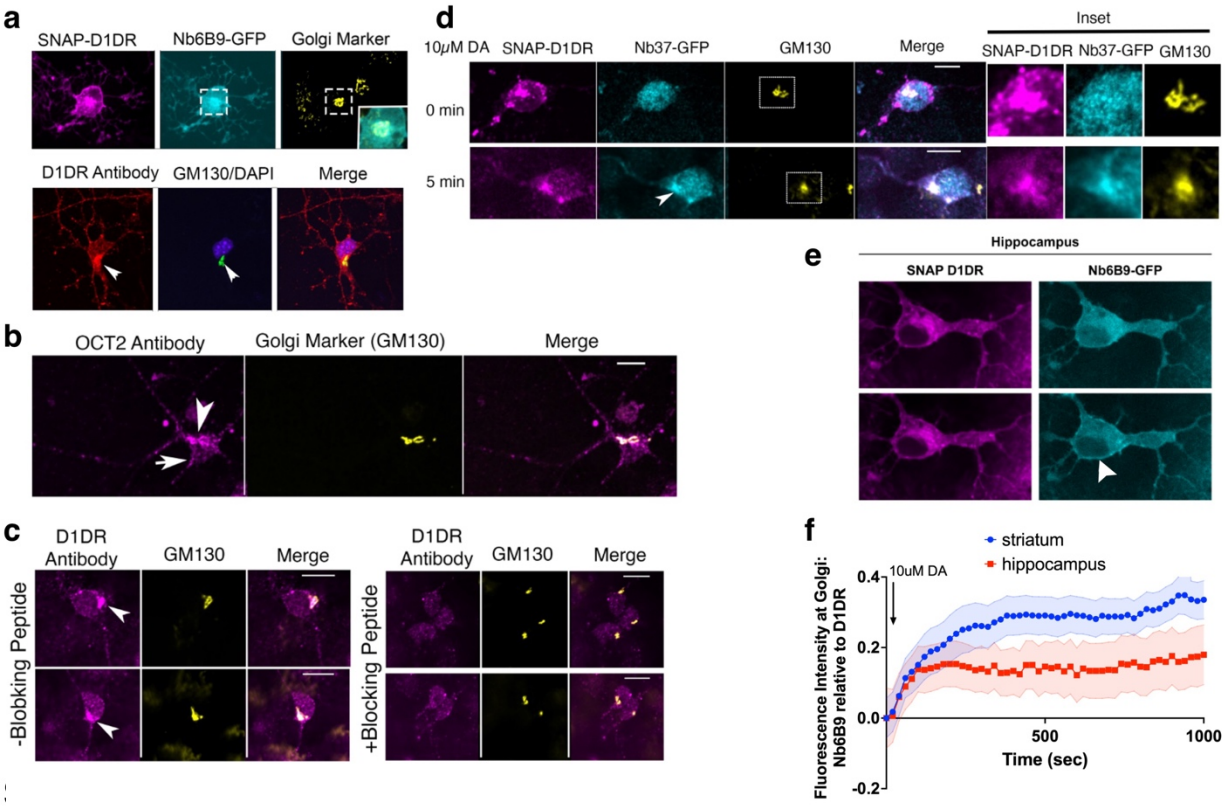
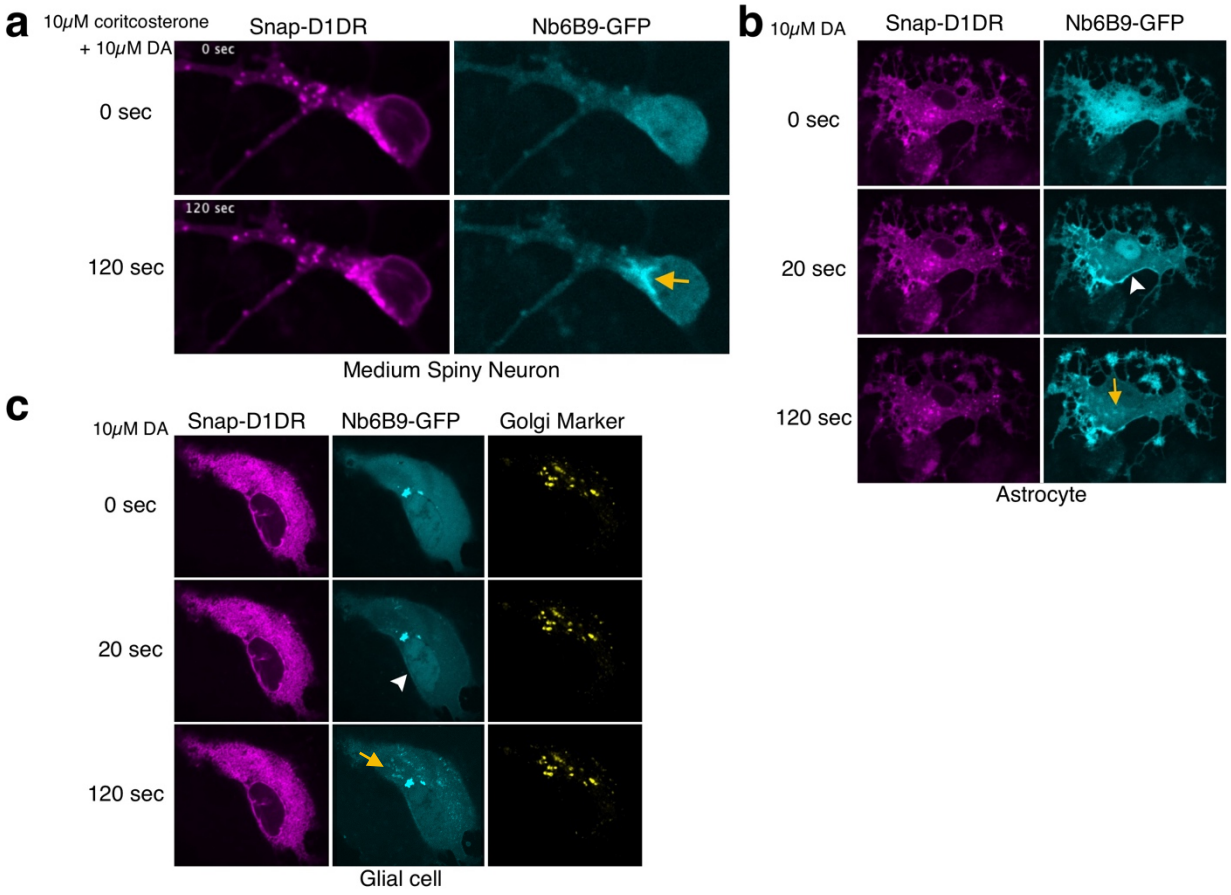


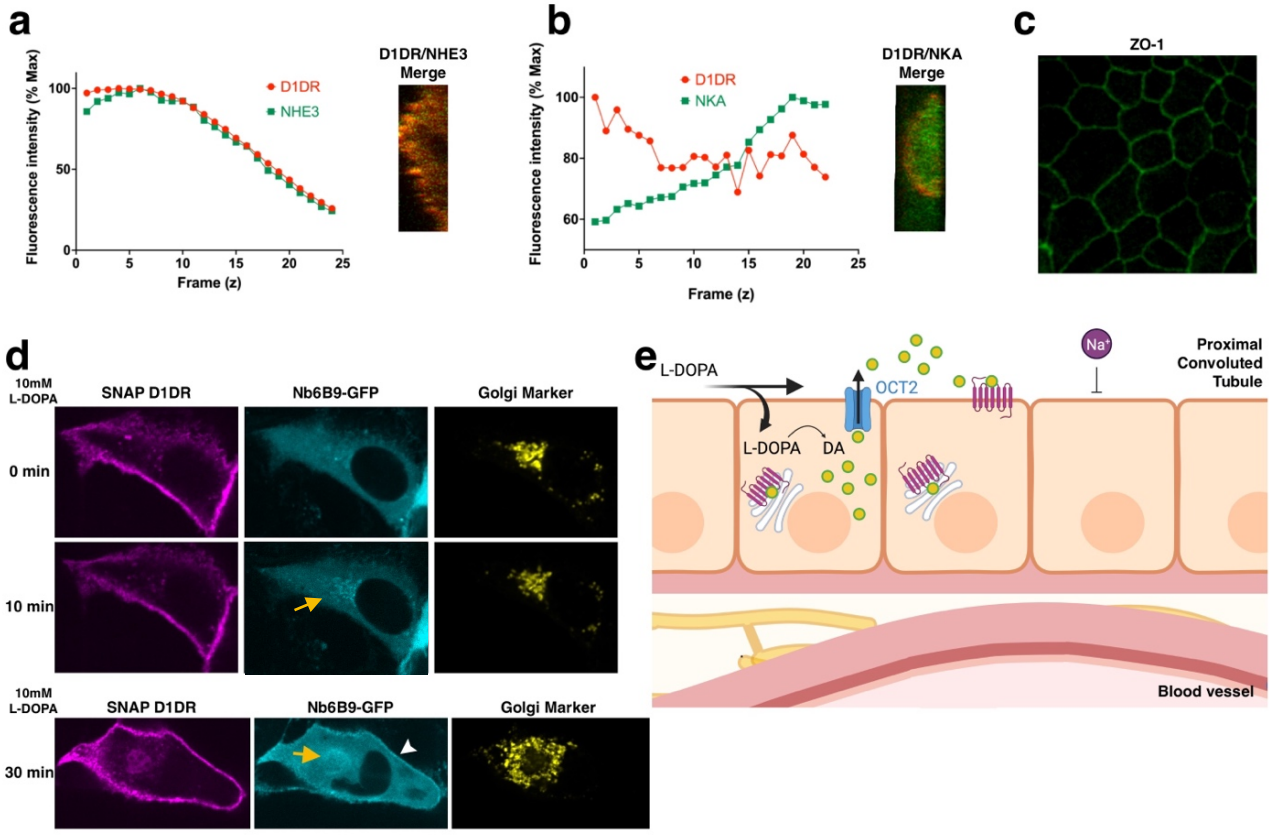
Figure 3.4 Activation of D1DR at the Golgi leads to sustained cAMP at the nucleus. a. Confocal images of representative HEK293 cells expressing Snap-D1DR, Pink Flamindo-NLS, and Golgi marker; Scale bar = 10μm. **b.** Normalized fluorescence intensity of Pink Flamindo-NLS relative to DAPI nuclear stain after treatment with 10nM dopamine or SKF81297. c. Proposed model of cAMP diffusion from plasma membrane and Golgi membrane; activation of D1DR at the Golgi results in a higher concentration gradient of cAMP near the nucleus which could allow for local regulation of transcriptional responses.



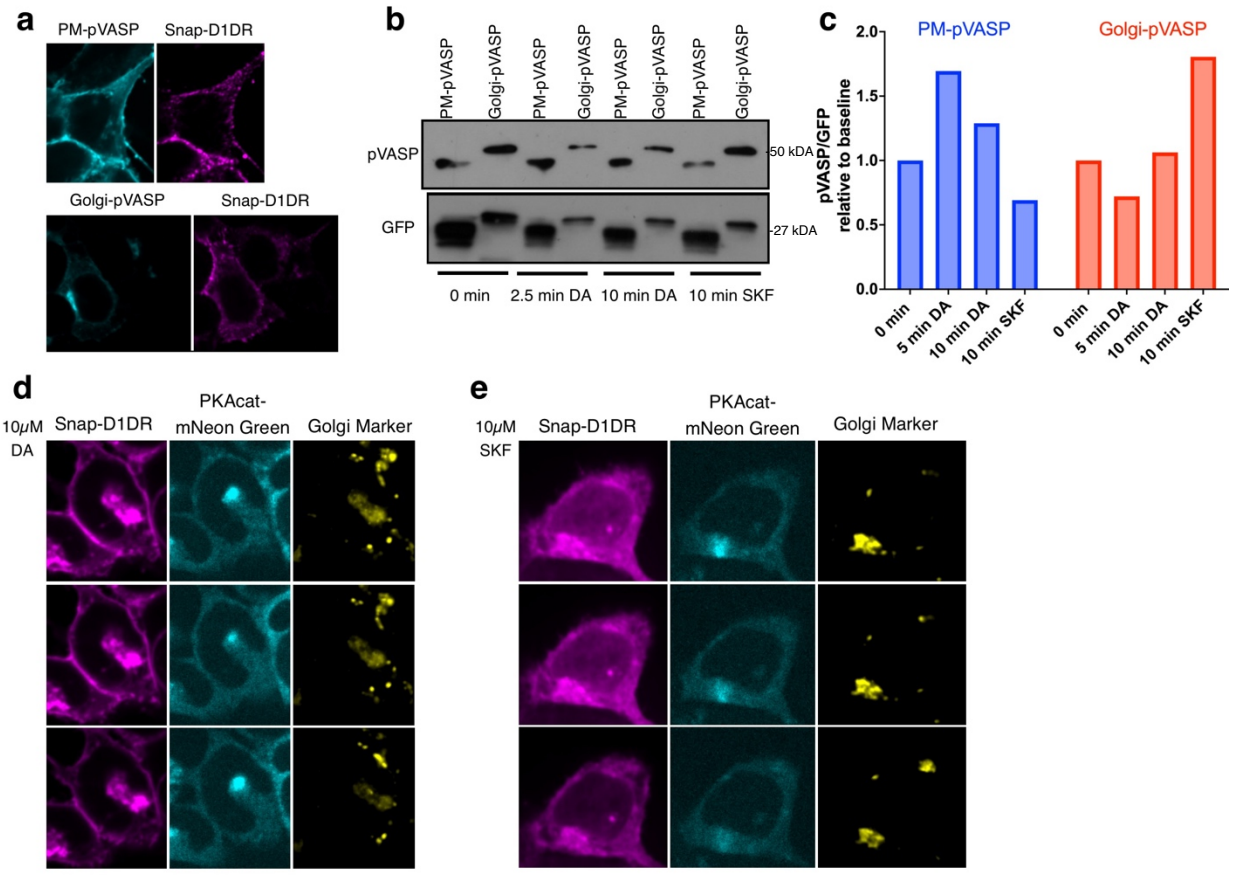
Supplementary Figure 3.1 a. Top panel, co-localization of Nb6B9-GFP at the Golgi after 10μM DA stimulation for 10 min. Lower panel, Endogenous localization of D1DR at the plasma membrane and the Golgi membranes. MSN cells were labeled with D1DR specific antibody and the Golgi antibody (GM130). Arrowhead indicates Golgi localizations. **b.** Endogenous localization of OCT2 at the plasma membrane and the Golgi membranes. **c.** Endogenous localization of D1DR at the plasma membrane and the Golgi membranes in the presence or absence of blocking peptide. MSN cells were labeled with D1DR specific antibody and the Golgi antibody (GM130). **d.** Representative MSN expressing Snap-D1DR and Nb37-GFP before and after 10μM dopamine addition. DA stimulates G protein activation at the Golgi in D1DR expressing MSNs (*n* = 10 cells, Pearson's coefficient = 0.34 and 0.62 before and after 10mM DA stimulation, 6 biological replicates). Arrowhead indicates active Gs at Golgi membrane; Right panels show zoomed images of insets for Snap-D1DR, Nb37-GFP and the Golgi marker (GM130). Scale bar = 10μm. **e.** Representative hippocampal neuron expressing Snap-D1DR and Nb6B9-GFP before and after 10μM dopamine addition. **f.** DA stimulates D1DR activation at the plasma membrane, and less so at the Golgi, in D1DR expressing hippocampal neurons.



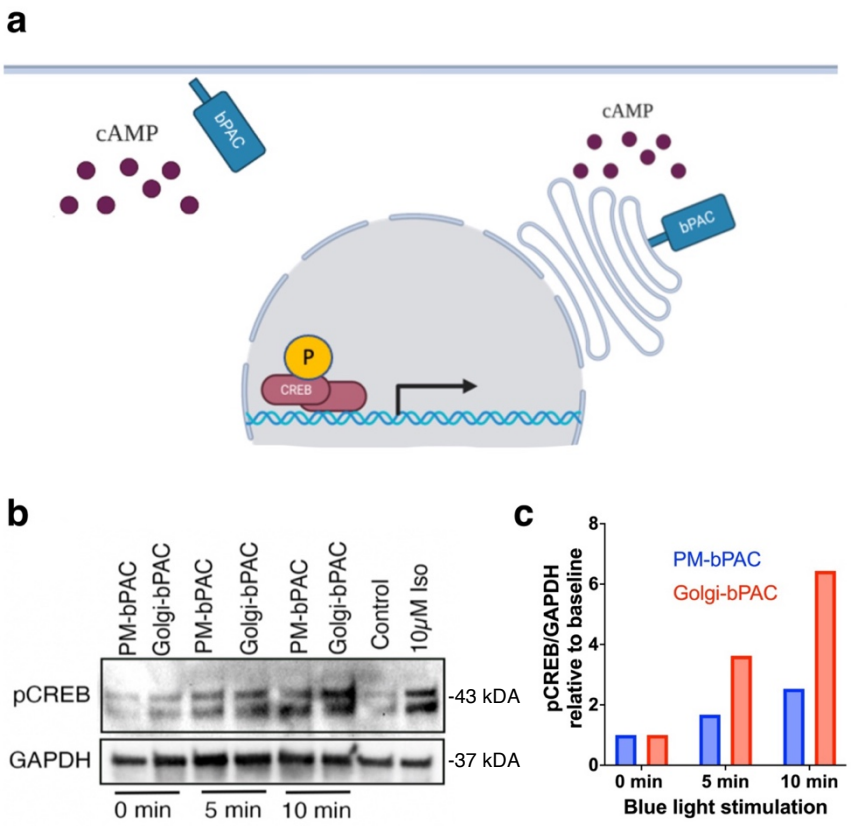
Supplementary Figure 3.2 a. Representative MSN cell expressing Snap-D1DR and Nb6B9-GFP, pretreated with 10μM corticosterone for 15 min, before and after 10μM dopamine addition. Inhibition of OCT3 does not block Golgi-localized D1DR activation at MSN. Non-neuronal primary cells, namely representative astrocyte cell (**b**) and glial cell (**c**) from brain section isolations before and after 10μM dopamine addition.



Supplementary Figure 3.3 a. Graphical representation and representative orthogonal image of fluorescence intensity of D1DR localization (red) and localization of sodium transporters NHE3 (a) or NKA (b) in green in polarized MDCK cells grown in 3D transwell cultures. **c.** Endogenous localization of ZO-1 at tight junctions of polarized MDCK cells grown in 3D culture. **d.** Representative non-polarized MDCK cells expressing Snap-D1DR, Nb6B9-GFP, and Golgi marker before and after 10mM L-DOPA addition. D1DR at the Golgi is activated by intracellular DA 10 minutes after L-DOPA addition (top panels), and can be transported out of cell to activate D1DR at the plasma membrane at later time points (lower panel). Yellow arrow indicates active D1DR at the Golgi; white arrowhead indicates active D1DR at the plasma membrane. e. Proposed model of compartmentalized dopaminergic signaling pathway in kidneys; L-DOPA derived from adrenal glands is transported through the glomerulus into proximal tubule cells of the nephron; DA is derived from L-DOPA intracellularly and is then transported into the lumen of the nephron to act on D1DR localized to the apical membrane of PCT cells; intracellular DA derived from L-DOPA can activate D1DR localized to the Golgi membrane of PCT cells.



Supplementary Figure 3.4 a. Representative HEK293 cell expressing Snap-D1DR and PKA reporter (pVASP) targeted to the plasma membrane (top panel) or Golgi membrane (bottom panel). **b.** Western blot probing phosphorylated VASP at the plasma membrane or Golgi before and after stimulation with 1μM dopamine or SKF81297 at indicated times. **c.** Quantification of western blot shown in b. **d.** Representative image of HEK293 knock-in cell line fluorescently tagging endogenous PKA catalytic subunit before and after addition of 10μM DA at 2 min (middle panel) and 10 min (bottom panel). **e.** Representative image of HEK293 knock-in cell line fluorescently tagging endogenous PKA catalytic subunit before and after addition of 10μM SKF at 2 min (middle panel) and 10 min (bottom panel).



Supplementary Figure 3.5 a. Model depicting experimental setup; HEK293 cells expressing bPAC targeted to either the plasma membrane and Golgi membrane are stimulated by blue light to produce local pools of cAMP; CREB phosphorylation is measured after stimulation with blue light. **b.** Western blot probing phosphorylated CREB before and after blue light stimulation at indicated times, and quantification (**c**) of western blot shown.

3.6 Materials and methods

Table 3.1 Key Resources

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Cell line	HEK293T	ATCC	ACS-4500	Mycoplasma Tested negative
Cell line	MDCK	ATCC	NBL-2	Mycoplasma Tested negative
Cell line	mpkCCD	ATCC	CVCL_R771	Mycoplasma Tested negative
Antibody	Rabbit Anti-Dopamine Receptor D1	Abcam	ab216644	1:100
Antibody	Mouse Anti-GM130	BD biosciences	610822	1:1000
Antibody	Rabbit Anti-SLC22A2	Abcam	ab170871	1:1000
Antibody	Rabbit Anti-SLC22A2	ABClonal	A14061	1:100
Antibody	GAPDH	Proteintech		1:10000
Antibody	HRP-conjugated rabbit IgG	GE Healthcare		1:10000
Antibody	Sheep anti-mouse IgG	GE Healthcare		1:10000
Antibody	Sheep anti-rabbit IgG	GE Healthcare		1:10000
Antibody	Mouse Anti-Myc	Santa Cruz Biotechnology	9E10	1:200
Antibody	Mouse phospho-VASP (Ser157)	Abcam	ab58555	1:1000
Antibody	Mouse Anti-ZO-1	Invitrogen	33-910	1:500
Antibody	Mouse Anti-Flag M1	Sigma	F3040	1:1000
Antibody	Mouse Anti-CREB	Cell Signaling Technology	86B10	1:1000
Antibody	Rabbit Phospho-CREB (Ser133)	Cell Signaling Technology	87G3	1:1000
Antibody	Rabbit Anti-Dopamine Receptor D1	Proteintech	17934-1-AP	1:100

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Peptide	D1DR blocking peptide	Proteintech	Ag12366	1:20
Conjugated dye	Snap-Cell 647	NEB		
Chemical compound, drug	Dopamine hydrochloride	Sigma		
Chemical compound, drug	SKF81297 hydrobromide	Tocris		
Recombinant DNA reagent	pCAG-Snap-D1DR	This study		pCAG vector
Recombinant DNA reagent	pCAG-Nb6B9-GFP pCAG-Nb37-GFP	This study		pCAG vector

Image analysis and statistical analysis

Images were saved as 16-bit TIFF files. Quantitative image analysis was carried out on unprocessed images using ImageJ software (http://rsb.info.nih.gov/ij). For measuring kinetics of Nb6B9–GFP and miniGs recruitment at the Golgi membrane over time in confocal images and kinetics of PKA-Cat GFP dissociation from the Golgi, analyses were performed on unprocessed TIFF images using custom scripts written in MATLAB (Jullie et al., 2020). Values were normalized by calculating the percent relative to the maximum value, then baseline corrected using Prism 6.0 software so that the first value of each condition was set to 0.

Isolation of murine striatal neurons

Primary striatal neurons were prepared from P1-P2 CD1 pups. In brief, striatum tissues isolated from brains in cold HBSS (w/o Mg^{2+}, Ca^{2+} and phenol-red) buffer with 10mM HEPES were treated by HBSS with 0.25%Trypsin and 10mM HEPES buffer at 37°C for 15 min. The digested striatum tissues were rinsed by neural plating media (DMEM with 10%FBS, 30mM HEPES, and PS) twice, and then dissociated by trituration using fire-polished Pasteur pipet in neural plating media. Suspended cells that pass through a 40μm strainer were collected by centrifuging at 350 x g for 5 min. Cells were plated at 10^3 cells per mm^2 on the 100□g/ml poly-D-lysine (Sigma) coated imaging dishes or coverslips in neural plating media. After 16-24hr, the culture media were replaced by neural differentiation media (Neural basal media with 10mM Glutamax, B27, and PS). The 50% media were replaced by fresh neural differentiation media every 3-4 days.

Live-cell confocal imaging

Live cell imaging was carried out using Nikon spinning disk confocal microscope with a 60 ×, 1.4 numerical aperture, oil objective and a CO_2 and 37 °C temperature-controlled incubator. A 488, 568 nm and 640 Voltran was used as light sources for imaging GFP, mRFP/mApple, and Snap-647 signals, respectively. Cells expressing both Snap-tagged receptor (2μg) and the indicated nanobody–GFP (200ng) were plated onto glass coverslips. Receptors were surface labelled by addition of Snap-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min, as described previously. Live cell images

where endocytosis was inhibited were carried out by incubating the cells in 30μM Dyngo 4a (ab120689) at 37 °C for 30 minutes before indicated agonist was added. HEK293 PKA-Cat-GFP knock-in cells were a generous gift from the Huang Lab. Indicated agonists (dopamine hydrochloride-Sigma, SKF81297 hydrobromide-Tocris) were added and cells were imaged every 20 s for 20 min in DMEM without phenol red supplemented with 30 mM HEPES, pH 7.4. NPEC-caged-dopamine (Tocris) was incubated for 10 min before cells were stimulated with 3.2 ?W/cm^2 blue light. Time-lapse images were acquired with a CMOS camera (Photometrics) driven by Nikon Imaging Software (NS elements).

Fixed-cell confocal imaging

Cells were permeabilized with saponin to reduce the cytoplasmic background, as described previously(Lobert & Stenmark, 2012). Briefly, HeLa cells were permeabilized with 0.05% saponin (Sigma) in PEM buffer (80mM K-Pipes, pH 6.8, 5 mM EGTA, 1mM MgCl2) for 5 min on ice. Cells were then fixed with 3% paraformaldehyde in PBS for 10 min and then quenched with 50mM Nh4Cl in PBS for 15 min. Primary antibodies D1DR antibody (ab216644) (1:100), D1DR (Proteintech 17934-1AP) (1:100) with or without D1DR blocking peptide (Proteintech Ag12366) (1:25), GM130 (BD biosciences 610822) (1:1000), and SLC22A2/OCT2 antibody (ab170871) or SLC22A2/OCT2 (ABClonal-A14061) (1:100), were diluted in PBS supplemented with 0.05% saponin. Striatal neurons

at DIV5 were fixed by 3.7% formaldehyde in PEM buffer for 15 min and then permeabilized by 0.3% Triton in PBS for 5 min at room temperature. D1DR and GM130 antibodies were diluted in TBS with 5% donkey serum and 0.1% Triton X-100. Confocal images were taken using Nikon spinning disk confocal microscope with a 60x 1.4 numerical aperture, oil objective.

Western blotting

Cells from HEK293, HEK293T and HeLa were lysed in extraction buffer (0.2% Triton X-100, 50mM NaCl, 5mM EDTA, 50mM Tris at pH 7.4 and complete EDTA-free protease inhibitor cocktail (Roche). Kidney and neural tissues from B6 adult mice were disrupted in RIPA buffer (50mM Tris at pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate 0.1% SDS and complete EDTA-free protease inhibitor cocktail). After agitation at 4°C for 30 min, supernatants of samples were collected after centrifuging at 15000 x rpm for 10 min at 4°C. Supernatants were mixed with SDS sample buffer for the protein denaturation. The proteins were resolved by SDS-PAGE and transferred to PVDF membrane and blotted for anti-SLC22A2 (ab170871-1:1000) or GAPDH (1:10,000) antibodies to detect OCT2 and GAPDH expression by horseradish-peroxidase-conjugated rabbit IgG, sheep anti-mouse and rabbit IgG (1:10,000 Amersham Biosciences) and SuperSignal extended duration detection reagent (Pierce).

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3.8 Acknowledgments

We thank D. Jullie, K. Silm, A. B. Lobingier, A. Manglik, E. Hernandez, D. Larsen, and S. Yee for assistance, advice and valuable discussion. These studies were supported by the National Institute on General Medicine (GM133521) to R.I and The American Heart Association (908933) to N.P.

3.9 Author Contributions

Natasha Puri conceived and executed project, contributed to interpreting results and writing. Ting-Yu Lin isolated and plated primary murine striatal neurons. Roshanak Irannejad helped designed the experimental strategy, contributed to interpreting results and writing.

Chapter 4: Discussions, speculations and future directions

Natasha Mukherjee Puri

4.1 Overview

GPCRs have been a widely studied protein superfamily for decades, and for good reason. A third of all pharmaceutical drugs target GPCRs, and they help regulate nearly all our bodily functions. The more recent evidence that some GPCRs can signal from distinct membrane compartments has shaken up an incredibly well-established field. Discovery of this novel signaling pathway has provided an explanation of why some drugs work better than others based on their ability to access intracellular receptor and will greatly impact the development of targeted therapies to treat dysfunctional GPCR signaling. Our work has added the D1 dopamine receptor to the growing list of compartmentalized GPCRs that play a major role in physiology and pathophysiology. In this chapter, I will speculate on what the work presented in this thesis could mean for the fields of GPCR biology and dopaminergic signaling.

4.2 Regulatory mechanisms of compartmentalized signaling

The earliest evidence of compartmentalized GPCR signaling came from data showing persistent cAMP signaling following robust internalization of the parathyroid hormone receptor and the thyroid stimulating hormone receptor. Since then, several GPCRs have been identified that signal from endosomes, including the beta 2 adrenergic receptor (β2AR), mu and delta opioid receptors, and D1 dopamine receptor (D1DR) (Calebiro et al., 2009; Ferrandon et al., 2009; Kotowski et al., 2011; Tsvetanova & von Zastrow, 2014). It was much more recently discovered that the beta 1 adrenergic receptor (β1AR) and D1DR can signal from the Golgi apparatus (Irannejad et al., 2017; Lin et al., n.d.; Nash et al., 2019). The Golgi processes newly synthesized proteins, allowing for the necessary post-translational modifications to ensure proper function and trafficking. GPCRs, like all other proteins, are processed at the Golgi and then trafficked to the plasma membrane. Interestingly, we have found that β1AR and D1DR exist as a stable pool in the Golgi, independent of retrograde trafficking of receptor from the plasma membrane (Irannejad et al., 2017; Puri et al., 2022). These discoveries are intriguing. First, they identify a completely novel function of the Golgi as a signaling hub. Next, they push us to wonder about the characteristics of these distinct pools of receptor. In our studies, we overexpress receptor in order to measure a robust activation readout. In this synthetic system, it is reasonable high amounts of exogenous protein would get stuck at the Golgi. But endogenously, how the Golgi decides which

receptors are trafficked to the plasma membrane and which solely exist at the Golgi is unclear. There is evidence that palmitoylation allows transport of proteins to the cisternal rim of the Golgi for anterograde trafficking. It is possible that distinct PTMs allow for a stable pool of receptor to exist at the Golgi and a separate pool to target to the plasma membrane. Interestingly, endogenous PKA catalytic subunit (PKAcat) and overexpressed D1DR both colocalize with the cis-Golgi (Chapter 3). I speculate that once at the cis-Golgi, a portion of the newly synthesized D1DR is palmitoylated in order to be transported into the trans-Golgi network (TGN) for anterograde trafficking to the cell surface. If these separate pools do exist at the Golgi, it would be interesting to know whether the transient pool of receptor at the contributes to intracellular signaling. Given our findings that organic cation transporter 2 (OCT2) is necessary and sufficient for Golgi-D1DR activation by dopamine, another question that arises is whether a Golgi-D1DR pool exists in dopaminergic cells that do not express OCT2, such as in presynaptic neurons of the substantia nigra. If Golgi-D1DR is unique to OCT2 expressing cells, how this is regulated in different cell types? If not, then it is possible that dysfunctional dopaminergic signaling in disease states could be partially attributed to Golgi-D1DR activity in regions where it normally would not occur.

Evidence that Gαs-coupled receptors can signal from discrete membrane compartments has helped to explain some mysteries in the field. For example, cAMP is

highly compartmentalized due to membrane bound regulatory mechanisms like AKAPs that scaffold PDEs and PKA, yet it acts on effectors at the Golgi, nucleus, and other organelles that are spatially distant from the plasma membrane (Cheng & Grande, n.d.; Maurice et al., 2014; Mongillo et al., 2004; Saucerman et al., 2014). In addition to the plasma membrane, AKAPs have been shown to localize to the Golgi, endoplasmic reticulum, and nucleus as well, further preventing plasma membrane derived cAMP from acting at these locations. It is reasonable that receptor activation and subsequent cAMP production would regulate local effectors, and that regulatory machinery would exist at distinct sources of signaling as well. However, it is unclear how AKAPs and the proteins they scaffold are distributed at the endogenous level in different cell types. Based on evidence described in chapter 3 showing transient cAMP/PKA levels downstream of plasma membrane D1DR activation, it is possible that there are higher degrees of AKAP regulation at the plasma membrane to abrogate signaling there. Understanding the membrane distribution of other GPCR effectors, such as adenylyl cyclase and G proteins, would also help uncover the differences in signaling dynamics at distinct locations.

While chapter 2 focuses on Golgi-localized D1DR activation, our biosensors also showed active receptor at endosomes as well. This is a unique characteristic, even compared to other compartmentalized GPCRs. β1AR and β2AR have been shown to

signal from the Golgi and endosomes, respectively. D1DR is one of the first compartmentalized receptors to show the ability to signal from the plasma membrane, endosomes, and Golgi. While, perhaps egregiously, we do not comment on the role of endosomal-D1DR signaling, Kotowski et al showed that internalized D1DR in medium spiny neurons (MSNs) of the striatum do signal and contribute to a rapid cAMP response at early time points after activation. This in conjunction with our work shows that three separate phases of signaling exist in one relevant D1DR cell type. In chapter 3, we show that stimulating receptor at the plasma membrane does result in a rapid but transient diffusion of cAMP to the Golgi and nucleus. Given the dynamic mobility of endosomes, it is very likely that the rapid and early generation of cAMP from endosomes seen by Kotowski et al could be the source of transient cAMP found at the perinuclear region. There is also evidence that D1DR exists in yet another membrane compartment: primary cilia. Ciliary localization of D1DR is thought to be transient yet necessary for normal signaling patterns in MSNs, and its disruption is associated with obesity in mice due to reduced movement. Studying how and when all these distinct signaling hubs function is absolutely vital to understanding the nuances of dopaminergic pathways. Whether these discrete phases of compartmentalized signaling occur in other D1DR-expressing cells, such as hippocampal neurons and proximal tubule kidney cells, needs to be studied as well.

While GPCRs are traditionally categorized based on which G protein they couple to, recent evidence has shown that some GPCRs can couple to more than one G protein type. Jang et al recently described how the conformation of the G protein, especially in the intermediate state of nucleotide release, can help predict G protein selectivity. They show that D1DR is among the group of GPCRs that can couple to both Gαs and Gαi (Jang et al., 2023). This could also be a mechanism of regulating signal dynamics at distinct locations. Several questions are raised from this work, such as what the kinetics of dual coupling are, and at which membrane compartments does dual coupling occur. The distribution of Gαs and Gαi in different cell types and at different membrane locations needs to be studied in order to better understand how dual coupling impacts signaling in relevant cell types. Another important question that should be addressed is whether other dopamine receptor types can also signal from distinct membrane compartments. Given their homology, it is possible other dopamine receptor types are compartmentalized.

4.3 Organic Cation Transporters as transmitters of signaling cascades

The work presented in this thesis along with evidence showing Golgi-β1AR signaling mediated by organic cation transporter 3 (OCT3) provides the first evidence of this family of uptake2 transporters in mediating GPCR signaling. This revealed a novel role of OCTs in the mediating intracellular signaling and could implicate OCTs in mediating multiple distinct signaling cascades. In the brain, OCT2 has been shown to regulate mood and behavior related responses mainly through synaptic clearance of norepinephrine and serotonin. Mouse studies have implicated OCT2 in anxiety and depression-related behaviors through its interactions with these neurotransmitters (Amphoux et al., 2006; Koepsell et al., 2007). This suggests a potential role for OCT2 in facilitating other intracellular signaling cascades as well. OCT2 activity is also blocked by a number of drugs, ranging from drugs of abuse like amphetamines to drugs to treat diabetes, impacting OCT2 activity at the brain and kidney respectively. Those studying the consequences of these off-target effects should take into account the potential modulation of dopaminergic activity, and possibly other neurotransmission.

4.4 Consequences of compartmentalized dopaminergic signaling in neurons

D1DR is one of the major dopamine receptor types in the brain, involved in the regulation of learning, memory, and motor function. Our recent finding that D1DR can signal from distinct membrane compartments in medium spiny neurons (MSNs) of the striatum has massive implications in how dopaminergic signaling regulates brain function. Moreover, this evidence emphasizes the need to understand the location of dysfunctional signaling in disease states such as Parkinson's disease, addiction, and other psychiatric disorders such as schizophrenia and Huntington's disease. To understand the consequences of compartmentalized dopaminergic signaling in the striatum, it is imperative to identify whether downstream effectors are differentially regulated by different pools of D1DR. Additionally, the role of OCT2-mediated compartmentalized dopaminergic signaling in non-neuronal brain cells, such as glial cells and astrocytes, needs to be investigated.

Intracellular calcium signaling is facilitated by D1DR-induced cAMP/PKA and possibly phospholipase C (PLC) activity. PLC-activating D1DR has been proposed to be molecularly distinct from cAMP-activating D1DR, although this has not been confirmed. PLC activity mediates release of calcium from intracellular stores in the endoplasmic reticulum (ER). This activity is also modulated by an AKAP9/PKA complex that associates with the ER-localized ligand-gated Ca^{2+} channel in order to initiate Ca^{2+}

oscillations in MSNs (Banerjee & Hasan, 2005; Brini et al., 2014; Catoni et al., 2019; Slocum et al., 2022). D1DR-mediated cAMP/PKA activity also facilitates Ca^{2+} influx via gated ion channels on the cell surface that are necessary to maintain Ca^{2+} oscillations. Interestingly, disrupting AKAP9 interactions at the ER impact the timing of initial Ca^{2+} flux and the frequency of subsequent Ca^{2+} oscillations. Given the perinuclear localization of AKAP9/PKA and Ca^{2+} release, it is reasonable to speculate that Golgi-D1DR is involved in regulation of intracellular Ca^{2+} signaling while D1DR at the plasma membrane facilitates Ca^{2+} influx to replete subcellular stores. It would be interesting to selectively activate D1DR at the Golgi or plasma membrane and measure cAMP and PLC mediated intracellular Ca^{2+} signaling. This could reveal a novel mechanism of action, identifying distinct pools of D1DR working in concert to facilitate a dopaminergic signaling pathway essential for neuronal function.

DARPP-32, a master regulator in MSNs of the striatum, is also a possible effector downstream of Golgi-specific D1DR regulation. Upon phosphorylation of Threonine34 by PKA, DARPP-32 becomes a potent inhibitor of phosphatase 1 (PP1) to block attenuation of D1DR-mediated signaling (Greengard et al., 1999; Yger & Girault, 2011). DARPP-32 is selectively expressed in MSNs of the striatum and has been shown to contribute to higher and more sustained D1DR-mediated cAMP/PKA responses compared to responses in pyramidal neurons of the cortex. PKA-dependent

phosphorylation of DARPP-32 and subsequent dephosphorylation by phosphatase 2A (PP2A), also activated by PKA phosphorylation, of Serine 97 residue near its nuclear export sequence results in rapid accumulation in the nucleus (Kuroiwa et al., 2008). Nuclear DARPP-32 is plays a role in gene expression by phosphorylation of histone H3. It is possible that cAMP/PKA responses at the Golgi play a role in the tightly controlled localization of DARPP-32 at perinuclear/nuclear regions. Interestingly, the kinetics of Thr34 phosphorylation are much faster than Ser97 dephosphorylation, suggesting that these responses could be regulated by distinct sources of cAMP/PKA.

DARPP-32 has also been attributed to mediate D1DR-dependent modulation of memory function (Essam & Kandil, 2023; Tropea et al., 2008). Our preliminary evidence that cAMP generated from the Golgi can result in increased CREB phosphorylation suggests that Golgi-D1DR activity could be regulating both DARPP-32 and CREB phosphorylation. However, we must consider the evidence from other GPCRs that endosomal signaling significantly contributes to the regulation of transcriptional responses, in particular CREB-mediated transcription. Given that D1DRs can also signal from endosomes, it is a possibility that endosomal and Golgi-D1DR activity work in conjunction to regulate CREB activity. Perhaps temporal modulation of CREB phosphorylation is executed by different pools of D1DR. Given that use of drugs associated with addiction disorders results in a rapid increase of nuclear DARPP-32 and

CREB activity, those studying the molecular mechanisms behind substance abuse should consider the role of increased Golgi-D1DR activity in drug-mediated transcriptional responses.

What is the role of tonic and phasic dopamine release in dynamic signaling cascades? Tonic dopamine regulates the indirect D2 pathway, acting on D2DR to decrease cAMP levels (Yapo et al., 2017; Dreyer et al., 2010). These low levels of synaptic DA release are thought to not act on D1DR, which has a lower affinity for its endogenous ligand compared to D2DR. Phasic bursts of dopamine, on the other hand, are thought to activate the direct pathway via D1DR (Dreyer et al., 2010). However, the transport of dopamine intracellularly following tonic and phasic dopamine release are unknown. Sustained cAMP/PKA responses in MSNs have been shown to be specifically mediated by phasic dopamine release (Castro et al., 2013). I hypothesize that large amounts of dopamine release specifically activate intracellular D1DR, whereas consistently low levels of dopamine potentially mediate basal levels of plasma membrane-D1DR activity. As previously mentioned, there is growing evidence that D1DR can couple to both Gαs and Gαi. How G protein selectivity is controlled in dopaminergic neurons is not known. In the striatum, medium spiny neurons are mainly divided into separate D1 and D2 neurons, but there exists a small but significant population of neurons expressing both D1DR and D2DR (Bateup et al., 2010). Understanding the distribution

of G protein types in the D1-specific and co-expressing dopaminergic neurons can help to reveal how G protein selectivity is regulated. Whether dual coupling of D1DR occurs in physiologically relevant cell types, and if D1DR preferentially couples to one G protein over another at different membrane compartments, will be an important area of study to uncover distinct dynamics of D1DR activity.

The mechanisms of intracellular dopamine regulation need to be identified in order to understand dopaminergic signaling dynamics. In previous chapters, we established that OCT2 is a key modulator of dopamine transport into cells. However, there are other mechanisms of dopamine removal or degradation that could be regulating intracellular dopamine concentrations. Monoamine oxidase (MAO), for example, is responsible for degrading dopamine. Catechol-O-methyltransferase (COMT) is also involved in the breakdown of dopamine. However, these enzymes have been mainly studied in presynaptic neurons, as they help terminate dopaminergic transmission. MAO and COMT activity in postsynaptic OCT2/D1DR-expressing neurons must be further investigated to explore their role in regulating subcellular dopaminergic signaling. Intracellular dopamine levels in disease states are also important to consider. Dopamine precursor L-DOPA is a common and effective treatment of Parkinson's Disease, a neurodegenerative disease characterized by degradation of midbrain dopaminergic neurons that project into the striatum. L-DOPA treatment provides

another source of dopamine when neurons of the substantia nigra can no longer mediate dopaminergic transmission through the synaptic cleft. Aromatic amino acid decarboxylase (AADC) converts L-DOPA into dopamine, thereby providing an exogenous source of intracellular dopamine in both pre and postsynaptic dopaminergic neurons. Prolonged L-DOPA treatment in PD patients can lead to L-DOPA induced dyskinesia, a motor disorder resulting in uncontrollable, hyperkinetic movement. It is possible that some of the symptoms of extended L-DOPA treatment are due to overstimulation of Golgi-localized D1DR. Furthermore, if non-OCT2 expressing dopaminergic neurons do have an inactive, subcellular pool of D1DR, L-DOPA treatment could be facilitating abhorrent dopaminergic signaling.

It remains to be seen whether there is a functional consequence of intracellular signaling in neurons beyond sustained cAMP responses. Perhaps Golgi-D1DR activity merely reinforces the signaling cascades generated from the cell surface. However, given the evidence that other compartmentalized GPCRs distinctly regulate direct downstream effectors and signaling pathways, along with evidence from the present work showing differences in dynamics of dopaminergic signaling cascades, it is reasonable to predict that nuances of spatiotemporal D1DR signaling is important in regulating neuronal physiology. Another method to study differences in effector regulation at different membrane locations is proximity labeling combined with mass

spectrometry. An incredibly useful tool, ascorbic acid peroxidase (APEX) has been used to resolve proteomes of individual organelles. Hobson et al describe APEX2 targeting to distinct membrane compartments of the plasma membrane in presynaptic neurons. By differentially probing for the proteomes at either axons and synapses, they showed an importance of axonal localization of dopamine-associated proteins. Similarly utilizing APEX to study local effectors in other subcellular compartments such as the Golgi, endosomes, and cilia in postsynaptic D1DR-neurons would reveal which effectors are commonly and distinctly regulated by D1DR activity at each membrane location.

4.5 Consequences of compartmentalized signaling in kidney

D1DR plays a role in regulating blood pressure by blocking sodium reabsorption in proximal convoluted tubules. Renal dopaminergic signaling is activated by high salt intake; dopamine-stimulated D1DR blocks sodium reabsorption into the bloodstream to help remove excess salt from the body. Dysfunctional dopaminergic activity in the kidney has been implicated in essential hypertension, the major symptom being high blood pressure. Understanding the role of compartmentalized signaling in the regulation of sodium transport can help develop targeted treatments for hypertension.

Interestingly, OCT2 has been proposed to mediate dopamine secretion into the lumen of the proximal tubule, suggesting that dopamine derived intracellularly can act on D1DR on the apical surface. As a bidirectional transporter this is possible but considering OCT2 follows an electrogenic gradient, it is unclear how dopamine would be transported across the plasma membrane against the membrane potential. However, it is possible that very high concentration of intracellular dopamine could help facilitate transport extracellularly. This begs the question of whether dopamine can fluctuate in and out of the cell based on intracellular concentrations, creating complementary waves of plasma membrane and Golgi-D1DR activation. Mechanisms of dopamine degradation need to be investigated in order to understand how signal

cascades are regulated. Studying renal dopaminergic signaling should be of interest not only because of its role in regulation of blood pressure, but also due to the polarization and size of kidney cells themselves. Studying compartmentalized D1DR signaling cascades in the kidney will provide a chance to better understand cAMP diffusion dynamics in a large, three-dimensional cell type, and could reveal higher degrees of compartmentation unique to large cells.

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