UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato di Ricerca in Biomedicina e Neuroscienze
Dipartimento di Biomedicina, Neuroscienze e Diagnostica avanzata (BiND)
SSD BIO/16

G PROTEIN BIASED SIGNALING BY NON-CATECHOL DOPAMINE D1 RECEPTOR AGONISTS

LA CANDIDATA
Dott.ssa Ashley Nilson

IL COORDINATORE
Chiar.mo Prof. Fabio Bucchieri

IL CO-TUTOR
Chiar.mo Prof. John Allen

IL TUTOR
Chiar.mo Prof. Fabio Bucchieri

CICLO XXXII
ANNO CONSEGUIMENTO TITOLO 2018/2019

Acknowledgments

I would like to express my thanks to everyone who helped me through my education, especially my graduate education. First, I want to thank John Allen who has been a wonderful mentor and took me in when I needed a change. I have enjoyed our various scientific discussions that built upon my critical thinking skills. Thanks to all the students and professors who welcomed me during my stay in Palermo and allowing the learn about their science, especially Fabio Bucchieri. In addition, all my lab mates have had a major impact on me and our relationships with continue for years, especially Kathleen Farmer, Eric Wright, Julia Gerson, Sweta Raval, Manish Jain, and Daniel Felsing. Finally, I want to thank Joseph Harding and Leen Kawas, my undergraduate mentors, for listening when I had no one else to turn to. Your support and guidance meant a lot to me, especially when I transitioned labs.

There are too many people to name that have had an impact on my studies. Thank you everyone, including all my classmates, colleagues, and friends who shared these experiences with me.

I could not have done this without the unending and unconditional support of my parents, Lee and Colleen Nilson and my grandma, Barbara Harwood. My best friend Lisa Adair provided immense support and always supported me throughout this process. The support and care packages Cora Fisher sent were also greatly appreciated. Finally, I could not have done it without Allison because she provided so much support and encouragement, and much needed sustenance in the form of delicious meals.

G protein biased signaling by non-catechol dopamine D1 receptor agonists

Table of contents

List of tables

List of Figures

List of abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
GPCR	G protein coupled receptor
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
cAMP	cyclic adenosine monophosphate
D1R	dopamine D1 receptor
HEK293	human embryonic kidney 293
HTLA	HEK293 cells stably expressing tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene
AP-2	adaptor protein complex-2
ERK1/2	extracellular signal-regulated kinase1/2
MAPK	mitogen-activated protein kinase
FDA	Federal Drug Administration
D2R	dopamine D2 receptor
DOPA	dihydroxyphenylalanine
L-DOPA	L-dihydroxyphenylalanine
6-OHDA	6-hydroxydopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PAM	positive allosteric modulator
CREB	cAMP response element-binding protein
KO	knockout
β-Arr1/2	β-arrestin1/2
β-Arr1	β-arrestin1
β-Arr2	β-arrestin2
siRNA	small interfering ribonucleic acid
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
GFP	Green fluorescent protein

5-HT	5-hydroxytryptamine (Serotonin)
5-HT2A	Serotonin2A
IBMX	Isobutyl-methylxanthine
HA	hemagglutinin
ELISA	Enzyme-linked immunosorbent assay
EV	Empty vector
KO	Knockout
DOI	2,5-dimethoxy-4-iodoamphetamine
AUC	Area under the curve
ADHD	attention deficit hyperactivity disorder
D5R	dopamine D5 receptor
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ANOVA	analysis of variance
NA	no activity
NT	not tested
DMSO	dimethyl sulfoxide
E_{max}	maximal effect
EC_{50}	half-maximal effective concentration

G protein biased signaling by non-catechol dopamine D1 receptor agonists

Abstract

Dopamine is a catecholamine neurotransmitter with essential roles in voluntary movement, working memory, attention, and reward. Dopamine acts through five G protein coupled receptors with the D1 and D5 receptors (D1R) stimulating $G\alpha_{s/olf}$ activation and increasing neuronal excitability. Deficits in D1R signaling are implicated in Parkinson's disease motor deficits as well as cognitive deficits in schizophrenia and attention deficit hyperactivity disorder. For more than 40 years, academic and industry scientists have been searching for a drug-like D1R agonist, but this has remained elusive. The challenge in developing D1R selective agonists is that all previous agonists possess a common problematic chemical moiety, a catechol. Catechols are associated with poor oral bioavailability, poor brain penetration, and rapid metabolism in the serum. Very recently, the breakthrough discovery of the first non-catechol D1R selective agonists overcame the pitfalls associated with the catechols. Unexpectedly, the non-catechol agonists also selectively activate G protein signaling without engaging β-arrestin indicating that they are G protein biased. The primary goals for this study were to characterize novel signaling by non-catechol agonists and elucidate a mechanism of action for the G protein biased non-catechol agonists. First, the role of β-arrestin in D1R agonist-induced endocytosis was established in HEK293 cells that had β-arrestin1/2 knocked out by CRISPR/Cas9 genome editing. The knockout of β-arrestin1/2 eliminated D1R agonist-induced endocytosis. β-arrestin1/2 knockout significantly reduced D1R agonist-induced endocytosis in an ELISA assay that measures cell surface D1R. Furthermore, re-expressing either β-arrestin1 or 2 rescued D1R endocytosis in confocal imaging and cell surface ELISA assays. Together, these results indicate that β-arrestin1/2 are required for D1R agonist-induced endocytosis. Next, catechol and non-catechol D1R agonists were tested in

cAMP Glosensor and β-arrestin Tango assays to investigate potential biased signaling. The unbiased catechol D1R full agonist SKF-81297 was used as the reference compound in all following studies. The non-catechol D1R agonists dose-dependently increased cAMP production in HEK293 cells similar to the full agonist SKF-81297 (E_{max} 100%), but did not engage β-arrestin. Interestingly, one non-catechol agonist (PW441) robustly activated both cAMP (E_{max} = 92%, EC_{50} = 4.4 nM) and also fully recruited β-arrestin (E_{max} = 100%, EC_{50} = 100 nM). The catechol agonist A-77636 dose-dependently increased full cAMP production (E_{max} = 104%, EC_{50} = 3.1 nM) but was a super agonist for β-arrestin recruitment (E_{max} = 130%, EC_{50} = 35 nM). To determine the effect of G protein biased agonists on D1R endocytosis, the catechol and non-catechol D1R agonists were tested in imaging and cell surface ELISA assays. The non-catechol G protein biased agonists all induced significantly less total D1R endocytosis than the catechol agonist SKF-81297. The pure G protein biased agonists PF-1119 and PW464 maximally induced 5% and 11% loss of cell surface D1R, respectively. In contrast, the catechol A-77636 maximally induced 47% loss of cell surface D1R and induced significantly more total endocytosis than SKF-81297. Moreover, the efficacy for β-arrestin recruitment strongly correlates to the maximum receptor endocytosis in Spearman's correlation analysis ($r = 0.96$, $p<0.05$). Collectively, this study demonstrates the essential role of β-arrestin in D1R agonist-induced endocytosis and characterizes novel non-catechol agonists. In addition, the discovery of the first balanced non-catechol D1R selective agonist adds a unique tool for future *in vitro* and *in vivo* studies. These results further elucidate a mechanism of action for the G protein biased non-catechol agonists in which agonist binding induces G protein activation without also inducing D1R endocytosis. These results provide insights into the molecular mechanism of the G protein biased non-catechol agonists. While the clinical efficacy of the non-catechol agonists is currently being explored, the mechanism of action is not fully understood. This study explored novel derivatives and their downstream effects on D1R endocytosis.

1.0 INTRODUCTION

1.1 Neuronal signaling and synaptic transmission

Neurons are unique cells in our bodies that conduct information rapidly. This information is encoded by electrochemical signaling where electrical potential at axon terminals is rapidly converted into a chemical signal that bridges the gap between neurons. Synaptic signaling induces changes in membrane potential or metabotropic changes in the postsynaptic cell, initiating a new cellular response (*1, 2*). Neurons integrate vast quantities of information and can constantly change based on the amplitude of the signals and the frequency they are received (*1, 2*). Neuronal signaling requires tight control and regulation with abnormalities resulting in a variety of neurological disorders.

The brain is a plastic organ, meaning it changes in response to new information and responding to our ever-changing environment. The plastic changes occur at a network level by adding and pruning synapses to fine-tune the inputs a cell receives. Existing synapses can grow and become stronger when needed, and shrink when no longer required. Plasticity also occurs at the synapse level where neurons can fine-tune signaling in either the pre- or postsynaptic compartments (*1, 2*). On the presynaptic side, a synapse can be strengthened by adding to the number of vesicles containing neurotransmitters docked at the membrane increasing the release of neurotransmitters during synaptic transmission. On the postsynaptic side, adding or removing receptors to the membrane as well as modulating the receptors themselves (i.e. phosphorylation, G protein coupling) tunes the response in the postsynaptic neuron (*1, 2*). Together, the pre- and postsynaptic neurons fine-tune signaling to either potentiate or inhibit signaling.

Tight control and regulation of neuronal signaling continues at the molecular level with the tight regulation of receptors. Synaptic transmission occurs by activating either ionotropic or metabotropic receptors. Ionotropic receptors are channels that open to allow the influx or efflux of ions upon neurotransmitter binding while metabotropic receptors induce changes in intracellular signaling. Both ionotropic and metabotropic receptors are activated rapidly after ligand binding

and quickly desensitize (*1, 2*). For example, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic receptors open after glutamate binding but quickly desensitize thereafter. Desensitization is thought to aid in the unbinding of glutamate and the resetting of the receptor so that it can respond to a new signal (*3-5*). Metabotropic receptors follow the same principles of rapid activation and desensitization. Metabotropic receptors, also known as G protein coupled receptors (GPCRs), bind neurotransmitters resulting in the activation of a pre-coupled heterotrimeric G protein. The G protein then dissociates into Gα and Gβγ subunits, each of which initiates intracellular signaling through second messenger cascades (*1, 2*). β-arrestin binds to the activated GPCR sterically hindering further G protein activation and effectively desensitizing the receptor (*6, 7*). Many GPCRs are internalized allowing the neurotransmitters to unbind from the receptor and resetting the GPCR for new signaling (*8-10*). Receptor recycling back to the membrane refreshes the receptor available at the membrane for neuronal signaling. Regardless of the neurotransmitter or the type of receptor, neuronal transmission requires tight control and regulation of receptors for appropriate signaling.

1.2 G Protein coupled receptors and Dopamine D1 Receptor

1.2.1 GPCRs, a textbook overview of signaling

Before delving into GPCR signaling, a basic understanding of receptor signaling terminology is required. Receptors are typically cell surface sensors that allow cells to communicate and respond to environmental stimuli. A ligand is the stimuli that binds to the receptor to activate, inhibit, or otherwise modulate receptor signaling. Agonists are ligands that activate the receptor and induce an intracellular signaling cascade. Most endogenous ligands are agonists at their specific receptors. Antagonists, on the other hand, are ligands that bind a receptor and block or inhibit intracellular signaling at that receptor.

GPCRs are essential signaling proteins that are present in every cell type and tissue including the brain where they are fundamental to neurotransmission. GPCRs detect a vast array of stimuli ranging from light, odors, tastes, hormones, amino acids and peptides. Additionally, GPCRs are essential to numerous physiological processes in every tissue including neurotransmission in the brain and heart rate and contractibility in the heart among many others. Every neurotransmitter has a GPCR while only some have ionotropic receptors. Glutamate, γ-aminobutyric acid (GABA), and acetylcholine are a couple examples of neurotransmitters that bind to both ionotropic and metabotropic receptors. Dopamine, endocannabinoids, and many peptide neurotransmitters act primarily through GPCRs (*1, 2*). GPCRs are neuromodulators as they can alter excitability through G protein activated channels and can alter receptor phosphorylation and gene transcription. Normal physiology requires GPCRs such as the dopamine receptors for voluntary motor movement and diseases arise when GPCR signaling is not appropriate.

There are over 800 validated GPCRs within the human genome (*11*). GPCRs are cell surface receptors that have seven transmembrane alpha helices with variable C terminal tails extending into the cytoplasm. GPCRs are cell surface receptors that bind an external stimuli (i.e. dopamine) and transduce that signal to the intracellular surface to mount an internal signaling cascade. To transduce this signal, GPCRs bind heterotrimeric G proteins on the intracellular surface that are comprised of Gα, Gβ, and Gγ subunits. The heterotrimeric G proteins often pre-couple to GPCRs prior to ligand binding and pre-coupling increases affinity of many agonists for GPCRs (*12*). Upon stimulation, the GPCR changes conformation activating the coupled G protein. The exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in the Gα subunit activates the G protein. The G protein dissociates into Gα and Gβγ subunits that each induce downstream signaling cascades. Hydrolysis of GTP into GDP inactivates the Gα subunit and triggers re-association of the heterotrimeric G protein. Following G protein activation, β-

arrestin recruitment to the GPCR desensitizes further G protein signaling (*1, 2, 13*). This textbook overview of GPCR signaling leaves many details out to simplify and generalize GPCR signaling.

1.2.2 Heterotrimeric G proteins and second messenger cascades

GPCR signaling is considerably more complex than the simplistic overview above. For example, the heterotrimeric G protein is comprised of combinations of 21 Gα, 5 Gβ, and 12 Gγ subunits. Focusing in on the Gα subunits, the 21 isoforms can be broken down into four different classes, Gs, Gi, Gq, and G12 (Table 1) (*14-16*). Gs G proteins stimulate adenylyl cyclase to increase production of cyclic adenosine monophosphate (cAMP). Gs and Golf are members of this class. The Gi class is comprised of nine isoforms that inhibit adenylyl cyclase and thus cAMP production. The Gq family stimulates phospholipase Cβ to produce inositol trisphosphate that releases calcium from the endoplasmic reticulum. Finally, the G12 family interacts with RhoA to induce actin cytoskeleton rearrangement (*16*). The Gβγ subunits are often overlooked when it comes to GPCR signaling but they are no less important and contribute to the complexity of GPCR signaling. As mentioned above, the heterotrimeric G protein dissociates into Gα and Gβγ subunits after GTP binds the Gα. The Gβγ subunits also induce downstream signaling including interacting with phospholipase Cβ (*16, 17*). In addition, the Gβγ subunit can interact with G protein-gated inwardly rectifying potassium channels to hyperpolarize neurons and inhibit neuronal activity (*17*). Furthermore, the diversity of Gβ and Gγ subunits also indicate that they may play a role in fine-tuning GPCR signaling. Heterotrimeric G protein signaling is extremely complex but most GPCRs have a preference for which class of G protein they typically pair with although, even this is being questioned now using knockout approaches in cells (*18*). For instance, the β2-adrenergic receptor and dopamine D1 receptor couple to Gs/olf G proteins to stimulate cAMP production while dopamine D2 receptors couple to Gi/o G proteins.

Table 1. Gα G protein subunits, members of each class, and basic function.

Gα protein subunit	Members of class	Function
Gs	Gs, Golf	↑cAMP
Gi	Gi_1, Gi_2, Gi_3, Go_1, Go_2, Gz, Gt_1, Gt_2, Gt_3	↓cAMP
Gq	Gq, G_{11}, G_{14}, G_{15}	↑ inositol trisphosphate and Ca release
G_{12}	G_{12}, G_{13}	Actin cytoskeletal rearrangement through RhoA

1.2.3 β-arrestin: from classical desensitization to controversial signaling

The classical function of β-arrestin is to desensitize GPCRs to further G protein signaling, attenuating second messenger cascades despite the continued presence of the agonist. In fact, arrestin was named for its ability to "arrest" GPCR activity. In 1986, the quenching, or desensitization, of rhodopsin-activated phosphodiesterase activity was reported when an "intrinsic 48 kDa protein" bound to the outer rod segments (*19*). The 48 kDa protein was later named arrestin and is one of two visual arrestin proteins. Shortly afterwards in 1990, the Lefkowitz lab discovered a homologous protein that desensitized the β-adrenergic receptor, calling the protein β-arrestin (*6*). In total, there are four arrestin proteins, two visual arrestin proteins expressed in the rods and cones of the retina and two non-visual arrestins (*6, 20, 21*). Arrestin1 and arrestin4 are the visual arrestins while arrestin2 and arrestin3, also known as β-arrestin1 and β-arrestin2 respectively, are ubiquitously expressed throughout the body (*20, 22, 23*). Arrestins sterically hinder G protein binding explaining why arrestin binding blocks further G protein activation. G proteins bind to the intracellular loop 2, intracellular loop 3, and the C terminus while arrestin interactions are primarily through intracellular loop 2 (*24-27*). Crystal structures showing GPCRs bound to either G proteins or arrestins confirmed that they bind into the same pocket and

thus cannot both bind GPCRs at the same time (*28, 29*). Arrestins are cytoplasmic proteins that GPCRs rapidly recruit after agonist stimulation to desensitize G protein signaling.

β-arrestin recruitment to GPCRs occurs rapidly following receptor activation. Generally, β-arrestin does not bind to inactive receptors excluding some potential but low levels of constitutive activity. This indicates that something about receptor activation changes the affinity of β-arrestin for the GPCR. Agonist activation changes the conformation of the receptor, which may allow β-arrestin recruitment. Agonist binding also induces phosphorylation of many GPCRs as well. Phosphorylation of rhodopsin increases the affinity of arrestin for rhodopsin while unphosphorylated rhodopsin does not efficiently bind arrestin (*21, 30*). This observation dates back even further to the original publication on arrestin in 1986. Wilden et al. observed that arrestin quenched G protein activation on phosphorylated rhodopsin but had no effect in unphosphorylated conditions (*19*). Furthermore, β-arrestin recruitment to the β2-adrenergic receptor requires the receptor to be both active and phosphorylated (*31*). Interestingly, agonist-induced phosphorylation of the dopamine D1 receptor (D1R) increased receptor desensitization and trafficking suggesting that phosphorylation is important for β-arrestin recruitment to the D1R (*32, 33*). In contrast, truncating the C terminal tail of the D1R eliminated receptor phosphorylation but did not significantly alter desensitization or trafficking (*24, 34*). To reconcile these disparate finding, the authors suggested that removing the C terminal tail may open the previously hidden β-arrestin binding site that C terminal phosphorylation would also open. Adding another layer of complexity, the phosphorylation of distinct residues control D1R desensitization and endocytosis (*35*). This study, however, did not investigate β-arrestin recruitment, so it is unclear if both these phosphorylation sites recruit β-arrestin despite the different outcomes or if other mechanisms are also involved. Phosphorylation appears to be important for GPCR arrestin recruitment resulting in classical desensitization, but arrestin recruitment is also important for receptor endocytosis.

The role of arrestin in GPCR signaling has further expanded to include receptor endocytosis. The general process begins with the recruitment of β-arrestin to the activated and phosphorylated GPCR. β-arrestin then recruits the receptor complex to clathrin-coated pits resulting in receptor endocytosis. The internalized receptor unbinds the agonist, is dephosphorylated, and recycled back the membrane to resensitize the cell. Alternatively, the receptor could be downregulated by degradation in the lysosome. The essential role of β-arrestin in agonist-induced GPCR endocytosis is documented by multiple approaches. The Caron lab in 1996 used a dominant negative β-arrestin mutant to inhibit β2-adrenergic receptor endocytosis expanding the role of β-arrestin beyond desensitization (*36*). Subsequent studies using siRNA in human embryonic kidney 293 (HEK293) cells and β-arrestin1 and 2 knockout mouse embryonic fibroblast cells confirmed the role of β-arrestins in GPCR agonist-induced endocytosis (*37, 38*). With the essential role of β-arrestins in GPCR endocytosis established, researchers began looking at the mechanism of how β-arrestin induces receptor endocytosis.

β-arrestins induce GPCR endocytosis by recruiting the receptor-β-arrestin complex to clathrin-coated pits. Indeed, β-arrestin interacts with adaptor protein complex-2 (AP-2) and clathrin (*39-41*). AP-2 is an adaptor for clathrin-mediated endocytosis and these results indicate that GPCR bound β-arrestins interacts both directly with clathrin and indirectly through AP-2. Kim and Benovic used site-directed mutagenesis of β-arrestin1 and β-arrestin2 to map the binding site of clathrin and AP-2 to the C terminus of β-arrestins, but the sites did not overlap. The authors further determined that the clathrin binding site was essential for endocytosis while the AP-2 site had a less significant role in endocytosis (*42*). Further support for the role of the C terminus in endocytosis came from structural studies of β-arrestin bound GPCRs. Upon binding the GPCR, the C terminus of β-arrestin changes conformation making it more accessible for clathrin and AP-2 binding (*43, 44*). After receptor endocytosis, the receptor needs to be sorted into either recycling

pathways for receptor resensitization or degradation pathways. For a review of resensitization and sorting see (*45*) and dephosphorylation see (*46*) but for the purpose of this dissertation, it is enough to say that these processes are tightly regulated and essential for GPCR signaling although out of the scope of this discussion. At this point, researchers linked β-arrestins to both GPCR desensitization and endocytosis through clathrin-mediated endocytosis.

In addition to desensitization and receptor endocytosis, β-arrestins also may induce their own G protein independent signaling cascades. In 1999, Luttrell et al. first reported the activation of extracellular signal-regulated kinase1/2 (ERK1/2) mitogenic signaling through src kinase activation and that src was recruited by β-arrestin to the β2-adrenergic receptor (*47*). Furthermore, β-arrestin2 coimmunoprecipitated with another mitogen-activated protein kinase (MAPK) pathway including c-Jun amino-terminal kinase 3 suggesting that β-arrestin2 was acting as a scaffold for the activation of mitogenic signaling (*48*). An additional MAPK pathway, c-RAF1-MEK1-ERK1/2 pathway used β-arrestins as scaffold after stimulating the angiotensin II type 1a receptor (*49*). Together, these studies provided the foundation for the scaffolding role of β-arrestins in GPCR MAPK signaling. These studies changed the perception of β-arrestins from shutting off GPCR signaling to also inducing their own signaling. Numerous studies followed the ones above supporting β-arrestin dependent but G protein independent signaling (*7, 50-53*). However, recently the β-arrestin dependent activation of ERK1/2 has become controversial. Studies using genome-editing techniques to knockout G proteins (Gs/olf, Gq, and G12/13) in combination with pertussis toxin, a Gi inhibitor, demonstrate no ERK1/2 activation (*54-56*). Furthermore, genetic deletion of β-arrestin1 and 2 did not alter ERK1/2 phosphorylation compared to the parent cell line (*56*). The newer studies indicate that β-arrestin modulates ERK1/2 activation but does not independently (from G proteins) activate ERK signaling. The disparity between the older studies that used pharmacological inhibitors and siRNA strategies and the newer genetic deletion strategies raises a controversial topic that is currently being argued in the literature. In

fact, Luttrell and colleagues from the Lefkowitz lab recently sent in a rebuttal paper defending β-arrestin signaling (*57*). Regardless of the outcome of this debate, β-arrestins will remain essential regulators of GPCR signaling.

1.2.4 GPCRs are drug targets

GPCRs are important drug targets due their vital and varied roles in physiological processes throughout the entire body. The orthosteric binding pocket confers drug specificity when targeting GPCRs. The orthosteric binding pocket will only bind specific ligands and is relatively unique to each GPCR excluding some highly homologous GPCRs. The unique orthosteric binding pocket allows drugs to be targeted specifically to a GPCR and also activate or inhibit intracellular signaling. As cell surface receptors, GPCRs are easier to target than many intracellular proteins since a drug does not need to pass through the membrane to interact with GPCRs. Furthermore, GPCR signaling is amplified as it proceeds downstream. For instance, each GPCR activates multiple G proteins that activate adenylyl cyclase that greatly amplifies that signal by producing large quantities of the second messenger cAMP and so forth. Thus, the signal generated from one ligand results in a greatly amplified cellular response. In addition to signal amplification, GPCRs are involved in nearly every aspect of physiology in both health and disease. As such, both academic and industry groups are currently exploring GPCRs as drug targets.

GPCRs are a large class of cell surface receptors with more than 800 validated members (*11*). As of 2017, GPCRs are the target of more than 30% of Federal Drug Administration (FDA) approved drugs with approximately 108 distinct GPCRs represented. An addition 321 agents were in clinical trials with 66 of those representing novel GPCR targets (*58, 59*). Furthermore, GPCR drugs comprise approximately 27% of the global drug market prior to 2017 earning $180 million annually (*59, 60*). Based on these numbers alone, one can conclude that GPCRs are important and validated drug targets. Drugs that target GPCRs include both agonists (activators) and

antagonists (inhibitors). A classic example for GPCR antagonists are typical antipsychotic medications that work, in part, by inhibiting dopamine D2 receptors (D2R) (*61*). The antipsychotic efficacy of the typical and atypical antipsychotics correlates with their ability to bind to the D2R (*62*). Aripiprazole is as effective as other atypical antipsychotics for managing positive symptoms but also improves negative symptoms. Interestingly, aripiprazole is a partial D2R agonist and is thought to exert its effect by blocking excessive D2R signaling (*63*). However, aripiprazole also binds to several other neurotransmitter GPCRs (*63*), a common phenomenon for antipsychotic medications. Regardless, antipsychotics all work, at least in part, by inhibiting excessive D2R signaling. Another example of medicines that target GCPRs are anticholinergics that target the muscarinic receptors. Many of the anticholinergics are non-selective between the muscarinic subtypes but they all work as antagonists to block the activity of acetylcholine. Anticholinergics treat a wide array of conditions including allergies, chronic obstructive pulmonary disease, motion sickness, Parkinson's disease, and are used by ophthalmologist to dilate pupils during exams. Scopolamine is a non-selective muscarinic receptor antagonist used to treat post-operative nausea and is often applied as a patch placed behind the ear (*64*). There are numerous additional examples of drugs that target GPCRs. GPCRs comprise an important class of therapeutics that continues to grow with the development of new medications and novel GPCRs targets. As mentioned above, there are 108 distinct GPCRs targeted by FDA approved medications with approximately 66 additional novel GPCR targets currently in development.

1.3 Biased GPCR signaling

1.3.1 What is biased signaling?

The basic paradigm of GPCR signaling begins with an agonist binding the receptor leading to the activation of the coupled G protein. The G protein then initiates a downstream signaling cascade. β-arrestin binds the GPCR desensitizing the receptor to further G protein signaling and inducing receptor endocytosis. With this basic paradigm, a receptor is either "on or off" and

activates or inhibits both G protein and β-arrestin pathways. To my knowledge, most endogenous agonists act in this fashion and activate both signaling pathways relatively equally. Agonists that equally activate G protein and β-arrestin signaling are also known as balanced agonists. However, in the last ten to fifteen years, discovery of agonists that selectively activate one pathway over another have opened new and exciting therapeutic opportunities. Biased agonists are GPCR agonists that preferentially activate one pathway over another. For example, an agonist that selectively induces G protein signaling with reduced or absent engagement of the β-arrestin pathway is a G protein biased agonist. Conversely, β-arrestin biased agonists are agonists that selectively activate the β-arrestin pathway. In the literature, selectively activating one pathway over another is called "functional selectivity" or "biased agonism" (*65-67*). Researchers in both academics and industry have immense interest in developing biased agonists.

The molecular basis of biased agonism is not clearly defined and may be different for distinct receptors and agonists. However, biased agonism can be broken down into biased agonists, biased receptors, and biased systems (*66*). For example, an agonist may induce the receptor to signal in a biased fashion. In this case, different agonists may induce different conformations of the GPCR resulting in balanced agonism is some cases but biased agonism in others. The bias depends on the agonist. In addition, the receptor may be biased and any agonist that binds elicits a biased signaling response. The CXCR7 receptor signals primarily through β-arrestin without activation of G protein signaling (*68*). β-arrestin may act as a scaffold for MAPK signaling, independent from G protein signaling (*49*). Finally, the system may produce bias by differentially expressing effectors and co-factors for GPCR signaling. An example of this is the differential expression of GPCR kinases in different tissues and cell types (*69, 70*). System bias means that an agonist could be biased in one cell type while balanced in another cell type depending on the expression of proteins such as GPCR kinases. While receptor and system bias are interesting and fundamental topics, this dissertation will focus exclusively on biased agonists

using both balanced and biased agonists at the human D1R. Furthermore, developing biased agonists hold more promise as a therapeutic strategy over trying to change receptor or system bias due to the relative ease of manipulating agonists over changing receptors themselves or the system.

1.3.2 Biased agonists offer new therapeutic opportunity

Biased agonism offers control over receptor signaling at a level previously unattainable. Selectively activating or inhibiting G protein or β-arrestin signaling allows researchers the ability to fine-tune drug action and the resulting physiological effects. However, researchers first need to understand what contributions to signaling and physiology each pathway has before advancing biased therapeutics. Behavior and physiology are the culminating result of many cellular processes and G protein signaling may contribute to one behavior while β-arrestin signaling contributes to another. However, it is also possible that both G protein and β-arrestin signaling are required for some processes. For instance, Rose and colleagues from the Caron lab recently developed a technique to investigate G protein versus β-arrestin signaling of the D2R *in vivo*. Most behaviors they studied depended on both G protein and β-arrestin signaling including locomotion and rearing while nestlet shredding only depended on G protein signaling (*71*). Perhaps a better example for the therapeutic potential for biased signaling is the μ-opioid receptor. G protein biased agonists at the μ-opioid receptor provide analgesia with reduced respiratory suppression and constipation (*72-75*). Together, these results indicate that G protein signaling is important for analgesia while β-arrestin signaling is important for adverse effects at the μ-opioid receptor. The development of biased agonists is accelerating research on G protein and β-arrestin signaling contributions to behavior as researchers now have the tools available to interrogate these questions *in vivo*. Biased agonists have the potential to fine-tune GPCR signaling creating medicines with increased efficacy or reduced side effect profiles.

1.4 The function of dopamine D1 receptors

1.4.1 Dopamine receptors

Dopamine is an essential neurotransmitter that works through five distinct GPCRs. The dopamine D1 and D5 receptors both couple to the Gs family of G proteins to stimulate adenylyl cyclase and increase cAMP production. The D1 and D5 receptors are practically pharmacologically indistinguishable leading many people to group them into the D1-like receptors family (*76*). Dopamine D2, D3, and D4 receptors are the D2-like receptor family. D2-like receptors couple to the Gi family of G proteins to inhibit adenylyl cyclase and decrease cAMP production. Thus, dopamine can either stimulate or inhibit cAMP production depending on if it binds D1-like or D2-like receptors. Medium spiny neurons in the direct pathway express the D1R while the D2R is expressed in the medium spiny neurons of the indirect pathway(*76*). Together, the D1R and D2R are integral for proper motor control and movement among other fundamental processes.

As mentioned, the D1R is a Gs/olf coupled GPCR. D1R stimulation increases the production of cAMP leading to activation of protein kinase A and cAMP response binding protein (CREB), among other signaling process (*77*). CREB is a transcription factor that alters gene transcription when it is activated by phosphorylation at serine 133 (*78*). In addition, the D1R recruits β-arrestin to desensitize the receptor and may be involved in regulating D1R endocytosis (*24, 76*).

1.4.2 Dopamine in the body

The D1R is expressed in multiple locations in the body including in the brain, retina, cardiovascular system and the kidneys (*24*). The D1R is abundantly expressed in the brain, especially in the striatum and prefrontal cortex (described below). Furthermore, the kidneys express all dopamine receptor subtypes including the D1R. In the kidneys, the D1R inhibits sodium transport to facilitate sodium excretion. Through this role, the D1R participates in

regulating blood pressure and is implicated in hypertension. In fact, the only current FDA approved D1R-selective agonist is fenoldopam that acts on the peripheral D1R to reduce hypertension in acute inpatient hospital settings (*79*). While it is easy for neuroscientists to forget about peripheral receptors, a potential pitfall for D1R agonists is hypotension when targeting central nervous system D1Rs. Thus, it is essential to test centrally active D1R agonists for their effects on blood pressure as well as the brain.

1.4.3 The role of dopamine and the D1R in the brain

The brain takes in and processes a plethora of information to create our perception of the world and control how we interact with it. Dopamine is a critical neurotransmitter with roles in voluntary motor control, cognition, working memory, attention, motivation, and reward processing among others (*76, 80-90*). There are four major dopaminergic tracts in the brain, nigrostriatal, mesocortical, mesolimbic, and tuberoinfundibular pathways. The nigrostriatal pathway stretches from the substantia nigra to the striatum where it modulates voluntary motor pathways. The substantia nigra contains dopamine-producing cells that degenerate during Parkinson's disease leading to difficulties in initiating voluntary motor movements. The mesocortical pathway extends from the ventral tegmental area to the prefrontal cortex where is plays a role in modulating cognition. Prefrontal cortical D1Rs are essential for working memory and attention (*82, 87-89, 91-93*). D1R antagonists induce working memory deficits in healthy young adults (*94*). In addition, patients with schizophrenia had increased D1R in the prefrontal cortex compared to age-matched control subjects. The authors suggest that the increased D1R is a failed compensatory mechanism designed to increase D1R signaling in the prefrontal cortex of patients with schizophrenia (*95*). Furthermore, D1R agonists rescued working memory deficits in patients with schizophrenia (*85*). Together, these studies suggest that dopamine is essential to working memory and indicate that targeting dopamine neurotransmission is a promising therapeutic target for the treatment of cognitive deficits in schizophrenia. The third major dopaminergic pathway is

the mesolimbic pathway that extends from the ventral tegmental area to the amygdala and nucleus accumbens. The mesolimbic pathway is important for reward and motivation. Numerous substances of abuse alter the mesolimbic pathway including nicotine, cocaine, and morphine among others (*96-98*). Modulating dopamine signaling in the mesolimbic pathway may be a therapeutic strategy for treating substance use disorders. Although, each type of substance affects the pathway differently and may require substance-specific therapies. The fourth major dopaminergic pathway is the tuberoinfundibular pathway that extends from the arcuate nucleus of the hypothalamus to the infundibular region (median eminence). The tuberoinfundibular pathway primarily expresses D2R that control prolactin release from the pituitary gland (*99*). Dopamine is undoubtedly an essential neurotransmitter in the brain in control of a multitude of functions. When dopamine signaling goes awry, distinct clinical disorders appear depending on the location of the altered signaling underscoring the importance of appropriate dopaminergic signaling in the brain.

1.5 Neuropharmacology of the D1R

1.5.1 Dopamine is a catecholamine

Dopamine, along with norepinephrine and epinephrine, are catecholamines named for the shared catechol structure. A catechol is a dihydroxyphenyl chemical moiety. Dopamine synthesis consists of a series of enzymatic reactions that starts with the conversion of tyrosine into dihydroxyphenylalanine (DOPA) and ultimately ending in dopamine. Phenylalanine can be used instead of tyrosine if there is a shortage of tyrosine (*100*). Tyrosine hydroxylase is a commonly used marker for dopamine producing neurons and is the enzyme responsible for converting tyrosine to DOPA. Interestingly, a standard of care for Parkinson's disease is treatment with L-DOPA which dopamine producing neurons in the substantia nigra uptake and convert into dopamine.

1.5.2 D1R drug development efforts

1.5.2.1 Benzazepines

For more than 40 years, academic and industry scientists have been attempting to develop drugs targeting the D1R. The first selective D1R agonist discovered was SKF-38393. Interestingly, SKF-38393 was first discovered for its ability to increase renal perfusion and later tested in the brain on a 6-hydroxydopamine (6-OHDA) lesion model of Parkinson's disease (*101, 102*). SKF-38393 is a partial agonist compared to dopamine. SKF-38393 gave rise to the benzazepines, a class of ligands based on a shared chemical structure and are widely used in experimental settings. In addition, all benzazepine agonists include a catechol moiety like dopamine. Dozens of benzazepine derivatives were developed; however, poor oral bioavailability and a propensity to cause seizures limited the therapeutic development of these agonists (*103*). SKF-81297 is a full D1R agonist in the benzazepine class but was not developed until approximately ten years later. The benzazepine pharmacophore also includes D1R antagonists such as SCH-23390 and ecopipam (*104, 105*). The benzazepine agonists are still widely used in lab settings today to study D1R signaling.

1.5.2.2 Dihydrexidine

The next advancement in D1R agonist development was dihydrexidine. Dihydrexidine was the first full D1R selective agonist that was also brain penetrant (*106, 107*). Although dihydrexidine is also a catechol agonist with poor pharmacokinetics, the ability to cross the blood-brain barrier made it a valuable agonist for investigating the potential of D1R-targeted therapies. Dihydrexidine was the first D1R agonist to improve motor deficits associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioning, a model of Parkinson's disease, in non-human primates (*108*). At the time, the D2R was thought to mediate the antiparkinsonian effects of dopamine agonists, but dihydrexidine provided evidence that the D1R played a critical role. Due this

antiparkinsonian activity, dihydrexidine advanced into Phase I and Phase II clinical trials for Parkinson's disease. Dihydrexidine was ultimately pulled out of clinical development due to poor pharmacokinetics, dose limiting hypotension, and tachycardia (*109*). A pro-drug was developed with a similar structure to dihydrexidine called, ABT-431. Similar to dihydrexidine, ABT-431 had antiparkinsonian properties but still lacked oral bioavailability (*110*). Aside from the antiparkinsonian effects, dihydrexidine also possesses cognitive enhancing effects, primarily on working memory. In young and aged non-human primates, dihydrexidine improved cognition. The authors further determined that the cognitive improvements observed in dihydrexidine treated non-human primates was mediated specifically by the D1R, since the D1R selective antagonist SCH-23390 blocked the effect of dihydrexidine (*111*). Further, in a proof-of-concept study, dihydrexidine improved working memory deficits in patients with schizophrenia (*112*). These results indicate that D1R agonists can improve working memory deficits associated with neuropsychiatric disorders. Again, the catechol moiety of dihydrexidine prevented clinical development due to poor pharmacokinetics, but dihydrexidine advanced D1R research as the first brain penetrant D1R selective and full agonist.

1.5.2.3 Benzazepine derivatives: A-68930 and A-77636

After the development of dihydrexidine, A-68930 and A-77636 were developed and overcame the oral bioavailability issues observed with other catechols. A-68930 and A-77636 are selective D1R agonists with full efficacy compared to dopamine. Both A-68930 and A-77636 possess antiparkinsonian properties in animal models. A-68930 and A-77636 induced rotational behavior in 6-OHDA lesioned rats and improved motor deficits in MPTP lesioned non-human primates (*113-115*). Additionally, A-77636 improves spatial working memory in non-human primates at low to moderate doses (*116*). A-77636 is orally bioavailable overcoming the previous limitation of catechol agonists (*113*). However, several early studies also observed that A-77636 produced profound and rapid tolerance even by the second dose (*113, 117, 118*). A-77636 binds

the D1R near irreversibly, which may explain, in part, the rapid D1R desensitization and *in vivo* tachyphylaxis. A-68930, but not A-77636, induced seizures precluding it from further *in vivo* testing (*114*). However, A-77636 is still widely used in experimental settings including *in vitro* and animal models (*86, 105*). Despite overcoming bioavailability issues associated with other catechol agonists, A-77636 induced rapid tolerance preventing its advancement into the clinic.

1.5.2.4 Catechols have problems: metabolism and pharmacokinetics issues

Despite more than 40 years of effort by academic and industry groups, there are still no FDA-approved brain penetrant and centrally active D1R selective agonists. The common chemical structure shared by all previous D1R selective agonists is the catechol moiety. The catechol agonists, as a class, are associated with poor oral bioavailability, rapid metabolism in the serum, and tolerance. While these setbacks prevented the advancement of a catechol agonist into the clinic for neurological disorders, the catechols greatly advanced our understanding of the role D1Rs have in health and disease. Catechol agonists were vital in establishing the critical role of the D1R in voluntary movement and cognition including working memory and attention. However, new drug-like agonists that can overcome the pitfalls of the catechol agonists are needed before D1R agonists can enter the clinic.

Recently, the discovery of non-catechol D1R selective agonists provided a breakthrough for the treatment of Parkinson's disease and neuropsychiatric disorders. The first non-catechol agonist lead was originally discovered at Pfizer in a large high throughput screening campaign (*86*). Subsequently, Pfizer generated numerous analogs of the lead to optimize both full and partial non-catechol agonists and recently described the analogs in publications and patents (*86, 119-122*). The non-catechol D1R agonists overcome the problems associated with catechol agonists and have good oral bioavailability, serum half-life, and interestingly, decreased tolerance (*86*). However, it remains to be seen if a non-catechol D1R agonist will become an approved medication, but some have advanced through Phase I and Phase II clinical studies at Pfizer and

more recently at Cerevel, a spinoff biotechnology company (*123-126*). Interestingly, the underlying signaling mechanisms of the non-catechol agonists are still not fully understood and will be discussed in great detail below.

1.5.2.5 D1R positive allosteric modulators

In addition to the recently discovered non-catechol agonists, D1R positive allosteric modulators, are also currently in development. Positive allosteric modulators (PAM) are ligands that bind outside of the orthosteric binding pocket and increase the efficacy and/or affinity of agonists that bind the receptor. A PAM allosterically modifies a receptor to increase the binding affinity or efficacy of the endogenous ligand. PAMs keep the spatial and temporal resolution of neurotransmission and increase receptor sensitivity to the ligand (leftward shift potency) and/or increase the maximum signaling efficacy through second messengers (*127-129*). Several companies are pursuing D1R PAMs as a potential therapeutic strategy to enhance endogenous dopamine signaling. Eli Lilly has several Phase I and Phase II clinical trials evaluating LY3154207. The Phase II trial is targeting cognitive deficits in Parkinson's disease dementia. Furthermore, Astellas has another D1R PAM currently in Phase II clinical trials for cognitive impairment in schizophrenia (*105, 130*). Several other D1R PAMs are also being actively investigated. D1R PAMs are another active avenue for the discovery of clinically relevant therapies for Parkinson's disease and schizophrenia among others. Maintaining the spatial and temporal signaling of the endogenous ligand dopamine may prove therapeutically beneficial. However, PAMs may not be as effective in the late stages of neurodegenerative diseases such as Parkinson's disease when the cells that produce dopamine have degenerated. D1R PAMs are not catechols and many possess good pharmacokinetics, overcoming a pitfall that has hampered the development of D1R targeted therapeutics. Together with the non-catechol D1R agonists, D1R PAMs are an exciting and active area of research with the potential to alleviate motor and cognitive deficits.

1.6 Hypothesis and aims

For more than 40 years, researchers have searched for drug-like agonists for the D1R. The catechol moiety common to all previous agonists prevented the development of D1R agonists with good pharmacokinetics. The recent discovery of non-catechol D1R agonists is a breakthrough that has created drug-like molecules with good pharmacokinetics and decreased tolerance (*86*). Here, we extend these findings by characterizing several non-catechol agonist analogs and use these ligands to determine the effects of D1R G protein biased signaling on receptor endocytosis. We further validate the dependence of D1R endocytosis on β-arrestin using advanced CRISPR/Cas9 knockout studies. The hypothesis and aims are outlined below.

Aim 1: The first aim tests the hypothesis that β-arrestin1/2 are required for agonist-induced D1R endocytosis. The initial experiments aim to verify the knockout of β-arrestin1 and β-arrestin2 from HEK293 cells using CRISPR/Cas9 genome editing. cAMP kinetic assays in the knockout cells examine the effect of β-arrestin1/2 knockout over a 60-minute treatment on cAMP levels. Further, desensitization assays in parent HEK293 and β-arrestin1/2 knockout cells confirm a role for β-arrestin1/2 in D1R desensitization. Next, confocal imaging analysis on parent HEK293 and β-arresin1/2 knockout cells investigates agonist-induced endocytosis. Finally, a cell surface ELISA assay quantifies agonist-induced endocytosis by measuring D1R receptors remaining at the membrane after agonist treatment. Together, these studies aim to determine if β-arrestin is essential for D1R signaling and endocytosis.

Aim 2: Aim 2 tests the hypothesis that D1R G protein biased agonists will induce less receptor endocytosis than balanced agonists. The first studies characterize G protein and β-arrestin signaling by several catechol and non-catechol D1R agonists. HEK293 cells are treated with agonists to investigate the effect of balanced or G protein biased D1R agonists on receptor endocytosis using confocal imaging. Finally, catechol and non-catechol agonists effects on

receptor endocytosis is quantified using the cell surface ELISA assay. The goal of these studies is to profile the balanced and G protein biased D1R agonists and to determine the effect of biased signaling on D1R endocytosis.

1.7 Goals of this study

The overall goals of this study are to investigate G protein biased signaling by non-catechol D1R agonists. This can be broken down into three separate goals detailed below.

1) Determine if β-arrestin1/2 are required for D1R agonist-induced endocytosis and determine the role of β-arrestin1/2 in regulating cAMP production (i.e. desensitization).
2) Characterize G protein and β-arrestin signaling for several catechol and non-catechol D1R agonists.
3) Determine the effect of G protein biased agonism on D1R endocytosis.

These studies build on the concepts of GPCR biased signaling and extend our knowledge of D1R G protein biased agonists. These results provide a foundation for the comparison of well-established catechol agonists and the more recent non-catechol agonists and open the door to further mechanistic studies investigating the molecular mechanisms of G protein biased signaling. Furthermore, these studies characterize several potent and efficacious balanced and G protein biased non-catechol agonists, providing opportunities in the future to explore the *in vivo* effects of D1R balanced and G protein biased signaling.

2. METHODS

2.1 Materials and Cell Culture

The commercially available agonists/chemicals were purchased from the following suppliers: SKF-81297 (Sigma), SKF-38393 (Tocris), SKF-77434 (Tocris), ascorbic acid (Sigma). D1R non-catechol agonists, which are not commercially available, were synthesized at the University of Texas Medical Branch by Dr. Jia Zhou's laboratory. PF-1119, PF-2334, PF-6142 were synthesized by according to protocols described in Gray, Allen et al (*86*). 6-(4-(Furo[3,2-c]pyridin-4-yloxy)-2-methylphenyl)-1,5-dimethylpyrimidine-2,4(1H,3H)-dione (**Cmpd 19**) and 6-(4-((3-(Difluoromethoxy)pyridin-2-yl)oxy)-2-methylphenyl)-1,5-dimethylpyrimidine-2,4(1H,3H)-dione (**Cmpd 24**) were synthesized as described previously by Wang, Felsing et al. (*131*) and in previous patents by Pfizer, Inc (*120-122*). 6-(4-(Furo[3,2-c]pyridin-4-yloxy)-2-methylphenyl)-5-methylpyrazin-2-amine (**Cmpd 1**), 4-(4-(Imidazo[1,2-a]pyridin-5-yl)-3-(trifluoromethyl)phenoxy)-furo[3,2-c]pyridine (**Cmpd 2**), and 7-(4-(Imidazo[1,2-a]pyridin-5-yl)-3-(trifluoromethyl)phenoxy)-thieno[2,3-c]pyridine (**Cmpd 3**) were synthesized according to synthetic protocols described by Martini et al (*132*).c All synthesized compounds were verified for purity (>95%) and chemical structures verified using NMR and MS analytical techniques (*86, 131*).

Parent HEK293 cells and β-Arr1/2 KO cells were a generous gift from Asuka Inoue (Tohoku University) (*55*) and were maintained in DMEM supplemented with 10% fetal bovine serum (Omega) and 1% penicillin/streptomycin (Gibco). The β-Arr1/2 KO cells were generated by using CRISPR/Cas9 genomic editing to delete *ARRB1* and *ARRB2* (the genes encoding β-Arr1 and β-Arr2) sequentially from the Parent HEK293 cell line. Stable D1 HEK293 (D1-HEK) cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% MEM NEAA (Gibco), 25 mM HEPES (Gibco), and 500 ug/ml Geneticin (Gibco). HTLA cells (HEK293 cells stably expressing tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene) were maintained in DMEM supplemented with 10% fetal bovine

serum, 1% penicillin/streptomycin, 2 ug/ml Puromycin (Gibco), and 100 ug/ml Hygromycin (Thermofisher).

2.2 SDS-PAGE and western blotting

Parent HEK293 and β-Arr1/2 KO cells were harvested and pelleted in cold PBS. Cell pellets were lysed in 1X RIPA buffer, pH 7.5 (25 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1% NP-40 alternative, 1% sodium deoxycholate, 0.1% SDS and 10% glycerol). Protease inhibitor cocktail and 1 mM PMSF were added to RIPA buffer just prior to cell lysis. Cell pellets were incubated on ice for 15 minutes in the RIPA buffer, followed by sonication with a microtip for 15 seconds on ice. The cell lysates were centrifuged at 12000g for 10 minutes, the supernatants were collected and protein concentration determined by bicinchoninic acid assay. Ten percent SDS-PAGE was performed and then transferred to PVDF membranes. Membranes were blocked in 5% milk in 1X tris buffered saline Tween-20 buffer (TBST) (25 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) at room temperature for 1 hour. Membranes were incubated overnight at 4˚C with the indicated primary antibody. Primary antibodies were β-Arr1 (1:1000), β-Arr2 (1:1000) and β-actin (1:1000). The membranes were washed 3 times in TBST and then incubated with secondary antibody for 1 hour at room temperature. Anti-rabbit IgG conjugated to horseradish peroxidase-conjugated (HRP) (1:1000) was used to detect β-Arr1 and β-Arr2. HRP signal was detected by chemiluminescence (SuperSignal West Pico Substrate). Membranes were reprobed with β-actin, followed by goat anti-rabbit IgG IR800 (Abcam, 1:2000) incubation. Membranes were scanned using LI-COR Odyssey FC Imager (Cambridge, UK).

2.3 D1R endocytosis confocal imaging

To investigate the β-arrestin dependence of agonist-induced endocytosis, we conducted antibody feeding which is an imaging based endocytosis assay. Parent HEK293 cells and β-Arr1/2 KO cells were plated into 6-well plates at 400,000 cells per well. After 24 hours, the cells were

transfected with 0.25 μg HA-D1R plasmid per well in a 6-well plates using 7.5 ul Lipofectamine2000 as per manufacturer's protocol and returned to the incubator overnight. The following morning cells were plated on poly-L-lysine coated coverslips in 24-well plates at 50,000 cells per well and returned to the incubator for 24 hours. The cells were then serum starved for 1 hour in DMEM without serum or antibiotics. After aspirating the media, ice-cold 1X HBSS with 20 mM HEPES was added gently to the cells and incubated on ice for 15 minutes. Anti-HA conjugated to Alexa488 (1:200, Cell Signaling) diluted in HEPES/HBSS was then added to the cells for 45 minutes in the dark on ice. After 3 washes in ice-cold HEPES/HBSS for 5 minutes each, 10 μM SKF-81297 or A-77636 was added to the cells which were returned to 37°C for 60 minutes in the incubator. The cells were fixed immediately in 4% paraformaldehyde for 20 minutes, followed by 3 washes in PBS for 5 minutes. The coverslips were mounted on slides using Vectorshield mounting medium with DAPI (Vector laboratories Inc.). Cells were imaged with a 63X oil objective on a Leica True Confocal Scanner SPE and Leica Application Suite Advanced software (Leica Microsystems, Germany). Z-stacks were obtained with 0.2μm steps with a zoom factor of 1.5. Representative images were adjusted for brightness and contrast for publication.

To further validate that the deficit in agonist-induced D1R endocytosis in the β-Arr1/2 KO cells is dependent on β-arrestin and control for potential off-target effect of CRISPR/Cas9 mediated KO of β-Arr1/2, we expressed β-arrestin1 and/or β-arrestin2 in the β-Arr1/2 KO cells. Parent HEK293 and β-Arr1/2 KO cells were transfected with 0.5 μg empty vector in addition to 0.25 μg HA-D1R as indicated. β-Arr1/2 KO cells were transfected with 0.5 μg β-Arr1, 0.5 μg β-Arr2, or 0.5 μg of both β-Arr1 and β-Arr2 as indicated in addition to 0.25 μg HA-D1R. Antibody feeding was conducted identically to the above.

For agonist-induced endocytosis experiments, parent HEK293 cells were transfected with 0.25 μg HA-D1R plasmid. Antibody feeding was then conducted using 10 μM of the indicated

agonists (dopamine, SKF-81297, A-77636, PF-1119, PF-2334, PF-6142, Cmpd 19, and Cmpd 24).

2.4 Cell surface enzyme-linked immunosorbent assay (ELISA)

We used the cell surface ELISA to quantify D1R endocytosis in the parent HEK293 and β-Arr1/2 KO cells. Parent HEK293 and β-Arr1/2 KO cells were plated in 6-well plates at 550,000 cells per well and incubated for 24 hours at 37°C. Parent HEK293 and β-Arr1/2 KO cells were transfected with 0.25 μg HA-D1R plasmid and 7.5 μl Lipofectamine2000 as per manufacturer's protocol per well of a 6-well plate and incubated overnight. The cells were then split into 96-well plates coated with poly-L-lysine at 65,000 cells per well and incubated for 24 hours. The cells were serum starved for 1 hour in DMEM then treated with 10 μM A-77636 for the indicated times (0-60 minutes) and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were washed 3 times with PBS for 5 minutes each, followed by blocking for 1 hour in 5% bovine serum albumin diluted in PBS at room temperature. The plates were incubated overnight at 4°C with rabbit anti-HA antibody (1:1000, Cell Signaling) diluted in blocking buffer. In the morning the cells were washed 3 times with PBS for 5 minutes each. Anti-rabbit HRP (1:1000; Cell Signaling) diluted in PBS containing 0.01% Hoechst fluorescence nuclear stain was added to the cells for 1 hour at room temperature. After 3 washes with PBS for 5 minutes each, Hoechst staining was read with an excitation 392 nm and emission 440 nm on a Synergy H4 plate reader (Biotek, Winooski, VT). The cells were then incubated in 3'3,5'5-tetramethylbenzidine liquid substrate (Sigma-Aldrich) in the dark at room temperature for 30 minutes and then absorbance was measured at 370 nm on the Synergy H4 plate reader. The background absorbance was determined by measuring non-transfected cells and was subtracted from each well. Each well was then normalized to the Hoechst signal to account for cell number variation. Following this, the results were expressed as % loss of cell surface HA-D1R with the 0 minute time point

considered 0% loss of the receptor at the membrane. The results were analyzed using GraphPad Prism 7 using a two-way ANOVA with Tukey's posttest.

To further validate the role of β-arrestin in agonist endocytosis, we expressed β-arrestin1 or β-arrestin2 in the β-Arr1/2 KO cells. Parent HEK293 and β-Arr1/2 KO cells were transfected with 0.5 μg empty vector in addition to 0.25 μg HA-D1R as indicated. β-Arr1/2 KO cells were transfected with 0.5 μg β-Arr1 or 0.5 μg β-Arr2 as indicated in addition to 0.25 μg HA-D1R. The cell surface ELISA was conducted identically to the above.

For agonist-induced endocytosis experiments, parent HEK293 cells were transfected with 0.25 μg HA-D1R plasmid. The cell surface ELISA was then conducted using 10 μM of the indicated agonists (dopamine, SKF-81297, A-77636, PF-1119, PF-2334, PF-6142, Cmpd 19, and Cmpd 24) added at the indicated times (0-120 minutes).

2.5 D1R cAMP agonist dose-response assay

D1-HEK cells were split into poly-L-lysine coated 6-well plates at 450,000 cells per well. After 24 hours incubation, the D1-HEK cells were transfected using 1.0 ug Glosensor plasmid and 10 ul Lipofectamine2000 (Thermofisher) as per manufacturer's protocol. The D1-HEK cells were transfected overnight and split the following morning into poly-L-lysine (Culturex) coated 96-well plates at 50,000 cells per well. Approximately 48 hours after transfection, the media was discarded and Glosensor reagent (Promega) diluted to 1% in 20 mM HEPES in 1X HBSS (Gibco) was added for 2 hours at room temperature. Agonist treatment consisted of eleven point dose-response curves that were incubated 10 minutes at room temperature. Agonist response (bioluminescence) was measured on the Microbeta2 plate reader (Perkin Elmer). The data were transferred to Graphpad Prism8 where the dose-response curves were analyzed using "dose-response – stimulation" nonlinear regression analysis with three parameters. The data were then normalized using SKF-81297 as 100% and the mean basal counts as 0%. Nonlinear regression was performed on the normalized data to express all agonists as a % of SKF-81297. Cmpd 1 and

3 were not soluble at high concentrations in 0.1% DMSO unlike all other tested agonists. Increasing the DMSO concentration to 0.5% increased solubility enough to conduce full dose-response curves. All other agonists were in 0.1% DMSO vehicle.

2.6 β-arrestin recruitment Presto-Tango assay

The procedure was adapted from Kroeze et al. (*133*). Briefly, 375,000 cells per well were split into 6-wells plates and incubated for 24 hours. The HTLA cells were then transfected using calcium phosphate transfections methods. Briefly, 1.4 ug of D1R-TANGO plasmid and 120 uM $CaCl_2$ (diluted with H_2O from 2 mM stock) were mixed in one centrifuge tube. In a separate tube, 2X HEPES buffered saline (HBS, 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 50 mM HEPES) was added in equal volume to the tube containing the DNA. The contents of the tubes were then mixed together and vortexed. The mixture was added immediately, dropwise, to the HTLA cells which were returned to the incubator overnight. The transfected HTLA cells were split into a poly-L-lysine coated 96-well plate at 80,000 cells per well and returned to the incubator. Forty-eight hours post transfection, the HTLA cells were treated with the indicated agonist using an 11 point dose-response curve. Due to the long treatment time, 100 μM ascorbic acid (Sigma, final concentration) was added to the 1X HBSS used to dilute the agonists. After 18-20 hours post-treatment, the cells were lysed using the Bright-Glo luciferase substrate (Promega) diluted 20-fold in 1X HBSS for 20 minutes at room temperature and read using the Microbeta2 plate reader to detect bioluminescence. The results were transferred to GraphPad Prism8 and analyzed identically to the Glosensor cAMP assay.

2.7 D1R cAMP desensitization

To assess D1R desensitization in the presence of A-77636, cells were transfected with 0.25 μg HA-D1R plasmid along with 1.0 μg of pGlosensor™-22F plasmid. Cells were cultured in DMEM containing 10% dialyzed FBS and 1% penicillin/streptomycin. After 12 hours of

transfection, 50,000 cells per well were seeded onto poly-L-lysine coated white-wall, clear bottom 96 well plates (Greiner Bio-One, Monroe, NC) overnight. Forty-eight hours post-transfection, the media was aspirated out and cells were serum starved with 90 μl of 1% Glosensor reagent in 1X HBSS + 20 mM HEPES buffer for 1 hour. To initiate desensitization, cell were treated with 10 μM A-77636 or vehicle at indicated time points (5 minutes – 240 minutes). Upon completion of desensitization time-course, media was discarded and cells were washed 4 consecutive times with 100 μl PBS for 5 minutes in an incubator. Cells were then loaded with 90 μl of 1% Glosensor reagent in 1X HBSS + 20mM HEPES buffer and re-challenged with 10 μM A-77636 for 10 minutes. As a negative control, a subset of cells was incubated with only vehicle, and rechallenged with either forskolin, A-77636, or vehicle. Luminescence was read on a Microbeta^2 Microplate Counter (PerkinElmer Life Sciences). Percent desensitization was calculated using the vehicle pre-treated cells with the A-77636 challenge as 0% desensitization. A two-way ANOVA with Bonferroni's multiple comparisons test was used to analyze the results from three independent experiments preformed in triplicate.

2.8 Spearman's correlation

Spearman's correlation was conducted on the β-arrestin recruitment E_{max} (x) and % loss of cell surface HA after 120 minute treatments (y). Spearman's correlation analysis was completed in GraphPad Prism8. A linear regression line based on the correlation analysis was plotted on the graph.

3. RESULTS - β-arrestin is essential for D1R agonist-induced endocytosis.

3.1 Introduction

GPCRs are widely expressed throughout the body including the brain (*134*). The D1R is an essential catecholamine GPCR in the brain responsible for many vital functions including voluntary movement, working memory, attention, and reward processing among others (*76, 80, 88*). GPCR signaling involves two fundamental pathways including G proteins and β-arrestins. β-arrestins are ~48kDa proteins found in the cytosol. Following activation of the GPCR and G protein, β-arrestins are recruited to the membrane where they bind to GPCRs preventing further G protein activation. Arresting G protein signaling is the canonical function of the arrestin protein family and the original function described resulting in the name, arrestin (*6, 19*). β-arrestin binds to GPCRs in the same pocket as the G protein to sterically hinder further G protein binding and activation (*6, 7, 21, 135*). In addition, β-arrestin can act as an adaptor for clathrin-mediated endocytosis of the receptor (*8-10*). After endocytosis, the receptor can be recycled back to the membrane, resensitized for a new round of signaling or degraded in the lysosome leading to receptor downregulation(*9*).

Advances in genomic editing and knockout (KO) strategies have allowed researchers to revisit the fundamental roles of β-arrestin1/2 (β-Arr1/2) and other transducers for a wide variety of receptors (*55-57*). Historically, researchers used small interfering RNA (siRNA) strategies to elucidate the function of proteins. Unfortunately, siRNA strategies maximally knockdown gene expression by 80% leaving at least 20% of the protein to potentially confound observed results (*50*). With the advancement of genome editing techniques, it is now possible to KO the expression of a specific gene to understand its function *in vitro* and *in vivo* (*55-57, 136, 137*). Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) genome editing is a powerful tool to study the fundamental roles of β-Arr1/2 without the confounding factor of residual

protein expression as with siRNA strategies (*138, 139*). However, CRISPR/Cas9 KO approaches can potentially have off-target effects (*140-142*). In addition, one group hypothesized that the cells may “rewire” their signaling pathways by changing the expression of other genes to compensate for the lost transducer (*57*). Therefore, CRISPR/Cas9 gene KO requires rigorous validation by re-expressing the deleted genes and rescuing observed deficits to address this potential caveat. Re-expressing the knocked out gene confirms that the deficit is specific to the knocked out gene and not due to any off-target effect or “rewiring” of the cells.

Tight control of signaling is important for proper neuronal transmission and the ability of cells to respond to diverse stimuli. The D1R is no exception to that rule and a multitude of proteins tightly regulate the D1R, including β-Arr1/2. The D1R recruits β-Arr1/2 upon agonist stimulation and undergoes endocytosis via clathrin-coated pits (*86, 143-145*). The D1R is internalized after agonist stimulation and is recycled back to the membrane as soon as 20 minutes after agonist removal in primary striatal neurons (*143*). Furthermore, D1R endocytosis occurred in an overlapping period to cAMP production and blocking endocytosis attenuated cAMP signaling. Interestingly, blocking recycling did not affect cAMP production during these short time courses (*144*). β-Arr1/2 recruitment to the D1R, as mentioned before, leads to D1R recruitment to clathrin-coated pits. Kim el al. used imaging with β-Arrestin1-Green fluorescent protein (GFP) and β-Arrestin2-GFP to show that D1R stimulation recruited both β-Arr1 and β-Arr2 to the membrane (*24*). They continued on to show, that D1R phosphorylation in the third intracellular loop is important for β-Arr2 recruitment but that the C terminal tail is not essential for β-Arr2 recruitment (*24*). These results suggest that D1R phosphorylation may be important for β-Arr1/2 recruitment. In addition, these studies indicate that both β-Arr1/2 are important for D1R signal regulation. Several studies have also raised the question of whether clathrin-mediated endocytosis is the only endocytosis mechanism for the D1R (*146-148*) . The D1R co-immunoprecipitated with caveolin-1 in COS-7 cells and rat brain extracts. Furthermore, the D1R contains a caveolin binding

motif and mutating the motif attenuated D1R caveolin-mediated endocytosis (*146*). Caveolin-mediated endocytosis is also dynamin-dependent which aligns with several of the studies above that used dynamin dominant negatives or dynamin inhibitors. Together, these studies suggest that multiple endocytic pathways may regulate the D1R. However, these studies were not conducted in a β-Arr1/2 null background and relied primarily on pharmacological inhibitors or imaging to investigate D1R endocytosis. Pharmacological inhibitors often have off-target effects and imaging may miss smaller effects due to the qualitative and not quantitative nature of the experiments. Thus, we decided to revisit the fundamental processes of D1R signaling including endocytosis using HEK293 cells with β-Arr1/2 knocked out by CRISPR/Cas9.

These studies show successful KO of β-arrestin1/2 (β-Arr1/2 KO) from HEK293 cells using CRISPR/Cas9 genome editing. First, to investigate the canonical role of β-Arr1/2 to arrest G protein signaling, D1R agonist-induced increases in cAMP were measured in a live-cell assay over a 60-minute time course. Using a unique kinetic assay conducted at 37°C, these studies show that β-Arr1/2 KO does not influence cAMP during the first 60 minutes of stimulation. Rather, the decrease in cAMP during the 60 minutes is partially due to phosphodiesterase activity. The kinetic experiments provide valuable insights into cAMP maintenance but the role of β-Arr1/2 was also determined in classic desensitization paradigms. Desensitization occurs when a cell is unable to respond to an agonist after prolonged stimulation. β-Arr1/2 KO reduced the ability of the D1R to increase cAMP after prolonged agonism indicating impaired D1R desensitization. Additionally, β-Arr1/2 KO eliminated D1R agonist-induced endocytosis and β-Arr1 or β-Arr2 re-expression rescued agonist-induced endocytosis. Finally, the role of β-Arr1/2 in agonist-induced endocytosis for another neurotransmitter receptor, the 5-HT2A receptor, was determined. The role of β-Arr1/2 is receptor specific as β-Arr1/2 KO did not eliminate 5-HT2A receptor endocytosis; however, β-Arr1/2 KO did significantly reduce 5-HT2A receptor agonist-induced endocytosis.

Together these results indicate that β-Arr1/2 are essential for D1R agonist-induced endocytosis and that the role of β-Arr1/2 is dependent on the specific receptor being studied.

3.2 Results

3.2.1 CRISPR/Cas9 knockout of β-arrestin1/2 in HEK293

β-Arr1/2 are essential proteins involved in regulating GPCR signaling. To begin with, the role of β-Arr1/2 in D1R signaling was validated using CRISPR/Cas9 genome editing to KO β-Arr1/2 expression in HEK293. This approach rigorously validates the function of β-Arr1/2 in D1R signaling without the confounding factors of siRNA strategies (i.e. 20% protein expression remaining). We received β-Arr1/2 KO and the Parent HEK293 cell lines as a generous gift from Dr. Asuka Inoue at the University of Tohoku in Japan. CRISPR/Cas9 genome editing was used to first delete *ARRB1*, the gene for β-Arr1. Subsequently, the gene for β-Arr2, *ARRB2*, was deleted with CRISPR/Cas9 as described in the methods. Western blotting analysis on three clones of β-Arr1/2 KO cells confirmed successful KO of β-Arr1/2 (Figure 1). Immunoblotting demonstrated successful KO of both β-Arr1 and β-Arr2 in clones 9, 10, and 11, which all had no immunoreactivity to either β-arr1 or β-arr2 antibodies. Western blotting detected β-Arr1 and β-Arr2 in the Parent HEK293 cells. Together, these results showed successful KO of both β-Arr1 and β-Arr2 in the KO cells. Clone 9 for was used in all subsequent experiments involving the β-Arr1/2 KO cells.

3.2.2 β-arrestin1/2 knockout does not alter cAMP production for 60 minutes following agonist stimulation

The original role of the arrestin family is to desensitize the receptor to further G protein signaling by binding to the receptor. To test this role, a unique kinetic assay was developed in

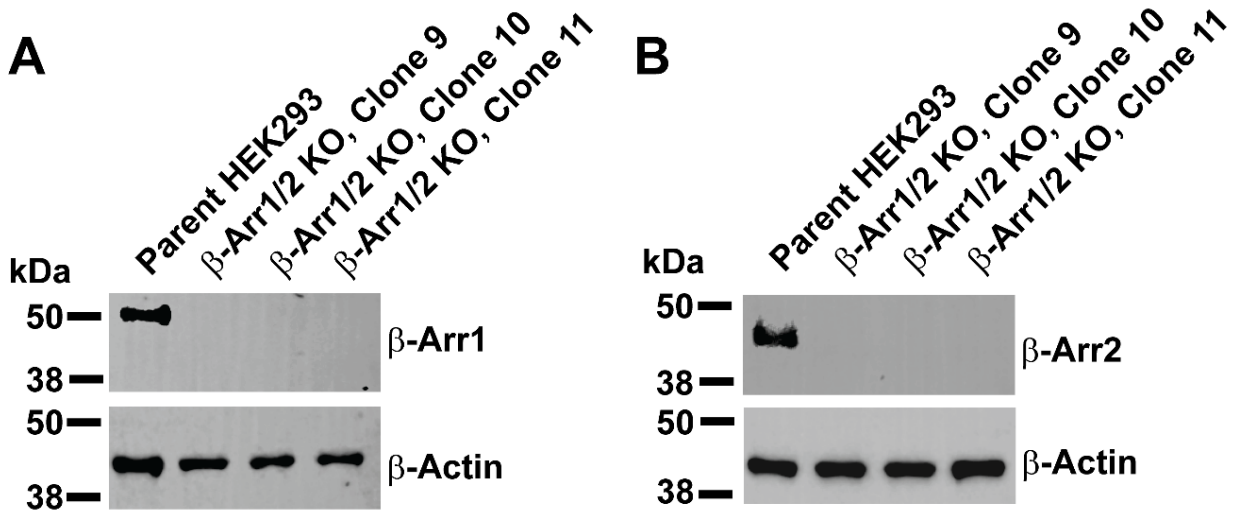

Figure 1. β-arrestin1/2 knockout in HEK293 cells using CRISPR/Cas9 genome editing. β-arrestin1 and 2 were sequentially knocked out using CRISPR/Cas9 genome editing generating three clonal cell lines. Parent HEK293 and β-arrestin1/2 knockout (β-Arr1/2 KO) cells were analyzed by western blotting as described in methods. **A)** Western blotting of Parent HEK293 and β-Arr1/2 KO cells demonstrates successful deletion of β-arrestin1 (β-Arr1) in clone 9, 10, and 11. Parent HEK293 cells had immunoreactivity for β-Arr1 while β–Arr1/2 KO clone 9, 10, and 11 did not. **B)** Parent HEK293 cells showed immunoreactivity to β-arrestin2 (β-Arr2). In β-Arr1/2 KO clones 9, 10, and 11 there was no immunoreactivity for β-Arr2 confirming successful knockout of β-Arr2. Representative blots shown from three independent experiments. Western blotting kindly contributed by MK Jain.

our laboratory that uses a dynamic live-cell cAMP sensor, Glosensor, to monitor cAMP levels over 60 minutes of continuous agonism at saturating concentrations of D1R agonists. The kinetic based assay differs from previous assays in that it is performed at 37°C whereas most cAMP assays are measured at room temperature. In addition, most cAMP assays are endpoint assays and few measure dynamic cAMP levels over time. Here, dynamic cAMP levels were measured and followed over a 60-minute period. We hypothesized that β-Arr1/2 KO would increase the duration of cAMP signaling due to the role of β-Arr1/2 in preventing further G protein signaling and cAMP production. However, there was no difference observed between the Parent HEK293 cells or the β-Arr1/2 KO cells (Figure 2A & B). A-77636 treatment sharply increased cAMP in both Parent HEK293 and β-Arr1/2 KO cells as expected after agonist addition. Interestingly, cAMP levels decreased at the same rate in Parent HEK293 and β-Arr1/2 KO cells. Since β-Arr1/2 KO did not influence the decay of cAMP levels, a phosphodiesterase inhibitor, Isobutyl-methylxanthine (IBMX), was added to the Parent HEK293 cells at the same time as agonist treatment. Phosphodiesterases are enzymes responsible for cleaving cAMP and helping to restore cAMP levels to basal conditions. IBMX treatment elevated cAMP levels indicating that phosphodiesterase activity is partially responsible for the decrease in cAMP levels during this 60-minute period (Figure 2A & B). Taken together, these results show that the initial decrease in cAMP levels is due to phosphodiesterase activity rather than β-Arr1/2.

3.2.3 β-arrestin1/2 knockout reduces D1R cAMP desensitization

The kinetic experiments above are unique and measure continuous cAMP levels. It is unclear by this assay, if re-stimulating the D1R would elicit another wave of G protein signaling or if the receptor is desensitized, presumably by β-Arr1/2. Thus, classic desensitization experiments that stimulate the D1R from 30-240 minutes were performed following which cAMP was measured after challenging the D1R with repeated agonism. The D1R desensitized slowly in

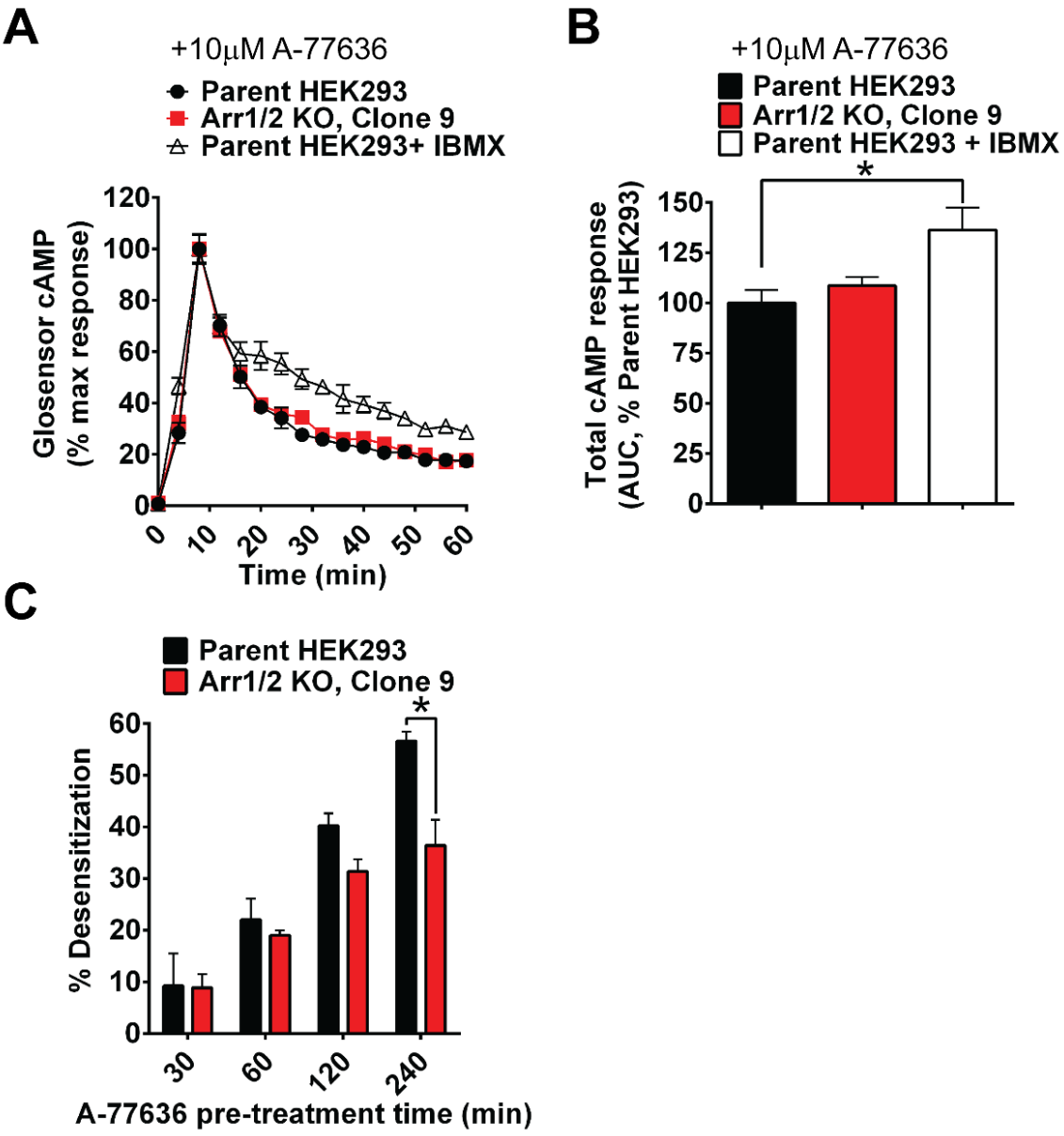
A
+10μM A-77636
Parent HEK293
Arr1/2 KO, Clone 9
Parent HEK293+ IBMX
Glosensor cAMP
(% max response)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)
B
+10μM A-77636
Parent HEK293
Arr1/2 KO, Clone 9
Parent HEK293 + IBMX
Total cAMP response
(AUC, % Parent HEK293)
150
125
100
75
50
25
0
*
C
Parent HEK293
Arr1/2 KO, Clone 9
% Desensitization
60
50
40
30
20
10
0
*
30
60
120
240
A-77636 pre-treatment time (min)

Figure 2. β-arrestin1/2 knockout does not alter cAMP kinetics for 60 minutes following agonist stimulation but does reduce D1R desensitization. Parent HEK293 and β-arrestin1/2 knockout (β-Arr1/2 KO) cells were treated with 10 μM A-77636, a full agonist at the D1R. cAMP levels were measured at four minute intervals for 60 minutes at 37°C. The kinetic cAMP assay measures cAMP levels in live cells at endogenous temperatures over time. In addition, classic desensitization experiments were conducted that measure the ability of the cells to produce cAMP after prolonged stimulation with A-77636. The differences between the assays is that the kinetic assay measures cAMP immediately following agonist addition for 60 minutes while the desensitization assay adds the agonist for 30-240 minutes followed by a washout and re-stimulation with A-77636, at which time cAMP production is measured. **A)** Both Parent HEK293 and β-Arr1/2 KO cells increase cAMP rapidly in response to 10 μM A-77636. cAMP levels then decline despite the continued presence of the agonist. We tested a phosphodiesterase inhibitor, 100 μM isobutyl-methylxanthine (IBMX), to determine if phosphodiesterase activity was responsible for the decline in cAMP levels. Phosphodiesterase inhibition increased cAMP levels but does not fully explain the decreased cAMP levels in Parent HEK293 and β-Arr1/2 KO cells. **B)** Area under the curve (AUC) analysis to determine the total cAMP response over the 60-minute period indicated β-Arr1/2 KO cells were not significantly different from Parent HEK293 cells. Parent HEK293 cells treated with IBMX had significantly higher total cAMP levels than Parent HEK293. **C)** β-Arr1/2 KO disrupts D1R cAMP desensitization. β-Arr1/2 KO cells produced significantly more cAMP (less desensitization) after 240 minutes of 10 μM A-77636 compared to Parent HEK293 cells. Data presented as Mean ± SEM, n=3, *, $p<0.05$ vs. Parent HEK293; **B)** One-way ANOVA with Tukey's multiple comparisons test. **C)** Two-way ANOVA with Bonferroni's multiple comparisons test. Desensitization kindly contributed by DE Felsing.

Parent HEK293 cells, consistent with previously published results obtained in primary neurons (*86*). Further, β-Arr1/2 KO significantly reduced D1R desensitization after 240 minutes (Figure 2C). At earlier time points, 30-120 minutes, β-Arr1/2 KO did not significantly alter D1R desensitization suggesting that the D1R is a slow desensitizing receptor and that β-Arr1/2 has a role in classic desensitization of the D1R. Taken together with the cAMP kinetic results, these results demonstrate that β-Arr1/2 KO does not affect rapid cAMP levels (0-120 minutes); however, β-Arr1/2 does decrease D1R G protein signaling after sustained stimulation.

3.2.4 β-arrestin1/2 knockout eliminates agonist-induced endocytosis

β-Arr1/2 also acts as a scaffold for AP-2, a protein adaptor for clathrin, which recruits the β-Arr1/2-receptor complex to clathrin-coated pits, typically in an agonist-dependent manner (*40, 41*). The result is receptor endocytosis that can lead to either receptor recycling back to the membrane or receptor degradation and downregulation in the lysosome. Thus, D1R agonist-induced endocytosis was investigated in the β-Arr1/2 KO cells. Using a confocal imaging based assay called antibody feeding, the D1R was visualized for agonist-induced endocytosis. Antibody feeding is an assay that labels receptors on the membrane of cells using an anti-hemagglutinin (HA) Alexa488 conjugated antibody while the cells are chilled on ice to prevent endocytosis. After labeling membrane receptors, the cells are warmed to 37°C in the presence of saturating concentrations of agonist for 60 minutes before being fixed and imaged. Alexa488 signal inside the membrane indicates receptor endocytosis and usually appears a punctate signal. Both Parent HEK293 and β-Arr1/2 KO Control treated cells had membrane localization for the D1R (Figure 3). However, Parent HEK293 cells display agonist-induced D1R endocytosis after treatment with 10 μM SKF-81297 or A-77636 (Figure 3A). In contrast, SKF-81297 or A-77636 treatment did not induce D1R endocytosis in the β-Arr1/2 KO cells (Figure 3B), suggesting that β-Arr1/2 KO blocked D1R agonist-induced endocytosis.

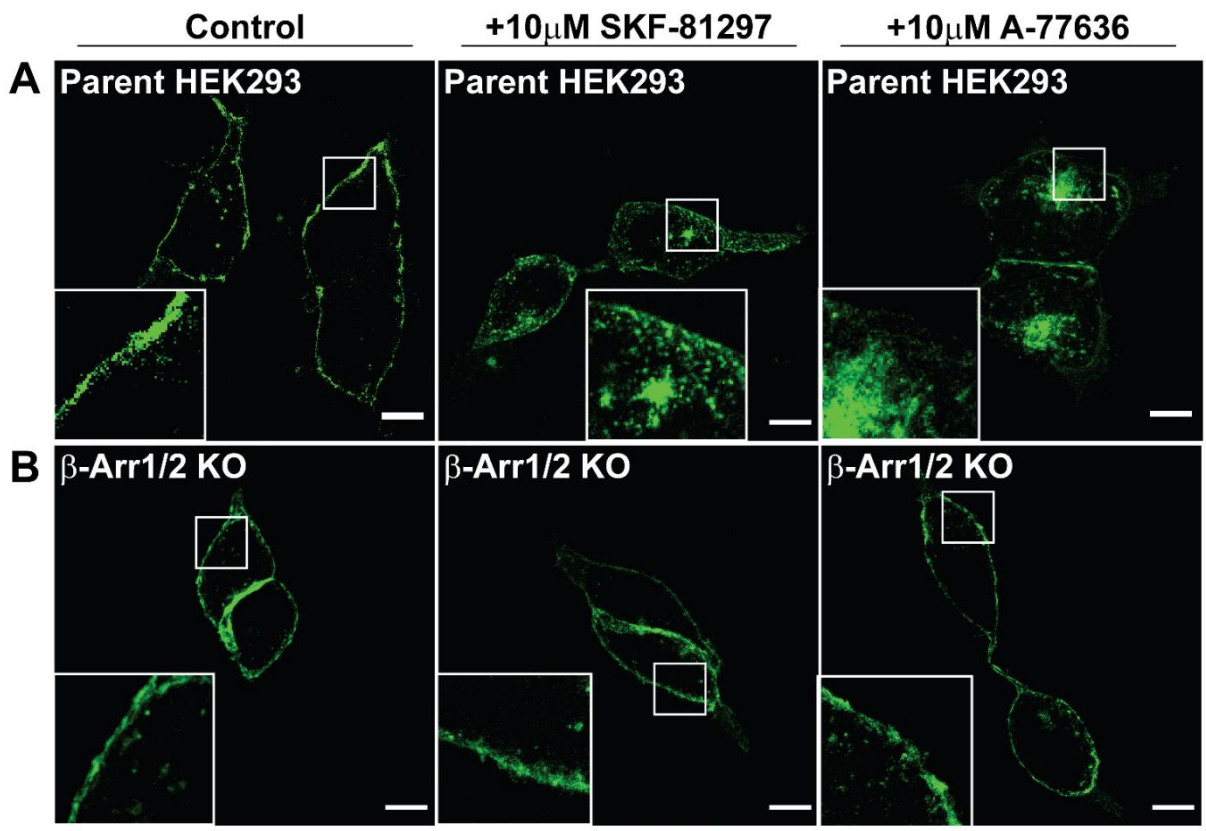
Control
+10μM SKF-81297
+10μM A-77636
A
Parent HEK293
Parent HEK293
Parent HEK293
B
β-Arr1/2 KO
β-Arr1/2 KO
β-Arr1/2 KO

Figure 3. β-arrestin1/2 knockout eliminates agonist-induced endocytosis. HEK293 and β-arrestin1/2 knockout (β-Arr1/2 KO) cells were transfected with 0.25 μg HA-D1R. After 48 hours, the cell surface D1Rs were labeled with an anti-HA antibody conjugated to Alexa488 on ice to prevent endocytosis. The labeled cells were warmed to 37°C in the presence of 1X HBSS with 20 mM HEPES (Control), 10 μM SKF-81297 or 10 μM A-77636 for 60 minutes and fixed. The coverslips were mounted on slides and imaged by confocal microscopy. **A)** Treatment with either SKF-81297 or A-77636 induced D1R endocytosis in the Parent HEK293 cells while control treatment did not induce endocytosis. Punctate and perinuclear staining is visible in SKF-81297 and A-77636 treated cells. The D1R is largely located at the membrane in untreated Control cells. **B)** Treatment with SKF-81297 or A-77636 did not induced endocytosis in the β-Arr1/2 KO. Control, SKF-81297, and A-77636 treated cells had membrane localization of the D1R. Images are representative of >30 cells from three independent experiments. Scale bar = 10 μm. Indicated sections are enlarged to show D1R localization.

To further investigate the role of β-Arr1/2 in D1R agonist-induced endocytosis, D1R endocytosis was quantified using the cell surface ELISA. The cell surface ELISA quantifies the level of receptor at the membrane of cells using an anti-HA antibody to detect the HA-D1R at the membrane under non-permeabilizing conditions. The cells were treated with 10 μM A-77636 for 0-60 minutes as indicated, then fixed. The cell surface ELISA detects the amount of receptor remaining at the membrane after the indicated treatment time allowing us to quantify D1R endocytosis over time. The data were normalized to untreated cells (time 0, data not shown) and presented as percent loss of cell surface HA. Saturating concentrations of A-77636 induced D1R endocytosis in the Parent HEK293 cells but not in the β-Arr1/2 KO cells (Figure 4). After 60-minute treatment with A-77636, 36% of D1R at the membrane was lost in Parent HEK293 cells, indicating A-77636 induced D1R endocytosis. In contrast, A-77636 induced less than 10% endocytosis of the D1R in the β-Arr1/2 KO cells even after 60 minutes of continuous agonism. These results provide an orthologous validation of the confocal imaging assay and quantify D1R agonist-induced endocytosis in the Parent HEK293 and β-Arr1/2 KO cells. Together, these studies indicate that β-Arr1/2 KO eliminates agonist-induced endocytosis of the D1R.

3.2.5 β-arrestin1 and 2 re-expression rescued D1R agonist-induced endocytosis

CRISPR/Cas9 genome editing is a powerful strategy that is allowing researchers to re-investigate the fundamental role of β-Arr1/2. Previous siRNA strategies maximally reduce protein expression by 80% leaving at least 20% to potentially confound results and interpretations. CRISPR/Cas9 genome editing overcomes this problem by deleting the gene; however, CRISPR/Cas9 gene editing can also have off-target effects. In addition, one group has claimed that CRISPR/Cas9 gene deletion may cause the cells to re-wire, or change gene expression and activities of other proteins, to compensate for the lost gene (*57*). Thus, β-Arr1 and β-Arr2 were re-expressed in the β-Arr1/2 KO cells to determine if β-Arr1/2 are specifically responsible for

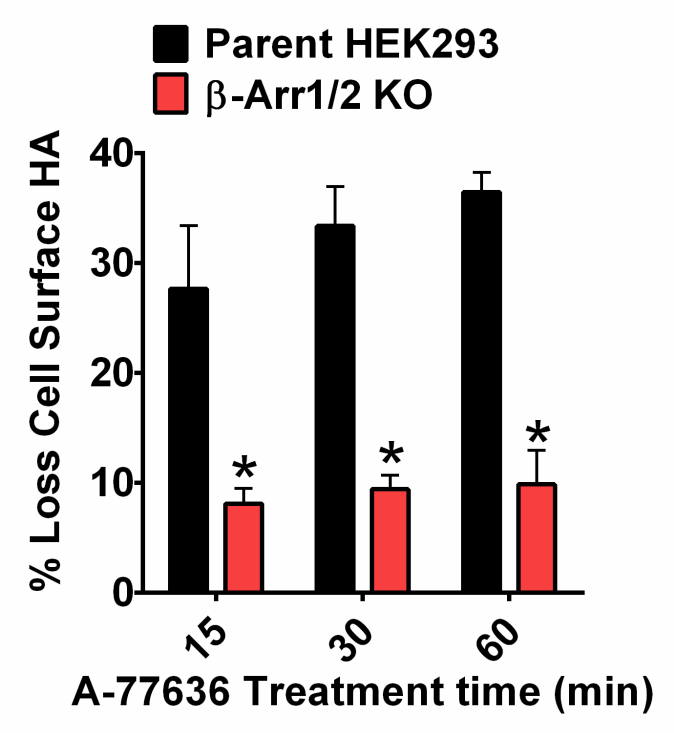

Figure 4. β-arrestin1/2 knockout prevents agonist-induced D1R endocytosis. Parent HEK293 and β-arrestin1/2 knockout (β-Arr1/2 KO) cells were transfected with 0.25 μg HA-D1R. Forty-eight hours later, the cells were treated with 10 μM A-77636 for the indicated times then fixed. An ELISA assay using an anti-HA antibody was conducted in non-permeabilizing conditions. Absorbance measurements were obtained and the data analyzed as described in the methods. Treatment with A-77636 induced time dependent D1R endocytosis in the Parent HEK293 cells. In contrast, the β-Arr1/2 KO cells did not display agonist-induced D1R endocytosis over time. At 15, 30, and 60-minute time points, β-Arr1/2 KO cells had significantly less endocytosis than the Parent HEK293 cells. Data presented as Mean ± SEM, n=3, *, $p<0.05$ vs. Parent HEK293; Two-way ANOVA with Bonferroni's multiple comparisons test.

agonist-induced endocytosis and control of potential off-target effects. Furthermore, using β-Arr1/2 KO cells provides a null background to investigate the role of β-Arr1 independent from β-Arr2 and vice versa. First, western blotting analysis was conducted to ensure successful re-expression of β-Arr1 and β-Arr2 at comparable levels. β-Arr1/2 KO cells were transfected with empty vector (EV), β-Arr1, or β-Arr2 and lysed 48 hours after transfection. Western blotting analysis was conducted on the lysates as described in the methods. β-Arr1/2 KO cells were not immunoreactive to β-Arr1 or β-Arr2 (Figure 5 A & B, respectively). β-Arr1/2 KO cells transfected with β-Arr1 had immunoreactivity for β-Arr1 but not β-Arr2. In addition, β-Arr1/2 KO cells transfected with β-Arr2 did not have immunoreactivity for β-Arr1 but β-Arr2 was detected (Figure 5). This data indicated successful re-expression of β-Arr1 and β-Arr2 in the β-Arr1/2 KO cells and demonstrated relatively equal expression of β-Arr1 and β-Arr2.

To validate rigorously that β-Arr1/2 KO blocked D1R endocytosis and control for potential off-target effects of CRISPR/Cas9 genome editing, β-Arr1 and β-Arr2 were re-expressed in the β-Arr1/2 KO cells prior to conducting endocytosis imaging. Parent HEK293 and β-Arr1/2 KO cells were co-transfected with HA-D1R and EV. Control treated Parent HEK293 cells showed membrane localization of the D1R whereas A-77636 treated cells had punctate and perinuclear signal indicating agonist induce-endocytosis (Figure 6A). β-Arr1/2 KO cells had membrane localization of the D1R after 60-minute treatment with A-77636 indicating β-Arr1/2 KO prevented agonist-induced endocytosis (Figure 6A). Next, the HA-D1R and β-Arr1 or β-Arr2 were re-expressed in the β-Arr1/2 KO cells. Both β-Arr1 and β-Arr2 rescued D1R agonist-induced endocytosis, displaying punctate and perinuclear signal similar to that seen in the agonist treated Parent HEK293 cells (Figure 6B). This data indicated that either β-Arr1 or β-Arr2 are required for D1R agonist-induced endocytosis.

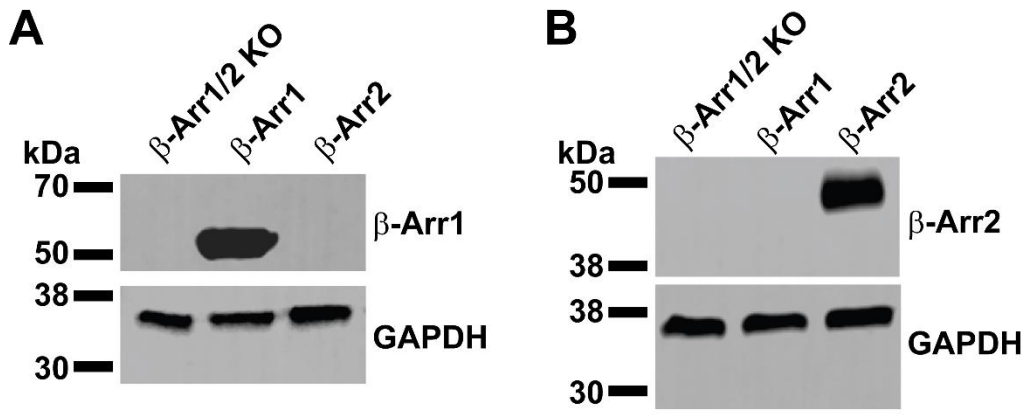

Figure 5. β-arrestin1 and 2 repression in the β-arrestin1/2 knockout cells. 0.5 μg of β-arrestin1 (β-Arr1) or β-arrestin2 (β-Arr2) was transfected into the β-Arrestin1/2 knockout (β-Arr1/2 KO) cells. After 48 hours, the cells were lysed in RIPA buffer and western blotting analysis was conducted against β-Arr1 and β-Arr2 as described in the methods. **A)** β-Arr1 re-expression was detected in the β-Arr1 transfected cells but not in the β-Arr1/2 KO cells or the β-Arr2 transfected cells. **B)** β-Arr2 immunoreactivity was detected in the β-Arr2 transfected cells but not in the β-Arr1/2 KO cells or the β-Arr1 transfected cells. Representative blots shown from three independent experiments. Western blotting conducted by MK Jain, used with permission.

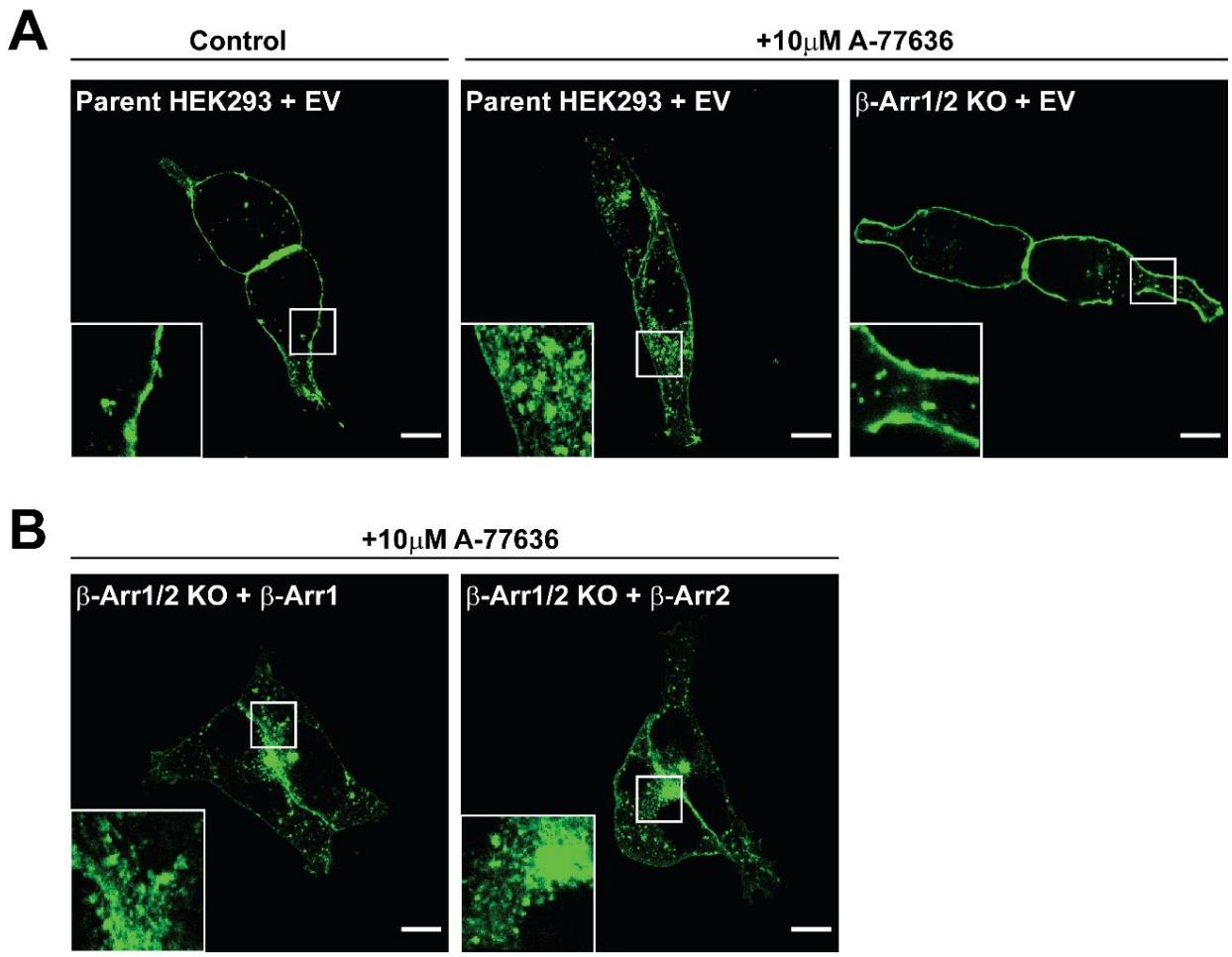
A
Control
+10μM A-77636
Parent HEK293 + EV
Parent HEK293 + EV
β-Arr1/2 KO + EV
B
+10μM A-77636
β-Arr1/2 KO + β-Arr1
β-Arr1/2 KO + β-Arr2

Figure 6. β-arrestin1 or 2 re-expression rescues agonist-induced D1R endocytosis.

Parent HEK293 and β-Arrestin1/2 knockout (β-Arr1/2 KO) cells were co-transfected with 0.25 μg HA-D1R and 0.5 μg empty vector (EV), 0.5 μg β-arrestin1 (β-Arr1), or 0.5 μg β-arrestin2 (β-Arr2). Forty-eight hours post-transfection, the cell surface D1Rs were labeled with an anti-HA Alexa488 conjugated antibody on ice to prevent endocytosis. The cells were warmed to 37°C in 1X HBSS with 20 mM HEPES (Control) or 10 μM A-77636 for 60 minutes and fixed. The coverslips were mounted on slides and imaged by confocal microscopy. **A)** A-77636 induced D1R endocytosis in the Parent HEK293 cells compared to Control treated Parent HEK293 cells. β-Arr1/2 KO prevented D1R agonist-induced endocytosis, consistent with previous results. **B)** Re-expressing either β-Arr1 or β-Arr2 in the β-Arr1/2 KO cells rescued D1R agonist-induced endocytosis. β-Arr1 and β-Arr2 transfected cells have punctate and perinuclear localization of the D1R similar to the A-77636 treated Parent HEK293 cells. Images are representative of >30 cells from three independent experiments. Scale bar = 10 μm. Inset is an enlargement of the indicated area.

D1R agonist-induced endocytosis was also quantified using the cell surface ELISA after co-transfecting HA-D1R and EV, β-Arr1, or β-Arr2 into Parent HEK293 and β-Arr1/2 KO cells. Treatment with A-77636 induced D1R endocytosis in the Parent HEK293 cells with 30% of the surface receptors removed from the membrane after 60 minutes (Figure 7). Consistent with the previous results, β-Arr1/2 KO cells transfected with EV did not undergo agonist-induced D1R endocytosis with less than 10% of the surface receptors removed after 60 minutes. β-Arr1/2 KO cells had significantly less endocytosis after 30 and 60-minute treatment with A-77636 compared to Parent HEK293 cells. In contrast, A-77636 induced D1R endocytosis in the β-Arr1/2 KO cells transfected with either β-Arr1 or β-Arr2 at comparable level to the Parent HEK293 cells (Figure 7). These results demonstrate the essential role that β-Arr1/2 has in D1R agonist-induced endocytosis. Furthermore, these results validated that β-Arr1/2 are required for D1R agonist-induced endocytosis and controlled for any potential off-target effects of CRISPR/Cas9 genome editing.

3.2.6 β-arrestin1/2 knockout reduced agonist-induced endocytosis for the 5-HT2A

To further investigate the role of β-Arr1/2 in GPCR agonist-induced endocytosis, another neurotransmitter binding GPCR, the serotonin or 5-hydroxytryptamine (5-HT) 5-HT2A receptor, was investigated in the β-Arr1/2 KO cells. The 5-HT2A receptor was chosen because there are reports that the 5-HT2A receptor undergoes both agonist-induced clathrin-mediated endocytosis and caveolin-mediated endocytosis, although this may be a cell-type specific phenomenon (*149-151*). Interestingly, in HEK293 cells, endocytosis inhibitors, a truncated β-Arr2 mutant and a dynamin dominant negative did not influence 5-HT2A receptor desensitization in contrast to the β2-adrenergic receptor (*151*). When measuring endocytosis specifically, the same group found that agonist and antagonist induced endocytosis was dynamin dependent and β-Arr1/2 independent (*149*). This differs from the D1R in that the present studies show that β-Arr1/2 KO

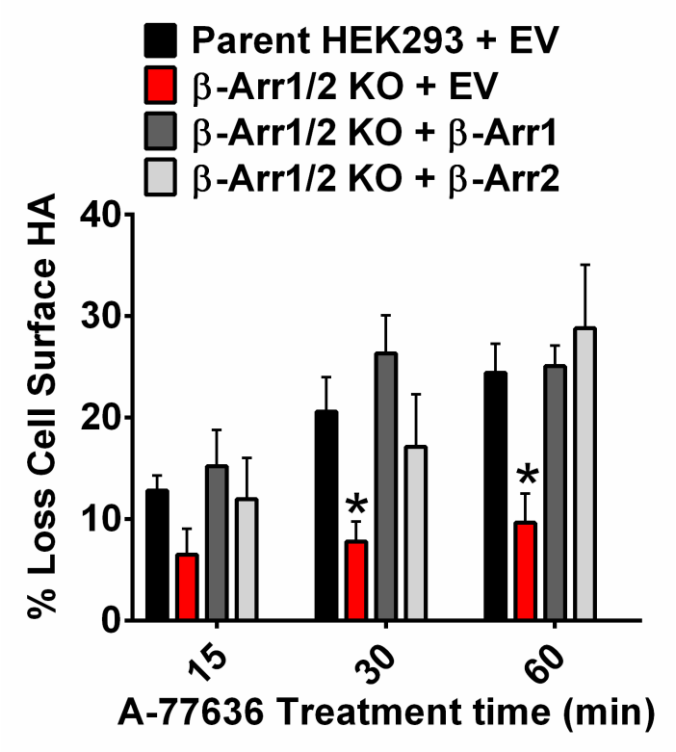

Figure 7. Re-expressing β-arrestin1 or 2 rescues D1R agonist-induced endocytosis.

Parent HEK293 and β-Arrestin1/2 knockout (β-Arr1/2 KO) cells were co-transfected with 0.25 μg HA-D1R and 0.5 μg empty vector (EV), 0.5 μg β-arrestin1 (β-Arr1), or 0.5 μg β-arrestin2 (β-Arr2). After 48 hours, the cells were treated with 10 μM A-77636 for 0-60 minutes as indicated then fixed. An ELISA was conducted in non-permeabilizing conditions to measure the D1R remaining at the cell membrane as described in the methods. Parent HEK293 cells underwent agonist-induced D1R endocytosis. The β-Arr1/2 KO cells had significantly less agonist-induced D1R endocytosis, consistent with previous results. Re-expressing either β-Arr1 or β-Arr2 rescued D1R agonist-induced endocytosis. Data presented as Mean ± SEM, n=4, *, $p<0.05$ vs. Parent HEK293 + EV; Two-way ANOVA with Tukey's multiple comparisons test.

eliminated agonist-induced endocytosis suggesting that the D1R exclusively employs clathrin-mediated endocytosis for agonist-induced endocytosis. Thus, we decided to investigate 5-HT2A receptor endocytosis using the β-Arr1/2 KO cells. 5-HT2A receptor endocytosis was quantified using the cell surface ELISA in the Parent HEK293 and β-Arr1/2 KO cells after treatment with the agonists, 5-HT or 2,5-dimethoxy-4-iodoamphetamine (DOI). 5-HT and DOI rapidly and robustly induced 5-HT2A receptor endocytosis in the Parent HEK293 cells (Figure 8A & C). Parent HEK293 cells endocytosed 52% and 40% of membrane 5-HT2A receptors after 60-minute treatments with 5-HT (Figure 8A) and DOI (Figure 8C), respectively. β-Arr1/2 KO attenuated 5-HT2A receptor endocytosis but did not fully prevent endocytosis with 29% and 22% of the receptor removed from the membrane after 60-minute treatments with 5-HT (Figure 8A) and DOI (Figure 8C), respectively. β-Arr1/2 KO cells had significantly less endocytosis after just 3-minute treatment with 5-HT (Figure 8A) and after 10-minute treatment with DOI (Figure 8C). Total endocytosis was quantified using area under the curve (AUC) analysis. β-Arr1/2 KO significantly reduced 5-HT2A receptor endocytosis over the 60-minute period for both 5-HT (Figure 8B) and DOI (Figure 8D). These results suggest that β-Arr1/2 has a role in 5-HT2A receptor endocytosis but there are also other mechanisms involved and may explain some of the conflicting reports on the role of β-Arr1/2 in 5-HT2A receptor endocytosis.

3.3 Discussion

Here, the β-Arr1/2 KO cells were used to re-investigate the fundamental role of β-Arr1/2 in receptor endocytosis for the D1R and 5-HT2A receptors. First, β-Arr1/2 KO did not influence cAMP levels following continued agonist stimulation in a unique kinetic assay. However, a classic desensitization assay indicated that the β-Arr1/2 KO cells had impaired desensitization after 240-minute treatment with agonist. Moving forward, these results demonstrate that β-Arr1/2 are required for D1R agonist-induced endocytosis and that either β-Arr1 or β-Arr2 can facilitate D1R

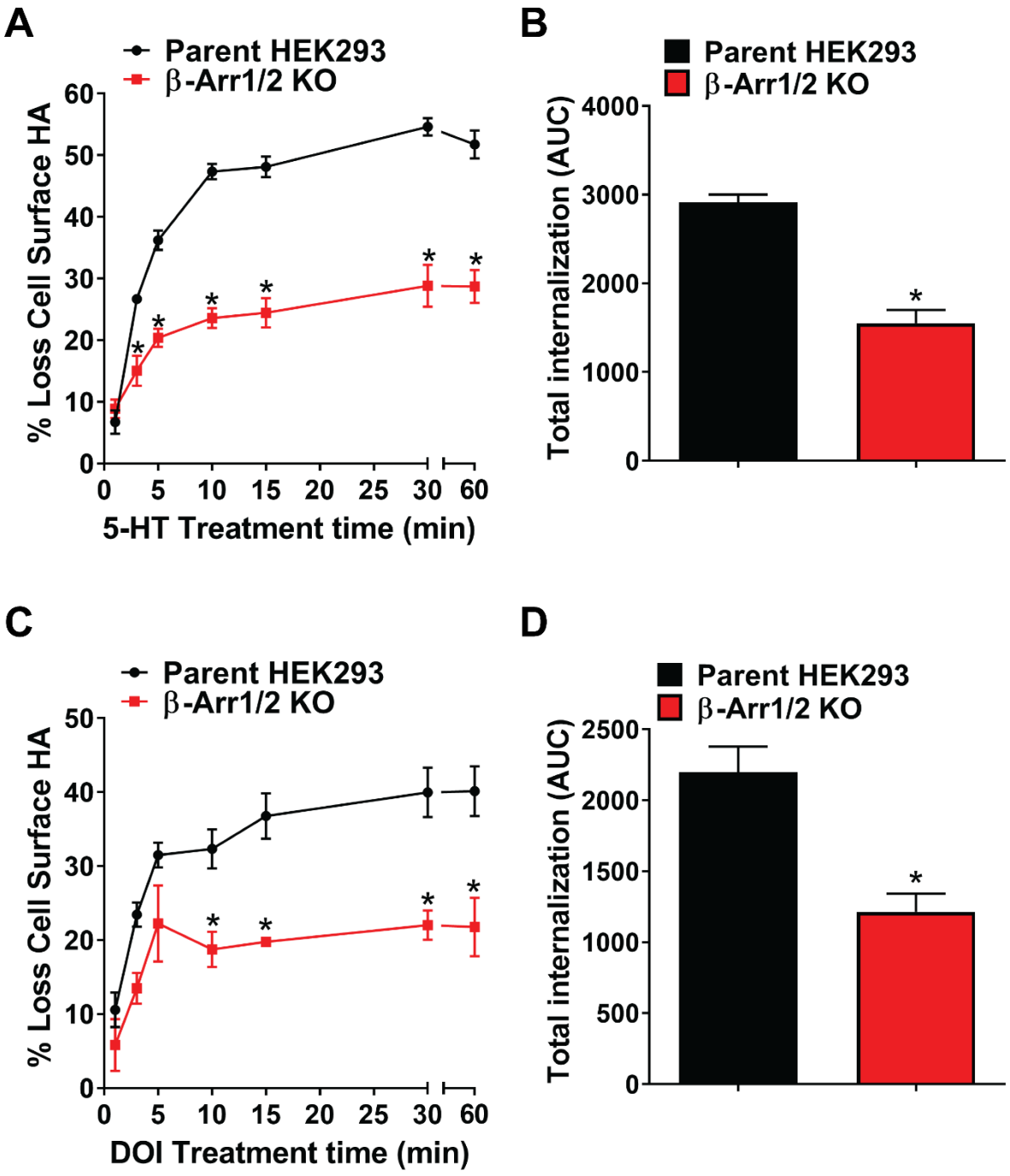
A
Parent HEK293
β-Arr1/2 KO
% Loss Cell Surface HA
5-HT Treatment time (min)
B
Parent HEK293
β-Arr1/2 KO
Total internalization (AUC)
C
Parent HEK293
β-Arr1/2 KO
% Loss Cell Surface HA
DOI Treatment time (min)
D
Parent HEK293
β-Arr1/2 KO
Total internalization (AUC)

Figure 8. Agonist-induced endocytosis of the 5-HT2A receptor is partially dependent on β-arrestin1/2. Parent HEK293 and β-arrestin1/2 knockout (β-arr1/2 KO) cells were transfected with 0.25 μg HA-5-HT2A receptor. Forty-eight hours after transfection, the cells were treated with 10 μM serotonin (5-HT) or 2,5-dimethoxy-4-iodoamphetamine (DOI) for 0-60 minutes. An ELISA was conducted with non-permeabilizing conditions to measure the receptor remaining at the membrane after treatment. **A)** 5-HT induced rapid endocytosis in the Parent HEK293 and β-arr1/2 KO cells. However, Parent HEK293 cells had significantly higher levels of endocytosis than the β-arr1/2 KO cells at 3, 5, 10, 15, 30, and 60 minutes suggesting β-arr1/2 plays a role in agonist-induced endocytosis but there may be other endocytosis mechanisms involved as well. **B)** Total endocytosis was measured using area under the curve (AUC) analysis. β-arr1/2 KO cells had significantly less total receptor endocytosis than Parent HEK293 cells. **C)** DOI, a partial agonist at the 5-HT2A receptor, induced rapid endocytosis in the Parent HEK293 and β-arr1/2 KO cells. Again, the Parent HEK293 cells had significantly higher endocytosis than the β-arr1/2 KO at 10, 15, 30, and 60 minutes. **D)** β-arr1/2 KO cells had significantly less total 5-HT2A receptor endocytosis compared the Parent HEK293. Data presented as Mean ± SEM, n=3, *, $p<0.05$ vs. Parent HEK293; Two-way ANOVA with Bonferroni's multiple comparisons test or Unpaired Student's t-test.

agonist-induced endocytosis. Interestingly, β-Arr1/2 KO attenuated 5-HT2A receptor agonist-induced endocytosis but did not block it to the same extent as the D1R. Together, these results support the role of β-Arr1/2 in D1R agonist-induced endocytosis and indicate that β-Arr1/2 are essential for D1R signaling. Furthermore, these results highlight the complexity of GPCR transducer interactions and show that β-Arr1/2 may have different functions in different receptors.

Historically, GPCR signaling and the function of the many protein involved was determined by pharmacological and genetic strategies (*55-57, 149, 151-154*). Pharmacological inhibitors often lack specificity and can have off-target effects whereas genetic strategies such as siRNA or dominant negative mutants are incomplete knockdowns. While both are useful tools to determine the basic function of protein, interpretation of the results must include the caveat that functional protein was still present during the assays. For example, siRNA knockdown methods are used to transiently knockdown the expression of a protein to study its function in GPCR signaling; however, siRNA strategies maximally achieve 80% knockdown allowing the remaining protein to influence signal transduction (*50*). With the advancement of genome editing techniques, it is now possible to KO the expression of a specific gene to understand its function *in vitro* and *in vivo* (*55-57, 136, 137*). CRISPR/Cas9 strategies have confirmed and extended our understanding of the function of many genes (*139*). Genome-editing techniques offer promising strategies to better understand GPCR-mediated signal transduction by providing a null background that may aid in interpreting results compared to incomplete knockdown with siRNA strategies.

CRISPR/Cas9 genome editing was used to KO β-Arr1/2 providing a null background in which to study the function of β-Arr1 and β-Arr2. After sequentially knocking out β-Arr1 and β-Arr2, successful KO of β-Arr1/2 was confirmed by western blotting. Next, D1R G protein signaling and the role β-Arr1/2 have in attenuating G protein signaling was elucidated in the β-Arr1/2 KO cells in cAMP kinetic assays. We hypothesized that β-Arr1/2 KO would increase cAMP levels

because β-Arr1/2 bind to GPCRs to prevent further G protein activation. Surprisingly, β-Arr1/2 KO did not influence cAMP production during a 60-mintue kinetic assay conducted at physiological temperatures. Instead, phosphodiesterase activity is, at least in part, responsible for the decrease in cAMP levels following D1R activation. This unique kinetic assay measures live-cell cAMP levels using the Glosensor over 60 minutes following agonist stimulation. It is one of the first cAMP assays to measure cAMP at 37°C and follow it for 60 minutes in the same cells. Thus, the kinetic assay allows researchers the unique opportunity to investigate the kinetics of G protein signaling dynamically in living cells. Interestingly, β-Arr1/2 KO reduced D1R cAMP production in desensitization assays. D1R cAMP desensitization measures the ability of the D1R to respond to repeated and prolonged agonism. The D1R was slow to desensitize reaching only 20% desensitization after 60 minutes of exposure to the D1R agonist and β-Arr1/2 KO significantly reduced desensitization after 240 minutes. This indicates that β-Arr1/2 have a role in regulating D1R G protein signaling after extended agonism and is consistent with the kinetic experiment in which β-Arr1/2 KO did not influence cAMP levels during the first 60 minutes of agonist exposure.

In addition, this study investigated the role of β-Arr1/2 in D1R agonist-induced endocytosis. β-Arr1/2 are adaptors for clathrin-mediated endocytosis and aid in the recruitment of the β-Arr1/2 bound GPCRs to clathrin coated pits (*39, 41*). This study demonstrated that β-Arr1/2 are required for D1R agonist-induced endocytosis but less so for the 5-HT2A receptor using the β-Arr1/2 KO cells. Interestingly, β-Arr1/2 have important roles in D1R and 5-HT2A receptor agonist-induced endocytosis, but 5-HT2A receptors can also undergo endocytosis in the absence of β-Arr1/2, albeit, to a lesser extent. Moreover, these results highlight that the function of β-Arr1/2 is receptor specific and that the CRISPR/Cas9-mediated β-Arr1/2 KO cells are a powerful tool to re-evaluate the fundamental role of β-Arr1/2 using modern techniques.

CRISPR/Cas9 genome editing is advancing the understanding of protein function through genetic KOs that are superior to siRNA strategies that only knockdown a protein. However, an important caveat to CRISPR/Cas9 is the potential for off-target effects (*141, 142*). To address this caveat, β-Arr1 or β-Arr2 were re-expressed in the CRISPR/Cas9 edited cells. Both β-Arr1 and β-Arr2 rescued D1R agonist-induced endocytosis. By re-expressing the β-Arr1/2, this study rigorously showed that the deficit in agonist-induced D1R endocytosis was specific to the KO of β-Arr1/2 and not due to an off-target effect. In addition, the β-Arr1/2 KO cells allow researchers to study the role of β-Arr1 independently from β-Arr2 and vice versa. Few studies separate the function of β-Arr1 from β-Arr2 and often single out β-Arr2 without also investigating β-Arr1. For example, many groups use β-Arr2-GFP constructs to study β-Arr2 recruitment to receptors and trafficking (*86, 155*). This study found that β-Arr1 and β-Arr2 have redundant roles for D1R agonist-induced endocytosis. Using the β-Arr1/2 KO cells to study β-Arr1 and β-Arr2 independently, could help to elucidate receptor specific roles and help to define their specific contributions to receptor signaling. For instance, β-Arr1 does not activate ERK signaling after binding the angiotensin II type 1A receptor whereas β-Arr2 binding potentiated ERK signaling (*37*). Furthermore, β-Arr2 KO significantly attenuated β2-adrenergic receptor sequestration but β-Arr1 KO did not in mouse embryonic fibroblasts when either β-Arr1 or β-Arr2 was knocked out (*38*). More studies are needed to determine the specific contributions of β-Arr1 and β-Arr2 to receptor signaling taking into account that each receptor may be regulated differently. These β-Arr1/2 KO cells are powerful tools to help tackle this question easily and efficiently.

The 5-HT2A receptor does not rely solely on β-Arr1/2 for agonist-induced endocytosis. The current studies show that β-Arr1/2 KO decreases, but does not prevent, 5-HT2A receptor agonist-induced endocytosis. As mentioned above, previous studies suggested that 5-HT2A receptor agonist-induced endocytosis was dynamin-dependent but β-Arr2-independent (*151*).

This study may have missed the effect of the β-Arr2 mutant used because qualitative imaging was the primary assay used to measure endocytosis. They would still observe receptor endocytosis since the 5-HT2A receptor can undergo β-Arr1/2 independent endocytosis as well, although to a lesser extent according to the present study. Furthermore, they did not consider the effect of β-Arr1 during the endocytosis imaging experiments (*151*). A couple years after this study, another group discovered that structurally distinct agonists, 5-HT and DOI, elicit distinct patterns of receptor endocytosis. In β-Arr1/2 KO mouse embryonic fibroblasts, 5-HT did not induce endocytosis at 1 μM whereas DOI did induce endocytosis (*156*). This adds another layer of complexity to agonist-induced endocytosis that includes cell-type, receptor-specific, and agonist-specific mechanisms for GPCR endocytosis.

4. RESULTS - Balanced and G protein biased non-catechol agonists: characterization and consequences on D1R endocytosis

4.1 Introduction

The D1R has fundamental roles in the control of voluntary motor movement, working memory, attention, and motivation among others. As such, the D1R is a validated drug target with the potential to treat cognitive and motor deficits in Parkinson's disease, cognition in schizophrenia, and attention in attention deficit hyperactivity disorder (ADHD) (*76, 80-87, 157*). For more than 40 years, academic and pharmaceutical groups have attempted to develop drug-like D1R agonists (*105, 131, 158*). All previous D1R agonists have a common pharmacophore known as a catechol. The catechol moiety is associated with poor oral bioavailability, short half-life, and tolerance, limiting clinical development (*84, 118, 157*). However, the catechol agonists allowed researchers to explore further the therapeutic value of D1R agonists. For instance, catechol agonists improve motor deficits associated with Parkinson's disease models. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model produces robust motor and cognitive deficits associated with the D1R. Administering catechol agonists relieves motor deficits in non-human primates previously lesioned with MPTP. However, the researchers also observed tolerance or a decline in efficacy in as little as two days with A-77636 or seven days with SKF-82958 (*118*). In addition, catechol agonists improved working memory in human patients with schizophrenia. However, the catechol used, dihydrexidine, required intravenous administration due to the aforementioned poor oral bioavailability and rapid metabolism (*112*). Furthermore, other proof-of-concept studies conducted in non-human primates show that D1R agonists can improve working memory deficits caused by antipsychotic medications, MPTP-lesioning, and aging (*89, 91*). Catechol agonists are useful tools and have greatly advanced our understanding of D1R-related behaviors and disorders but lack essential qualities for clinical development.

The D1R is a GPCR that couples to the Gs/olf G protein. Agonist binding activates the G protein by switching GDP to GTP in the Gs/olf subunit (*76*). The heterotrimeric G protein dissociates into Gs/olf and Gβγ subunits that can each interact with downstream proteins to initiate signaling cascades. The Gs/olf subunit activates adenylyl cyclase leading to the production of cAMP. cAMP is a second messenger that activates signaling cascades resulting in changes in neuronal excitability and gene transcription (*76*). Following G protein activation, β-arrestin binds the D1R and initiates endocytosis through clathrin-coated pits (*24, 86, 143-145*). In the previous chapter, we demonstrated that D1R agonist-induced endocytosis requires β-arrestin. After endocytosis, the D1R may be recycled back to the membrane for renewed signaling or possibly downregulated by degradation in the lysosome (*76, 143*).

Agonists can selectively activate one signaling pathway in favor of another. For instance, an agonist could initiate G protein signaling without engaging the β-arrestin pathway. This selective pathway activation is termed "biased agonism" or sometimes "functional selectivity" (*66, 86, 105, 159-162*). G protein and β-arrestin pathways can control different physiological processes. Selectively targeting one pathway over another allows researchers to fine-tune agonist signaling to elicit a particular therapeutic effect with potentially fewer side effects (*65, 66, 159, 163*). As such, there is immense effort by academic and pharmaceutical companies to discover biased agonists to improve the safety and/or efficacy of drugs (*74, 131, 132, 160, 163-165*). For example, the μ-opioid receptor is one such receptor with known clinical relevance in analgesia but also harmful side effects including respiratory suppression and constipation. Studies in β-arrestin2 knockout mice indicate that opioids such as morphine have prolonged analgesia, reduced tolerance, and decreased negative side effects (constipation, respiratory suppression) (*72, 73*). G protein biased μ–opioid receptor agonists reversed morphine tolerance in a mouse model (*74*). Indeed, the degree of G protein bias is suggested to correlate to the safety of the opioid (*75*). G protein biased μ–opioid receptor agonists proceeded into clinical trials where

Oliceridine (TRV-130) provided analgesia similar to morphine with reduced respiratory and gastrointestinal adverse events. The major limitation for oliceridine is that it must be administered intravenously limiting its use to inpatient settings (*166, 167*).

In addition, β-arrestin biased dopamine D2 receptor agonists provide further support that therapeutic efficacy can be separated from side effects. Antipsychotic medications targeting the dopamine D2 receptor induce catalepsy, which is an unwanted side effect. The β-arrestin biased agonist improved antipsychotic action at the dopamine D2 receptor without inducing catalepsy. The agonist, UNC9994, in an antagonist for the cAMP pathway but a partial agonist in the β-arrestin pathway. Furthermore, knocking out β-arrestin2 in mice eliminated the antipsychotic effects of UNC9994 (*163*). Accumulating evidence indicates biased agonists are therapeutically valuable and can fine-tune the effects of medicines to improve their efficacy or reduce side effects.

L-3,4-dihydroxyphenylalanine (L-DOPA) treatment is the standard of care for Parkinson's disease motor deficits. However, long-term L-DOPA treatment induces dyskinesia. Once L-DOPA is taken up by neurons in the substantia nigra, it is converted to dopamine and released during synaptic transmission to bind to dopamine receptors including the D1R. Interestingly, β-arrestin2 levels modulate L-DOPA induced dyskinesia. The dyskinetic effects of L-DOPA are exacerbated in β-arrestin2 knockout mice while overexpressing β-arrestin2 reversed dyskinesia (*165*). This study suggests that β-arrestin biased D1R agonists may be superior to balanced agonists such as dopamine for treating movement deficits in Parkinson's disease. However, no drug-like β-arrestin biased agonists are currently available to test this hypothesis. In addition, several benzazepine D1R agonists exhibit G protein bias. While the benzazepine agonists were discovered decades ago, the G protein bias of some of the agonists was not characterized until recently. In 2015, David Sibley's group characterized G protein signaling, β-arrestin recruitment, and D1R endocytosis for a wide array of D1R agonists including several benzazepine agonists.

Interestingly, SKF-38393 and SKF-77434 among others were partial agonists for cAMP production but did not engage β-arrestin or induce endocytosis (*162*). Although the results are interesting, catechol agonists such as the benzazepine agonists have poor oral bioavailability and short serum half-life making *in vivo* studies difficult. Very recently, the first non-catechol D1R agonists were identified at Pfizer, Inc. as part of a drug discovery and development program (*86, 119-122*). Of the nearly 3 million compounds screened, only one partial D1R non-catechol agonist hit was discovered. This lead to the generation of the first-generation non-catechol agonists described in Gray, Allen et al. (2018) (*86*). Remarkably, all of the first-generation non-catechol agonists are G protein biased with partial to full efficacy in increasing cAMP production while also exhibiting reduced or absent β-arrestin recruitment. Moreover, PF-2334 induced less *in vivo* tachyphylaxis in rodent and non-human primate models (*86*). The non-catechol agonists are a remarkable breakthrough that created the first drug-like selective agonists for the D1R that have potential for human clinical testing (*105*).

Here, we study several non-catechol D1R agonists and characterize their G protein signaling and β-arrestin recruitment activities. The downstream effects of balanced and G protein biased agonists on D1R agonist-induced endocytosis was also determined. Several non-catechol agonists developed at Pfizer and resynthesized in our lab were identified that have good potency and efficacy for D1R G protein stimulated cAMP production. Interestingly, many of the non-catechol agonists did not engage D1R β-arrestin recruitment, indicating they are G protein biased agonists. However, one non-catechol agonist unexpectedly had excellent G protein and β-arrestin recruitment efficacy. Two non-catechol agonists are of particular interest, one that is purely G protein biased (Cmpd 24) and one that is balanced (Cmpd 19). Furthermore, we tested a subset of the agonists for their ability to induce D1R endocytosis. G protein biased agonists do not induce endocytosis while balanced agonists do. D1R agonist-induced endocytosis strongly correlated to β-arrestin recruitment efficacy. Together these studies define the molecular signaling

mechanisms of catechol and non-catechol agonists. Finally, these results demonstrate that G protein biased agonists have different effects on downstream receptor endocytosis compared to balanced agonists.

4.2 Results

4.2.1 Catechol agonist dose-response curves for cAMP and β-arrestin recruitment

First, previously characterized catechol D1R agonists were tested to a) validate the assays for cAMP and β-arrestin recruitment in our lab with established agonists and b) to confirm previously published research suggesting that some of the benzazepine derivatives are G protein biased (*162*). SKF-81297 was a full agonist for cAMP production but a partial agonist for β-arrestin recruitment compared to the endogenous agonist dopamine in previous reports (*131, 162*). Two closely related benzazepine derivatives, SKF-38393 and SKF-77434 were G protein biased with high partial and partial agonism, respectively in the G protein pathway but no measureable activity for β-arrestin recruitment in previous studies (*162*). To replicate these results, dopamine, SKF-81297, SKF-38393, SKF-77434, and A-77636 were selected for Glosensor and β-arrestin recruitment assays. All efficacy measurements were normalized to 100% of SKF-81297 because the Presto-Tango β-arrestin recruitment assay is a gene-reporter based assay that requires long incubation times with the agonists. Thus, it was not feasible to use dopamine in the β-arrestin recruitment assay because it is unstable and becomes rapidly oxidized. Preliminary results with dopamine did not saturate in the dose-response curves likely due to this long incubation and rapid degradation (data not shown). Consistent with the previous studies, dopamine (E_{max} = 98%, EC_{50} = 38 nM), SKF-81297 (E_{max} = 100%, EC_{50} = 3.1 nM) and A-77636 (E_{max} = 104%, EC_{50} = 3.1 nM) were all potent full agonists for cAMP production. SKF-38393 (E_{max} = 85%, EC_{50} = 130 nM) was a high partial agonists while SKF-77434 (E_{max} = 43%, EC_{50} = 110 nM) as a partial agonist for cAMP production (Figure 9A, Table 1). In the β-arrestin recruitment assay, A-77636 (E_{max} = 130%,

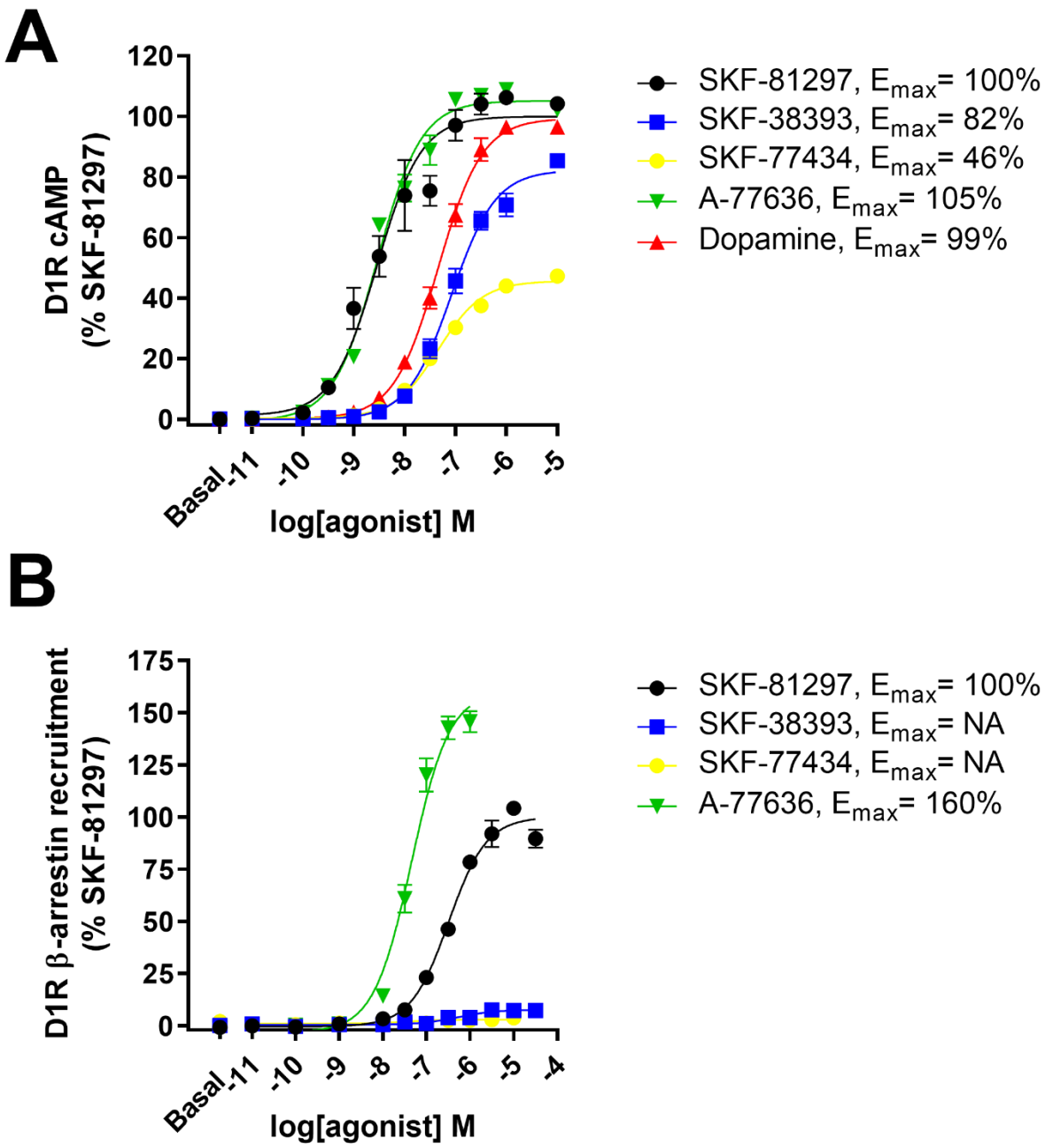
A
D1R cAMP
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
log[agonist] M
SKF-81297, Emax= 100%
SKF-38393, Emax= 82%
SKF-77434, Emax= 46%
A-77636, Emax= 105%
Dopamine, Emax= 99%
B
D1R β-arrestin recruitment
(% SKF-81297)
175
150
125
100
75
50
25
0
Basal
-11
-10
-9
-8
-7
-6
-5
-4
log[agonist] M
SKF-81297, Emax= 100%
SKF-38393, Emax= NA
SKF-77434, Emax= NA
A-77636, Emax= 160%

Figure 9. Dose responses of catechol D1R agonists in cAMP and β-arrestin recruitment assays. cAMP was measured using the Glosensor assay described in methods. β-arrestin recruitment was measured using the Presto-Tango assay described in methods. **A)** Dopamine and A-77636 are full agonists at the D1R compared to SKF-81297. SKF-38393 is a high partial agonist while SKF-77434 is a partial agonist. A-77636 and SKF-81297 had similar potency while all other agonists are rightward shifted. **B)** Compared to SKF-81297, A-77636 had higher efficacy and potency. SKF-38393 and SKF-77434 both had no activity in the β-arrestin recruitment assay suggesting they are G protein biased agonists. Representative plots shown from at least three independent experiments performed in triplicate. Light counts per second (bioluminescence) from both assays was normalized to 100% SKF-81297 response. NA = no activity.

EC_{50} = 35 nM) was a super agonist, and can be considered a β-arrestin biased agonist compared to SKF-81297. SKF-81297 (E_{max} = 100%, EC_{50} = 290 nM) was moderately potent in the β-arrestin recruitment assay. Interestingly, SKF-38393 and SKF-77434 both had no activity in the β-arrestin recruitment assay (Figure 9B, Table 1). All potency and efficacy measurements are summarized in Table 1. These results confirmed that SKF-38393 and SKF-77434 are G protein biased and are consistent with previously published studies. Since validating the cAMP and β-arrestin assays in our lab and confirming that several benzazepines are indeed G protein biased, the novel non-catechol agonists were characterized next.

4.2.2 First generation non-catechol agonists are G protein biased

As mentioned previously, catechol agonists have poor oral bioavailability, are rapidly metabolized, and often induce tolerance *in vivo*. Thus, to advance D1R agonists into the clinic new drug-like agonists are needed to overcome these pitfalls. The first-generation of non-catechol agonists were discovered by Pfizer (*86, 119-122*). An initial screen of nearly three million compounds identified one hit compound that was optimized to produce PF-1119, PF-2334, and PF-6142 among others. PF-1119 was a partial agonist for cAMP production whereas PF-2334 and PF-6142 were reported as high partial to full agonists. Interestingly, all three agonists had reduced β-arrestin recruitment compared to dopamine and SKF-81297 in total internal reflection fluorescence (TIRF) microscopy (*86*). Given that different assays were used to determine β-arrestin recruitment in previous studies (*86, 162*), it is impossible to compare the ligands directly from the published literature. Thus, we set out to compare all ligands together by measuring cAMP using the Glosensor cAMP assay and β-arrestin recruitment using the Presto-Tango β-arrestin recruitment assay. In cAMP studies to assess D1R G protein signaling, PF-1119 (E_{max} = 79%, EC_{50} = 58 nM) and PF-6142 (E_{max} =91%, EC_{50} = 22 nM) were high partial agonists compared to SKF-81297 while PF-2334 (E_{max} =98%, EC_{50} = 11 nM) was a full agonist for cAMP production

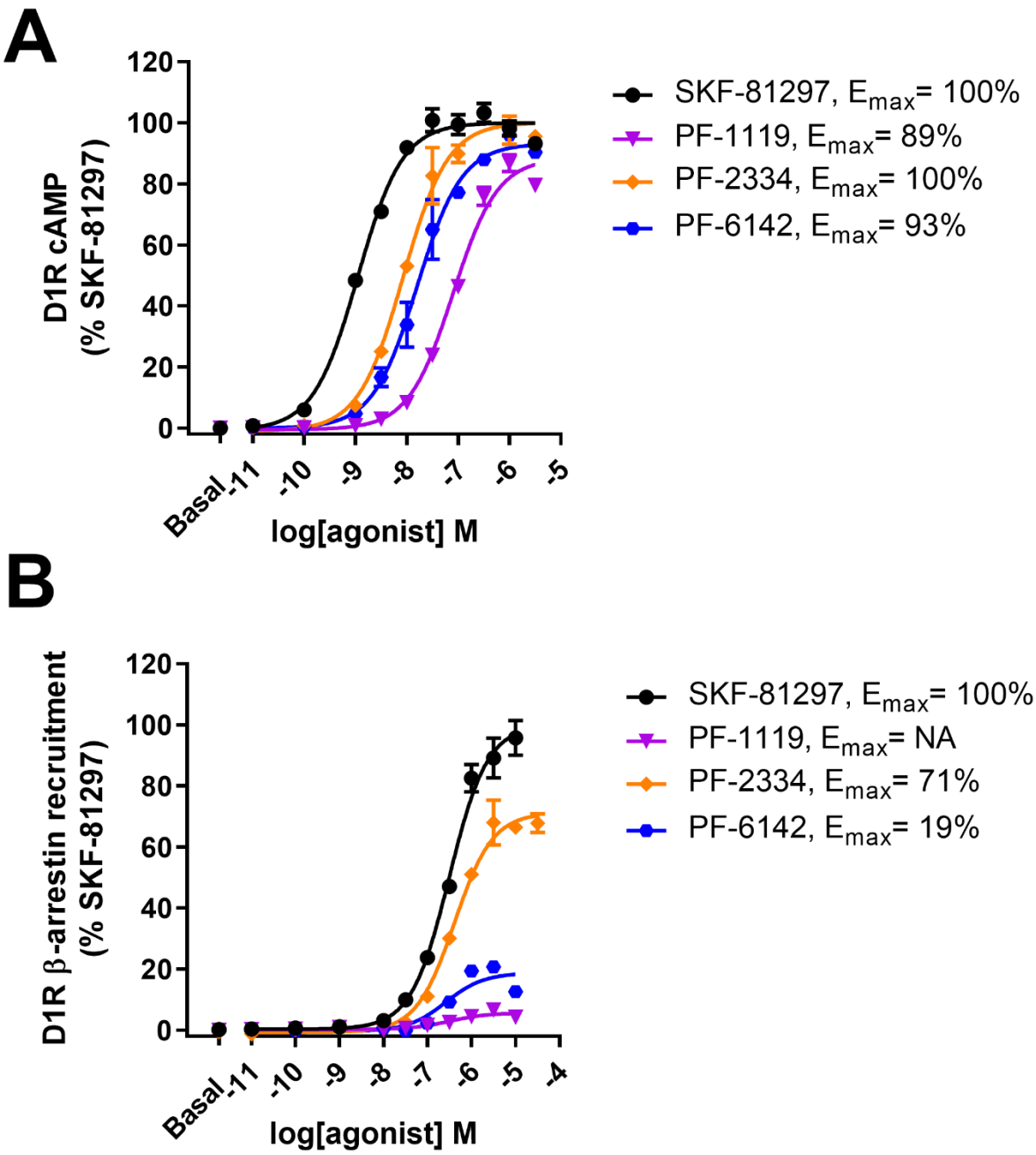
A
D1R cAMP
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
log[agonist] M
SKF-81297, Emax= 100%
PF-1119, Emax= 89%
PF-2334, Emax= 100%
PF-6142, Emax= 93%
B
D1R β-arrestin recruitment
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
-4
log[agonist] M
SKF-81297, Emax= 100%
PF-1119, Emax= NA
PF-2334, Emax= 71%
PF-6142, Emax= 19%

Figure 10. Dose response curves for the first-generation non-catechol D1R agonists in cAMP and β-arrestin recruitment assays. Glosensor and Presto-Tango assays were performed as described in the methods for cAMP and β-arrestin recruitment measurements, respectively. **A)** In the cAMP Glosensor assay, PF-1119, PF-2334, and PF-6142 are all full to high partial agonists compared to SKF-81297, an established full agonist at the D1R. The potency was rightward shifted by approximately one log unit for PF-1119, PF-2334, and PF-6142 compared to SKF-81297. **B)** For β-arrestin recruitment, the non-catechol agonists varied from partial agonists to no-activity in this pathway. PF-2334 was a partial agonist for β-arrestin recruitment while PF-6142 was a very low partial agonist. PF-1119 had no detectable activity for β-arrestin recruitment. Representative plots shown from at least three independent experiments performed in triplicate. Light counts per second (bioluminescence) from both assays was normalized to 100% SKF-81297 response. NA = no activity.

(Figure 10A, Table 1). PF-2334 (EC_{50} = 490 nM) had the highest efficacy of the first generation non-catechol agonists in the β-arrestin recruitment assay at 70% of SKF-81297 response. PF-6142 (EC_{50} = 245) was a very low partial at 20% of SKF-81297 while PF-1119 had no activity in the β-arrestin recruitment assay (Figure 10B, Table 1). These results indicate that the first-generation non-catechol agonists are G protein biased, albeit to varying degrees, and these results are consistent with the previous study of these ligands (*86*). Indeed, PF-1119 was a purely G protein biased agonist with no β-arrestin recruitment while PF-2334 had higher β-arrestin activity making it weakly G protein biased.

4.2.3 Second-generation non-catechol agonists include G protein biased and balanced agonists

The non-catechol agonists overcame many of the pitfalls associated with catechol agonists including poor oral bioavailability, short serum half-life, and tolerance (*86, 161*). However, none of the non-catechol agonists have balanced G protein and β-arrestin signaling and improvements can always be made to potency. Next, the non-catechol agonist derivatives resynthesized within our laboratory (*131*) and by Martini et al. (*132*) were characterized. These more recent "second-generation" agonists are derivatives of either PF-2334 or PF-6142. To begin with, several non-catechol agonists from Martini et al. (*132*) were analyzed in the Glosensor cAMP and β-arrestin recruitment assays. Martini et al. (*132*) derivatized PF-6142, creating 6-(4-(Furo[3,2-c]pyridin-4-yloxy)-2-methylphenyl)-5-methylpyrazin-2-amine (**Cmpd 1**), 4-(4-(Imidazo[1,2-a]pyridin-5-yl)-3-(trifluoromethyl)phenoxy)-furo[3,2-c]pyridine (**Cmpd 2**), and 7-(4-(Imidazo[1,2-a]pyridin-5-yl)-3-(trifluoromethyl)phenoxy)-thieno[2,3-c]pyridine (**Cmpd 3**). Cmpd 1 (E_{max} = 92%, EC_{50} = 470 nM) and Cmpd 3 (E_{max} = 85%, EC_{50} = 370 nM) were high partial agonists. Of the Martini et al. agonists, Cmpd 2 (EC_{50} = 180 nM) had the lowest efficacy at 78%, still a high partial agonist for cAMP production (Figure 11A, Table 1). These results contrast with Martini et al. (*132*) who indicated that the potency for cAMP production was much higher at 22 nM, 2.3 nM,

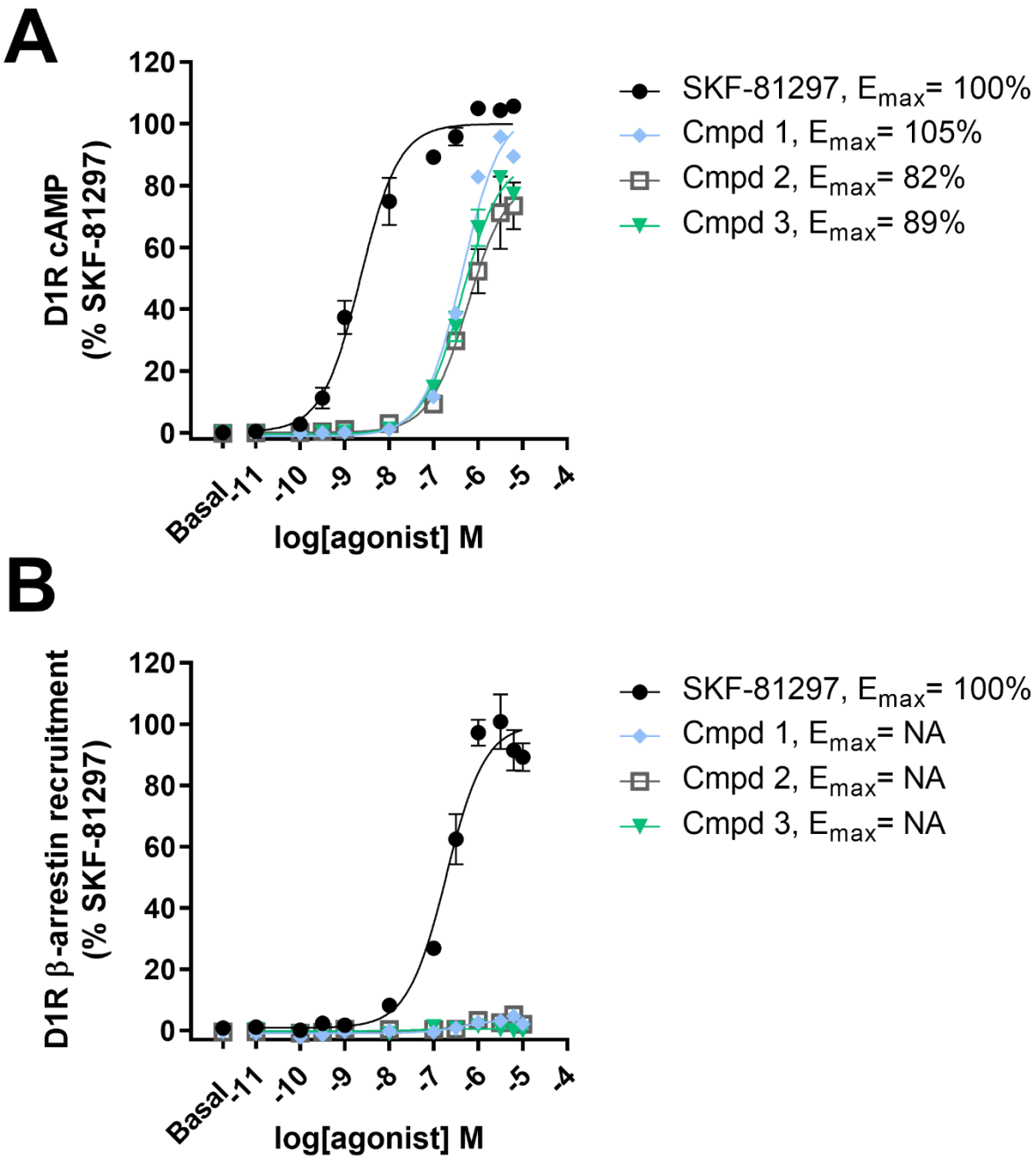
A
D1R cAMP
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
-4
log[agonist] M
SKF-81297, Emax= 100%
Cmpd 1, Emax= 105%
Cmpd 2, Emax= 82%
Cmpd 3, Emax= 89%
B
D1R β-arrestin recruitment
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
-4
log[agonist] M
SKF-81297, Emax= 100%
Cmpd 1, Emax= NA
Cmpd 2, Emax= NA
Cmpd 3, Emax= NA

Figure 11. Representative dose-response curves for second-generation non-catechol agonists from Martini et al. in cAMP and β-arrestin recruitment assays. cAMP and β-arrestin recruitment were measured with the Glosensor and Presto-Tango assays, respectively. Cmpd 1 and Cmpd 2 were not soluble at high concentrations in the standard 0.1% DMSO used for all the other agonists. Cmpd 1 and Cmpd 3 were dissolved in 0.5% DMSO. **A)** In the cAMP Glosensor assay, Cmpd 1 (E_{max} = 105%, EC_{50} = 440 nM), Cmpd 2 (E_{max} = 82%, EC_{50} = 580 nM), and Cmpd 3 (E_{max} = 89%, EC_{50} = 420 nM) are full to high partial agonists but are also rightward shifted in potency by approximately 2 log units compared to SKF-81297. **B)** Cmpd 1, Cmpd 2, and Cmpd 3 did not have activity in the β-arrestin recruitment assay. Representative plots and data shown from at least three independent experiments performed in triplicate. Light counts per second (bioluminescence) from both assays was normalized to 100% SKF-81297 response. For Mean ± SEM from the three independent experiments, see Table 1. NA = no activity, EC_{50} = half-maximal effective concentration (potency), E_{max} = maximum effect (efficacy).

and 2.2 nM for Cmpd 1, Cmpd 2, and Cmpd 3, respectively. Furthermore, Cmpd 1, Cmpd 2, and Cmpd 3 were not active in the β-arrestin recruitment assay (Figure 11B, Table 1). For Cmpd 1 and Cmpd 3, these data are consistent with Martini et al. (*132*) for previously reported D1R β-arrestin recruitment activity. Cmpd 1 and Cmpd 3 therefore appear G protein biased, which these data confirmed. However, Cmpd 2 was a balanced agonist in the Martini et al. (*132*) paper while these results indicate that Cmpd 2 does not have activity in the β-arrestin pathway. One potential explanation for the discrepancy between the present results and Martini et al. is that β-arrestin recruitment was measured using different assays, the Tango assay (here) and bioluminescence energy transfer assays in Martini et al. However, for all three agonists, the potency was rightward shifted in D1R cAMP assays, compared to the previously published study. The decreased potency may also explain why Cmpd 1, Cmpd 2, and Cmpd 3 were not active in the β-arrestin recruitment assay as it is common for β-arrestin assays to shift right in potency compared the cAMP assays.

Cmpd 19 and Cmpd 24 are also more recently described non-catechol agonists (*131*). Both Cmpd 19 (E_{max} = 92%, EC_{50} = 4.4 nM) and Cmpd 24 (E_{max} = 83%, EC_{50} = 76 nM) are potent, high partial agonists for cAMP production (Figure 12A, Table 1). Interestingly, Cmpd 19 (EC_{50} = 100 nM) potently recruited β-arrestin with 100% of SKF-81297 response. Cmpd 19 showed an unprecedented increase in D1R β-arrestin recruitment compared to any of the other non-catechol agonists studied. On the other hand, Cmpd 24 had no activity in the β-arrestin recruitment assay (Figure 12B, Table 1). Together, these results indicate that Cmpd 19 is a full balanced agonist for the D1R G protein and β-arrestin pathways, while Cmpd 24 is a purely G protein biased agonist.

4.2.4 G protein biased agonists do not induced D1R endocytosis

Agonist-induced D1R endocytosis requires β-arrestins, as shown in Chapter 3. Since several of the agonists are G protein biased and thus, did not recruit β-arrestin, we hypothesized that the G protein biased agonists would not induce D1R endocytosis. To determine the effects

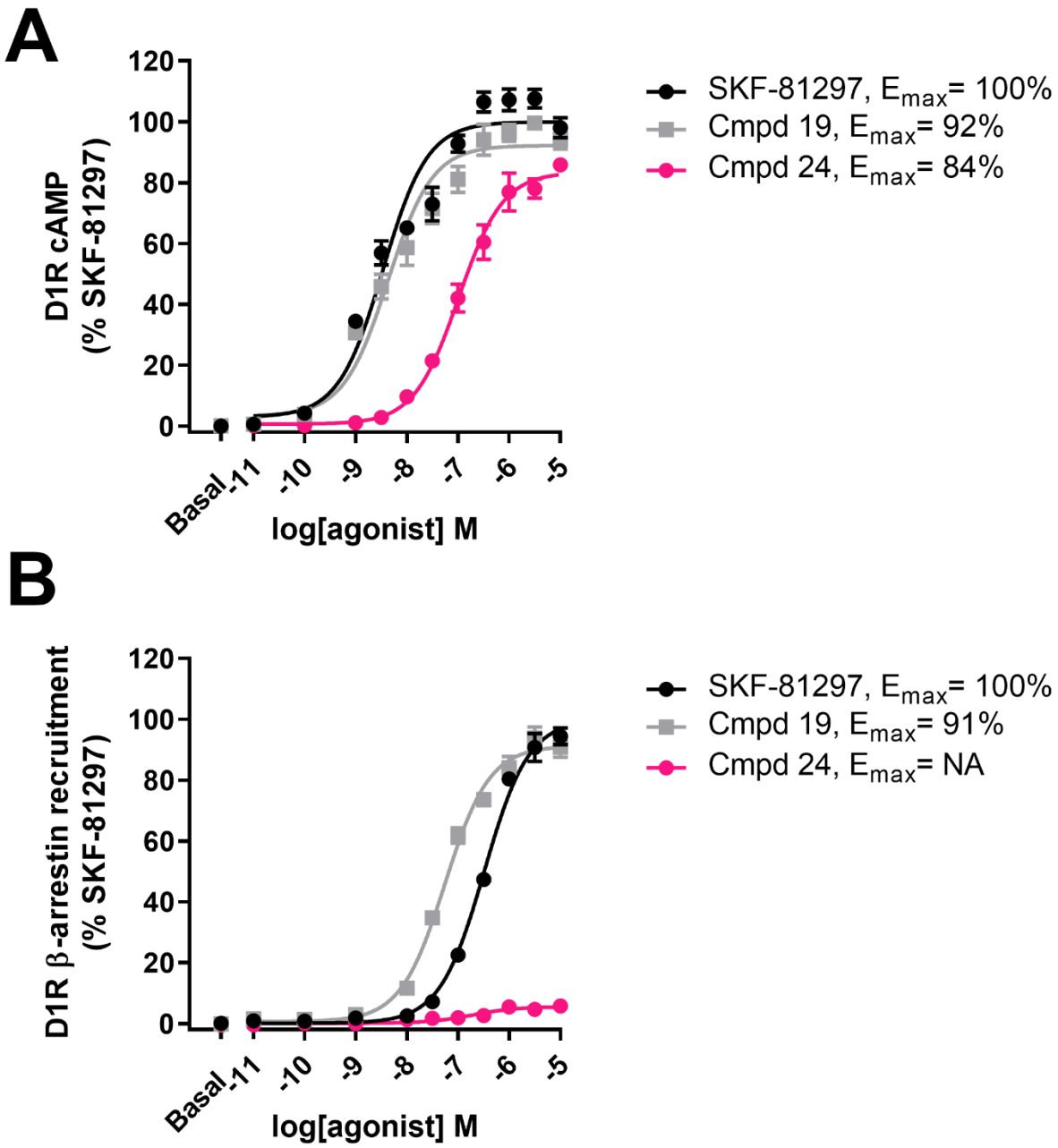
A
D1R cAMP
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
log[agonist] M
SKF-81297, Emax= 100%
Cmpd 19, Emax= 92%
Cmpd 24, Emax= 84%
B
D1R β-arrestin recruitment
(% SKF-81297)
120
100
80
60
40
20
0
Basal
-11
-10
-9
-8
-7
-6
-5
log[agonist] M
SKF-81297, Emax= 100%
Cmpd 19, Emax= 91%
Cmpd 24, Emax= NA

Figure 12. Representative dose-response curves for second-generation non-catechol agonists in cAMP and β-arrestin recruitment assays. Glosensor and Presto-Tango assays were performed as described in the methods for cAMP and β-arrestin recruitment measurements, respectively. **A)** Cmpd 19 and Cmpd 24 are both high partial agonists compared to SKF-81297. Cmpd 19 has comparable potency to SKF-81297 while Cmpd 24 is less potent than SKF-81297. **B)** In the β-arrestin recruitment assay, Cmpd 24 had no activity whereas Cmpd 19 was a full agonist. Cmpd 19 exhibited full agonism for cAMP and β-arrestin recruitment indicating it is a balanced agonist while Cmpd 24 is a G protein biased agonist. Representative plots shown from at least three independent experiments performed in triplicate. Light counts per second (bioluminescence) from both assays was normalized to 100% SKF-81297 response. NA = no activity.

Table 2. EC_{50} values and E_{max} for cAMP and β-arrestin recruitment of D1R agonists

	cAMP		β-arrestin recruitment	
Compound	**EC_{50} (nM)**	**E_{max} (% SKF-81297)**	**EC_{50} (nM)**	**E_{max} (% SKF-81297)**
SKF-81297	3.1 ± 1	100 ± 2	290 ± 22	100 ± 2
SKF-77434	110 ± 18	43 ± 2	NA	NA
SKF-38393	130 ± 27	85 ± 1	NA	NA
A-77636	3.1 ± 0.3	104 ± 1	35 ± 4	130 ± 10
PF-1119	58 ± 10	79 ± 4	NA	NA
PF-2334	11 ± 3	98 ± 3	490 ± 66	70 ± 5
PF-6142	22 ± 4	91 ± 4	245 ± 55	20 ± 1
Cmpd 1	470 ± 17	92 ± 7	NA	NA
Cmpd 2	180 ± 32	78 ± 1	NA	NA
Cmpd 3	370 ± 32	85 ± 4	NA	NA
Cmpd 19	4.4 ± 1	92 ± 2	100 ± 28	100 ± 5
Cmpd 24	76 ± 17	83 ± 1	NA	NA
Dopamine	38 ± 4	98 ± 1	NT	NT

The values are the Mean ± SEM from at least three independent experiments preformed in triplicate. EC_{50} = half-maximal effective concentration, E_{max} = maximum effect, nM = nano-molar, NA = no activity, NT = not tested.

of G protein biased agonists on D1R agonist-induced endocytosis, a subset of catechol and non-catechol agonists were tested in the antibody feeding endocytosis assay. SKF-81297 and dopamine are positive control agonists for D1R endocytosis. The results in Chapter 3 demonstrated that SKF-81297 and A-77636 induced D1R endocytosis. In addition, SKF-81297 and dopamine recruit β-arrestin and induce D1R endocytosis (*86, 162*). The catechol agonists, SKF-81297 and dopamine induced D1R endocytosis in the antibody feeding imaging assay. The antibody feeding assay labels cell surface receptors with an Alexa488-labeled antibody. Then, the indicated agonist was added and the cells are imaged after 60-minute treatments with the agonist. Internalized receptor/antibody appear as punctate signal inside the membrane. The vehicle treated cells had primarily membrane D1R without punctate and perinuclear signal (Figure 13A). SKF-81297 and dopamine induced punctate and perinuclear antibody pattern indicating agonist-induced endocytosis (Figure 13 B, C). To further investigate the downstream effects of G protein biased agonists on D1R agonist-induced endocytosis, PF-1119, PF-2334, and PF-6142 were also analyzed in the antibody feeding assay (Figure 13 D, E, & F). The first-generation non-catechol agonists did not induce substantial D1R endocytosis. Few puncta and no perinuclear receptor localization indicated that PF-1119, PF-2334, and PF-6142 did not induce receptor endocytosis to the same extent as the balanced catechol agonists SKF-81297 and dopamine. Finally, the non-catechol agonists, Cmpd 19 and Cmpd 24, were tested in the antibody feeding assay. Cmpd 19 is the only balanced non-catechol agonist and was compared to Cmpd 24, one of the pure G protein biased agonists, allowing a comparison between balanced and G protein biased non-catechol agonists. Cmpd 19 induced robust D1R endocytosis with punctate and perinuclear receptor localization. In contrast, Cmpd 24 did not induce D1R endocytosis with most of the receptor remaining at the membrane even after the 60-minute treatment (Figure 13 G & H). Together, these results consistently show that G protein biased agonists that do not engage β-arrestin, do not induce D1R endocytosis.

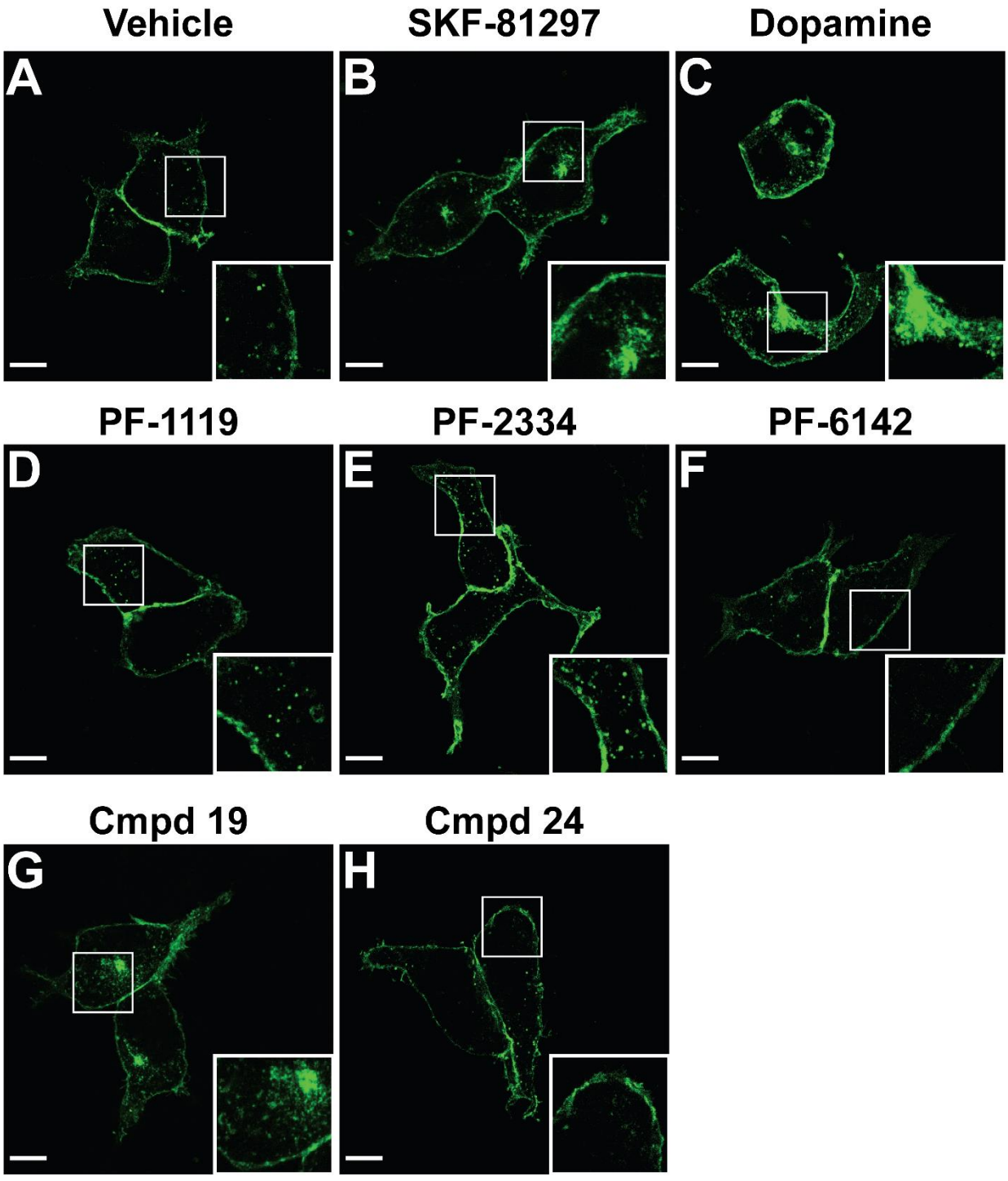
Vehicle
SKF-81297
Dopamine
A
B
C
PF-1119
PF-2334
PF-6142
D
E
F
Cmpd 19
Cmpd 24
G
H

Figure 13. G protein biased agonists do not induced endocytosis while balanced agonists do. HEK293 cells were transfected with N-terminally HA tagged D1R. The surface D1Rs were labeled with an anti-HA antibody conjugated to Alexa488 on ice to prevent endocytosis. The indicated agonists were added at saturating concentrations (10 μM) and the cells warmed to 37°C for 60 minutes, then fixed and imaged as described for Antibody feeding in the methods. **A)** Cells treated with vehicle for 60 minutes do not undergo endocytosis. **B, C)** Cells treated with the catechol agonists, SKF-81297 or dopamine, at saturating concentrations (10μM) have punctate and perinuclear HA-D1R indicating these balanced, catechol agonists induced endocytosis. **D-F)** In contrast, the non-catechol agonists PF-1119, PF-2334, and PF-6142 did not induce endocytosis with very few puncta observed. **G)** Cmpd 19 is the balanced non-catechol agonist, synthesized by our group. Cmpd 19 induced endocytosis similar to the balanced catechol agonists with punctate and perinuclear HA-D1R observed in the HEK293 cells. **H)** Cmpd 24, the pure G protein biased non-catechol agonist did not induced endocytosis in HEK293 cells. Representative images shown from three independent experiments. Similar results observed in >30 cells. Indicated areas enlarged in the lower right corner.

To further explore D1R agonist-induced endocytosis, a cell surface ELISA assay was used to quantify D1R agonist-induced endocytosis. The imaging results in Figure 13 are compelling evidence that G protein biased agonists do not induced endocytosis while balanced agonists do. However, to quantify D1R endocytosis over time after treatment with catechol and non-catechol agonists a cell surface ELISA assay was used. The cell surface ELISA quantifies the receptor remaining at the membrane after treatment. The three catechol agonists, SKF-81297, dopamine, and A-77636 all induced D1R endocytosis (Figure 14A). SKF-81297 and dopamine maximally induced 33% and 29% loss of cell surface HA, respectively, inducing similar amounts of D1R endocytosis (Figure 14A). Whereas A-77636 maximally induced 47% loss of cell surface HA (Figure 14A). This is not surprising since A-77636 is also a super agonist in the β-arrestin recruitment assay compared to SKF-81297. PF-1119 did not induce substantial D1R endocytosis with 5% loss of cell surface HA maximally. PF-6142 maximally induced 13% loss of cell surface HA while PF-2334 maximally induced 16% loss of cell surface HA (Figure 14B). These results align with the β-arrestin recruitment results in which PF-1119 did not recruit β-arrestin, PF-6142 was a very low partial agonist, and PF-2334 was a partial agonist. Finally, Cmpd 19 and Cmpd 24 were also tested in the cell surface ELISA. Cmpd 19 (28% loss of cell surface HA) induced similar levels of D1R endocytosis compared to SKF-81297 (33% loss of cell surface HA), which is consistent with Cmpd 19 being a balanced agonist (Figure 14C). Cmpd 24, the purely G protein biased agonist, maximally induced 11% loss of cell surface HA (Figure 14C). Total D1R endocytosis was also quantified using area under the curve (AUC) analysis. A-77636 induced significantly more total D1R endocytosis than SKF-81297 while the G protein biased agonists, PF-1119, PF-2334, PF-6142, and Cmpd 24 all induced significantly less total D1R endocytosis than SKF-81297 (Figure 14D). These results indicate that the balanced agonists induce similar amounts of endocytosis while the G protein biased agonists all induced less endocytosis. Further, A-77636 can be considered β-arrestin biased compared to SKF-81297 and A-77636 induced

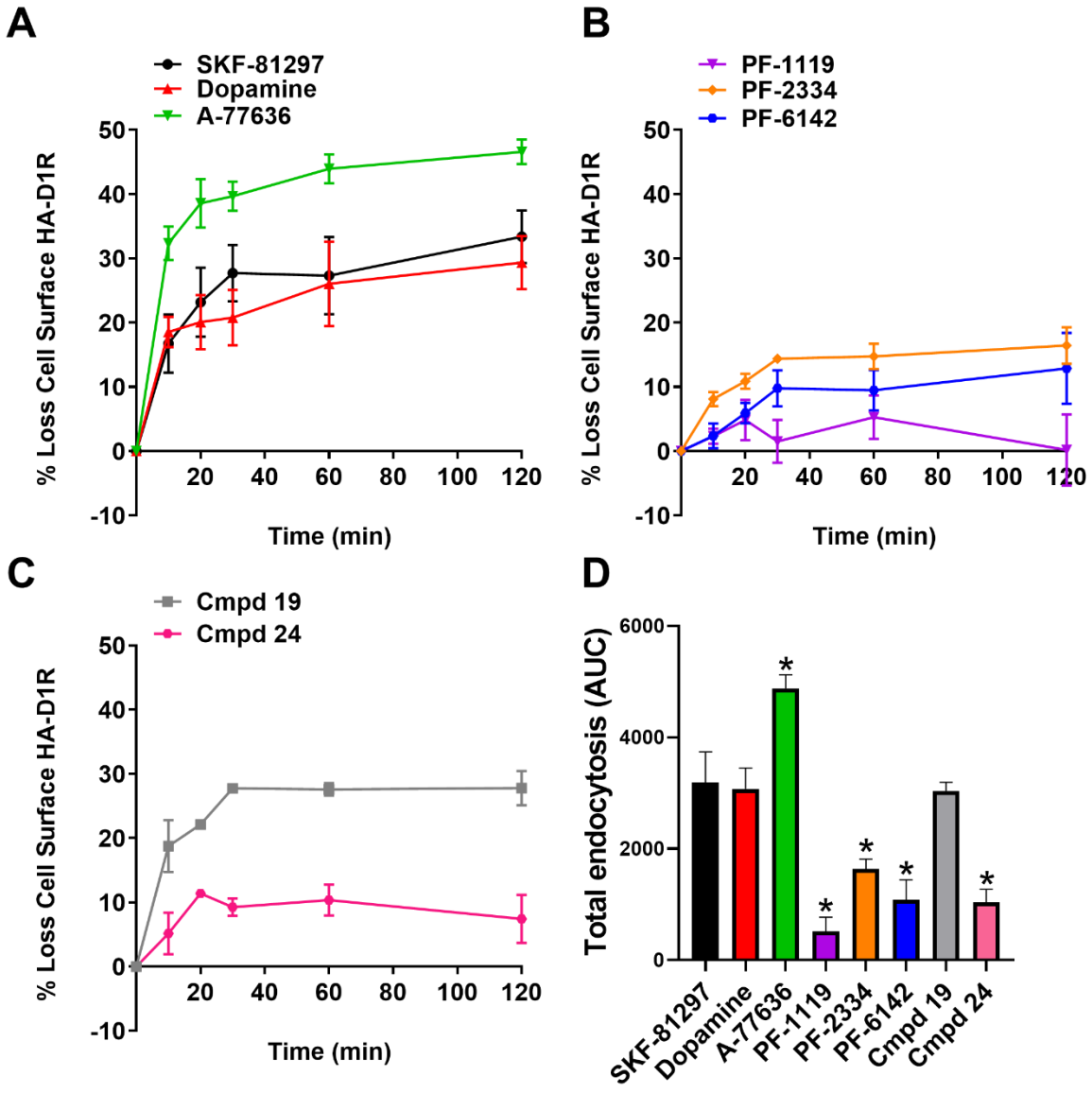
A
SKF-81297
Dopamine
A-77636
% Loss Cell Surface HA-D1R
Time (min)
B
PF-1119
PF-2334
PF-6142
% Loss Cell Surface HA-D1R
Time (min)
C
Cmpd 19
Cmpd 24
% Loss Cell Surface HA-D1R
Time (min)
D
Total endocytosis (AUC)
SKF-81297
Dopamine
A-77636
PF-1119
PF-2334
PF-6142
Cmpd 19
Cmpd 24

Figure 14. G protein biased agonists do not induce endocytosis while balanced agonists induced endocytosis. HEK293 cells transfected with HA-D1R were treated with saturating concentrations (10 μM) of the indicated agonist for 0-60 minutes and fixed. Anti-HA was used to detect HA-D1R remaining at the membrane after treatment under non-permeabilized conditions in an ELISA assay as described in the methods. **A)** Balanced catechol agonists, SKF-81297, dopamine, and A-77636 induced endocytosis after agonist treatment. A-77636 induced more endocytosis from 10 minutes onward than SKF-81297. SKF-81297 and dopamine induced comparable levels of endocytosis. A-77636 treatment reduced cell surface HA-D1R by 47% compared to SKF-81297 and dopamine which were 33% and 29%, respectively. **B)** The first generation non-catechol agonists did not induce endocytosis. PF-2334 reduced cell surface HA-D1R maximally by 16% while PF-1119 induced 5% receptor endocytosis. **C)** Cmpd 19, the balanced non-catechol agonist, induced endocytosis similar to the balanced catechol agonists. However, Cmpd 24 did not induce HA-D1R endocytosis with 11% of the receptor undergoing endocytosis at the maximum. **D)** Total D1R endocytosis (Area under the curve, AUC) analysis indicated A-77636 induced significantly more D1R endocytosis than SKF-81297. On the other hand, PF-1119, PF-2334, PF-6142, and Cmpd 24 induced significantly less total D1R endocytosis than SKF-81297. Only dopamine and Cmpd 19 were not significantly different from SKF-81297. Data presented as Mean ± SEM, n=3, *, $p<0.05$ vs. SKF-81297; One (**D**) or Two-way (**A-C**) ANOVA with Bonferroni's multiple comparisons test.

more endocytosis than SKF-81297. These results are consistent with the β-arrestin recruitment assay and the confocal imaging assay.

These results support the idea that β-arrestin recruitment and endocytosis are correlated. To test this hypothesis, Spearman's correlation analysis was conducted between the β-arrestin recruitment efficacy and D1R endocytosis at 120 minutes. β-arrestin recruitment strongly correlates with D1R endocytosis (Figure 15, $r=0.96$, $p<0.05$). The β-arrestin biased agonist, A-77636 (upper right corner) induced more endocytosis while the G protein biased agonist clustered in the lower left corner. Thus, the more an agonist engages β-arrestin, the more D1R endocytosis the agonist induces. Together, these results support our hypothesis that G protein biased agonists do not induce D1R endocytosis while balanced agonist do.

4.3 Discussion

To summarize, these results show for the first time, a direct comparison of several catechol agonists with non-catechol D1R agonists. These data validated the G protein bias observed with some catechol benzazepine agonists (SKF derivatives). Furthermore, these results indicate for the first time that A-77636 is a β-arrestin biased agonist compared to SKF-81297. In addition, this study determined that the non-catechol agonists are commonly G protein biased with one unexpected exception, Cmpd 19. Cmpd 1, Cmpd 2, and Cmpd 3 were not as potent for D1R cAMP as previously published by Martini et al. (*132*). In addition, Cmpd 2 was previously reported as a balanced agonist but these new results contradict that activity, since Cmpd 2 had no activity in the Presto-Tango β-arrestin recruitment assay (Figure 11). Due to problems with compound solubility and their lack of potency, these three compounds were not tested further. The non-catechols PF-1119, PF-2334, PF-6142, and Cmpd 24 were all potent full to partial agonists for increasing cAMP production but had reduced or absent β-arrestin recruitment, indicating that they are G protein biased. Of note, Cmpd 19 is defined as the only balanced non-catechol agonist

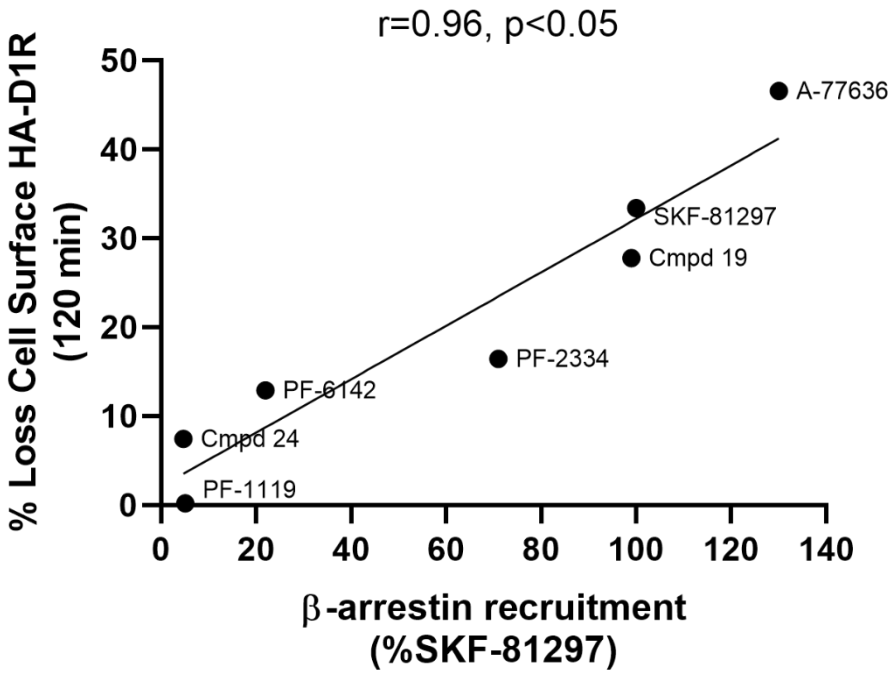

Figure 15. β-arrestin recruitment efficacy correlates to D1R agonist-induced endocytosis. β-arrestin recruitment E_{max} for each agonist was plotted with the percentage of D1R lost after 120-minute treatment with that agonist. Spearmen's correlation coefficient indicated a strong correlation between β-arrestin recruitment efficacy and D1R endocytosis ($r=0.96$, $p<0.05$).

tested. Additionally, we tested several of the catechol and non-catechol agonists in D1R endocytosis assays. These results indicate that the G protein biased agonists induced less endocytosis than their balanced counterparts did. These results strongly indicate that G protein bias decreases downstream agonist-induced D1R endocytosis. Taken together, these results characterized several non-catechol agonists that are both balanced and G protein biased and demonstrate that the degree of G protein bias influences agonist-induced D1R endocytosis.

To compare more recently synthesized non-catechol derivatives, three promising agonists were selected from the Martini et al. (*132*) report. As mentioned, Cmpd 1, Cmpd 2, and Cmpd 3 were much less potent than previously described for D1R cAMP activity. Furthermore, Cmpd 2 was not a balanced agonist and failed to recruit β-arrestin. Interestingly, this group followed up with a second publication in which they performed further medicinal chemistry and ended up validating the balanced D1R signaling activity of Cmpd 19 (Cmpd 10 in their article) (*161*). Cmpd 19 is an unprecedented and potent D1R balanced agonist for both D1R G protein and β-arrestin activity. In addition, Martini et al. extended our studies by showing that Cmpd 19 has good pharmacokinetic properties, brain penetrance, and is selective for D1R-like receptors. In the 6-OHDA unilateral lesion model of Parkinson's disease, Cmpd 19 also improved motor deficits in locomotor and rotation tests. They did not test a G protein biased agonist in these behavioral assays (*161*). The non-catechol agonists that are balanced (e.g. Cmpd 19) or G protein biased, are powerful chemical tools to study with *in vivo* behavioral assays due to their favorable pharmacokinetics and diverse signaling (i.e. balanced vs. biased).

The D1R is a validated drug target for a plethora of neurological disorders and more than 40 years of research effort focused on developing drug-like agonists. As previously mentioned, until very recently all D1R agonists were catechol agonists and suffered from poor oral bioavailability, rapid metabolism in the serum, poor brain penetrance, and tolerance (*84, 118, 157*). The non-catechol agonists overcome these pitfalls and are excellent candidates to advance

into *in vivo* assays. Most of the non-catechol agonists are G protein biased agonists with only one balanced agonist currently (Cmpd 19). Preclinical data suggests that β-arrestin biased agonists may be better for preventing L-DOPA induced dyskinesia; however, this remains untested as no non-catechol β-arrestin biased agonist has been reported to date. Increasing β-arrestin2 expression in rats and non-human primates reduced L-DOPA induced dyskinesia. It is worth pointing out that L-DOPA increases dopamine and does not specifically target the D1R and other mechanisms may also be involved in L-DOPA induced dyskinesia. Further, β-arrestin biased agonists may not be the best therapeutic route as A-77636 is a β-arrestin biased agonist in the present studies. A-77636 induces rapid tolerance in rodent and non-human primate model of Parkinson's disease (*86*). This rapid tolerance is not the same thing as the dyskinesia that presents after chronic treatment. Rapid tolerance is nonetheless a pitfall that prevents clinical development. A-77636 is a unique agonist in that it is the only β-arrestin biased agonist for the D1R, to my knowledge, and binds with high affinity, nearly irreversibly, to the D1R. This irreversible binding may also play a role in the rapid tolerance and until new β-arrestin biased agonists are discovered to test this, this question cannot answer this question.

Currently, the therapeutic potential of balanced and G protein biased agonists can be explored further. PF-2334, a G protein biased agonist, induced contralateral rotation in the 6-OHDA unilateral lesion model in rats. This rotational behavior was significantly higher after three days of repeated treatment compared to rats treated with A-77636 treatment. Similar results were obtained with the eye-blink rate in non-human primates (*86*). Eye blink rate is a convenient biomarker for D1R activation. These results suggest that G protein biased agonists do not induce tolerance as rapidly as catechol agonists such as A-77636. PF-2334 is actually one of the least G protein biased non-catechol agonist and it would be interesting to see if the pure G protein biased agonists such as PF-1119 or Cmpd 24 do not induce tolerance at all. Moreover, it is unknown currently if G protein biased agonists are better than balanced D1R agonists. Our recent

profiling of the first balanced non-catechol D1R agonist (Cmpd 19) will allow us to investigate if G protein biased or balanced agonism is beneficial in preclinical models. Previously, G protein biased agonists had to be compared to catechol agonists that are rapidly metabolized making comparisons difficult. Now, it is possible to compare G protein biased non-catechol agonists to the balanced non-catechol agonists eliminating the potential confounding factors, pharmacokinetics and brain penetrance. Furthermore, the efficacy of G protein biased and balanced non-catechol agonists can be tested in a variety of behavioral assays. For example, G protein bias may be more beneficial for cognition and working memory while balanced agonists may be better for motor disorders. The tools to answer these questions are now available with the balanced or biased D1R non-catechol agonists. The non-catechol agonists are a breakthrough after 40 years of searching for drug-like D1R agonists. There is great potential for the G protein biased non-catechol agonists.

5. DISCUSSION

5.1 Overall findings and interpretations

1) Surprisingly, β-arrestin does not influence the kinetics of D1R stimulated cAMP for the first 60 minutes of continuous agonism. Phosphodiesterase activity is partially responsible for cAMP decreases during this time period. However, D1R desensitization was reduced in β-arrestin1/2 knockout cells after four hours indicating β-arrestin1/2 still has a role in classic D1R/G protein/cAMP desensitization.
2) These results demonstrate that β-arrestin1 or β-arrestin2 are required for agonist-induced endocytosis. β-arrestin1/2 knockout eliminated D1R agonist-induced endocytosis. Following these studies, re-expression of β-arrestin1 rescued D1R agonist-induced endocytosis. Similar results were observed when β-arrestin2 was re-expressed in the knockout cells. Interestingly, β-arrestin1 and β-arrestin2 both rescued D1R agonist-induced endocytosis indicating that they have redundant functions, at least in regards to D1R agonist-induced endocytosis.
3) In addition, G protein/cAMP signaling and b-arrestin recruitment assays were used to characterize several catechol and non-catechol agonists. These results confirmed a previous report that several benzazepine catechol agonists are G protein biased. One catechol agonist, A-77636, appears to be β-arrestin biased, a previously overlooked characteristic of the agonist. Perhaps more interesting, the non-catechol agonists Cmpd 19 and Cmpd 24 had superior potency in the G protein pathway compared to other non-catechol agonists. Importantly, we clarified the activity of several D1R agonists with pure G protein bias and the first non-catechol agonist that was balanced was identified in these studies. Cmpd 19 is the first non-catechol agonist to exhibit balanced signaling and will be

an invaluable tool going forward to compare with the G protein biased non-catechol agonists.

4) To investigate the downstream effects of G protein biased agonists on D1R signaling, we investigated the effects of G protein biased agonists on D1R endocytosis. G protein biased agonists (that do not recruit β-arrestin) do not induce D1R endocytosis. The balanced catechol and non-catechol agonists induce D1R endocytosis and the β-arrestin biased catechol induced greater endocytosis than the balanced agonists.

5.2 Working models of the results

5.2.1 The role of β–arrestin in D1R endocytosis

The canonical role of β-arrestins in GPCR endocytosis is to bind the GPCR after G protein activation, desensitizing further G protein signaling and leading to endocytosis of the receptor through clathrin-coated pits. The results of these studies support this canonical role of β-arrestins for the D1R and advance upon previous studies that investigated D1R endocytosis. In the present study, β-arrestins are absolutely required for D1R agonist-induced endocytosis. Interestingly, the reliance of GPCRs on β-arrestins for agonist-induced endocytosis appears to be receptor specific as 5-HT2A receptor endocytosis is only partly dependent on β-arrestins. In this model, the D1R binds an agonist leading to G protein activation and increases in cAMP production through adenylyl cyclase. β-arrestins are then recruited to the D1R preventing further G protein activation and leading to receptor desensitization at extended time courses. The D1R/β-arrestin complex interacts with proteins in clathrin-coated pits and is internalized in an agonist dependent manner. Furthermore, this model is now clearly linked to the D1R using a robust genetic knockout model and supports previous studies that indicated the D1R is internalized through a dynamin dependent process. However, caveolin-mediated endocytosis has also been suggested as an endocytosis

mechanism for the D1R. The current study clearly demonstrates that this is not the case for HEK293 cells. The major caveat is that caveolin-mediated endocytosis was demonstrated in COS-7 cells indicating that caveolin may play a role in other cell types (*146*). Future studies addressing this caveat are outlined below. In addition to endocytosis, caveolin is a protein that aids in the formation of lipid rafts/microdomains. The lipid rafts can organize GPCR signaling by bringing signaling components (i.e. GPCRs and G proteins) together to promote signaling or sequester components to inhibit signaling (*168*). Thus, caveolin may still be important for the regulation of GPCR signaling even if it does not have a role in D1R agonist-induced endocytosis.

5.2.2 A model for D1R G protein biased agonism

Several catechol and non-catechol D1R agonists exhibit G protein biased agonism. G protein biased agonism is not a new concept and biased agonists are being pursued for multiple GPCRs including the μ-opioid receptor and the D1R, among others. The idea is that selectively targeting one signaling pathway over another may increase the efficacy or reduce on-target side effects. Using the D1R as an example, G protein biased agonists may be as effective as treatments such as L-DOPA (a dopamine precursor) while also reducing the tolerance associated with balanced agonists. The model for the D1R is that a G protein biased agonist activates the G protein pathway leading to improved voluntary motor control or improved working memory. The G protein biased agonists do not engage the β-arrestin pathway preventing D1R endocytosis and desensitization. The decreased desensitization could prolong D1R G protein-mediated signaling and reduce tolerance. Together with previously reported results, this model provides a potential explanation for how the G protein biased agonists improve voluntary movement without inducing profound tolerance like previous catechol agonists. A G protein biased non-catechol agonist is entering Phase III clinical trials and has excellent safety and tolerability in humans (*123, 125, 126, 169*). Further support for this model includes *in vivo* studies using A-77636. According to the present study, A-77636 is a β-arrestin biased agonist that robustly induces D1R endocytosis,

significantly more than balanced agonists do. A-77636 is associated with rapid and profound tolerance even by the second dose 24 hours after the initial dose. Thus, the propensity of an agonist to induce tolerance at the D1R may be tied to its ability to recruit β-arrestin and induce endocytosis. Future studies testing a range of agonists, β-arrestin biased to balanced to G protein biased, are needed to examine the relation between bias and desensitization/tolerance *in vitro* and *in vivo*.

5.3 Significance

5.3.1 A new mechanism of action for non-catechol D1R agonists

The non-catechol agonists are a breakthrough simply because they are the first drug-like D1R agonists. Interestingly, most of the non-catechol agonists are also G protein biased agonists. This study elucidated a new mechanism of action for the G protein biased agonists. The G protein biased non-catechol agonists are high partial to full agonists in the G protein pathway indicating that they will fully induce the G protein/cAMP/protein kinase A pathway leading to changes in protein phosphorylation and gene transcription. However, the novel aspect of their mechanism of action is a lack of β-arrestin recruitment that leads to decreased receptor endocytosis. The obvious implication of a lack of β-arrestin recruitment is a lack of receptor desensitization. The current studies provide evidence that G protein biased agonists would not induce D1R desensitization, since β-arrestin1/2 KO reduced D1R cAMP desensitization. This is likely due to decreased D1R β-arrestin recruitment as well as less D1R endocytosis leaving more D1Rs at the membrane to signal. Another interesting implication of the lack of endocytosis is that there is also likely less receptor downregulation. As mentioned above, GPCR endocytosis requires the GPCR then be sorted into recycling pathways or degradation pathways. The reduced β-arrestin recruitment and subsequent endocytosis of the G protein biased agonists also suggests that there are fewer receptors in the degradation pathways. Thus, the G protein biased non-catechol

agonists would likely also have reduced risk of downregulating the D1R receptor. This has great potential to increase D1R signaling without inducing drug tolerance.

Indeed, downregulating the D1R in the brain would produce profound negative consequences. A D1R antagonist increased cocaine taking in patients with cocaine use disorder and significantly increased depression, anxiety, and suicidal thoughts during a Phase III clinical trial of ecopipam for weight loss in obesity (*170, 171*). Inhibiting D1R signaling produces profound adverse effects. Furthermore, nicotine reduces D1R density in current smokers compared to healthy controls in the striatum (*172*). The authors suggested that the decreased D1R density may underlie sustained nicotine use (*172*). Cocaine, amphetamine, and methamphetamine all decreased dopamine release, the dopamine transporter, and downregulated the D1R in a meta-analysis that looked at withdrawal after 5 days to 3 weeks (*173*). The G protein biased non-catechol D1R agonists have great potential in treating these disorders by normalizing D1R signaling without exacerbating the hypodopaminergic state during withdrawal by further downregulating the D1R. Specifically, a partial D1R agonist could be used to normalize D1R signaling without the potential of producing a “high” since the efficacy is lower than dopamine. Moreover, a partial D1R agonist may prevent a “high” if the patient relapses and the partial agonist is at high receptor occupancy, preventing the binding of the fully efficacious dopamine. The G protein biased non-catechol agonists may be ideal candidates for elevating D1R signaling in these individuals.

5.3.2 Therapeutic implications for balanced and G protein biased agonists

The major implications for this research project are the potential therapeutic benefits of the identified non-catechol agonists. The non-catechol D1R agonists overcome the pitfalls associated with the previous catechol D1R agonists. Catechol agonists are associated with poor oral bioavailability, poor serum half-life, and rapid tolerance. The non-catechol agonists overcome these pitfalls. Furthermore, Phase I studies indicate the non-catechol agonists are safe and well-

tolerated in patients with schizophrenia and early-stage Parkinson's disease (*123, 125, 126*). Cerevel, a small biotechnology spin-off company from Pfizer, is taking the non-catechol agonists forward and recently completed Phase II studies (*124, 169*). The results of a Phase II clinical study of early-stage Parkinson's disease followed patients over 15 weeks and ended with favorable outcomes, reducing motor deficits with few adverse reactions. Based on these results Cerevel is planning a Phase III clinical trial for early and late-stage Parkinson's disease beginning in 2020 (https://www.cerevel.com/news/cerevel-therapeutics-announces-positive-results-from-a-phase-2-study-of-tavapadon-in-patients-with-early-stage-parkinsons-disease/) (*169*).

Surprisingly, most of the non-catechol agonists are G protein biased agonists. This thesis project also characterized the first balanced non-catechol agonist along with a purely G protein biased non-catechol agonist. Together these agonists are invaluable tools to study balanced and biased D1R signaling *in vitro* and in the future *in vivo* (detailed below). Here, we show that the G protein biased agonists do not induce D1R endocytosis while the balanced agonists do. Reduced or absent β-arrestin recruitment and subsequent D1R endocytosis may explain why the G protein biased agonists also do not induce the profound tolerance that previous catechol agonists such as A-77636 did. PF-2334 induced significantly less tachyphylaxis in a rat model of Parkinson's disease (*86*). PF-2334 also did produce tachyphylaxis in non-human primates after three days as measured by the eye-blink test (a convenient biomarker for D1R engagement) (*86*). PF-2334 was compared to A-77636 in these behavioral studies and interestingly PF-2334 is one of the least G protein biased non-catechol agonists. These results suggest that β-arrestin biased (A-77636) agonists engage more with β-arrestin leading to receptor endocytosis and potentially downregulating the D1R reducing efficacy in a little as one day. However, the G protein biased agonists do not induce endocytosis and likely do not downregulate the D1R leading to sustained therapeutic efficacy. As such, the G protein biased non-catechol agonists have the potential to alleviate motor deficits in Parkinson's disease, cognitive deficits in schizophrenia, attention deficits

in ADHD, and alter reward processing in substance use disorders. The non-catechol agonists are the first D1R drug-like agonists and took 40 years of concerted research effort to create these promising, novel agonists.

5.4 Future directions

The molecular effects of G protein biased signaling at the D1R is not clearly understood. In the present study, only one downstream effect of G protein biased signaling at the D1R was examined, endocytosis. This study demonstrated that G protein biased agonists do not induce endocytosis. It is logical that agonists that do not recruit β-arrestin also do not induce endocytosis considering that β-arrestins are required for agonist-induced endocytosis of many GPCRs. The current studies focused exclusively on the β-arrestin recruitment pathway. Future studies examining the effect of G protein bias on the G protein pathway are the next logical step to advance this research. For example, does less β-arrestin recruitment lead to prolonged G protein activation and reduced D1R desensitization? Furthermore, investigating downstream signaling events such as CREB phosphorylation would provide a more holistic picture of the effects that G protein biased agonists have on D1R signaling. We hypothesize that G protein biased agonists have prolonged CREB phosphorylation due to decreased D1R desensitization. Another future direction is to investigate dopamine D5 receptor (D5R) signaling with the G protein biased agonists. Sibley's group showed that while several benzazepine agonists where G protein biased at the D1R, the same benzazepine agonists were balanced at the D5R. Thus, it would be very interesting to see if this trend continues with the structurally distinct non-catechol agonists. Distinguishing the D1R and D5R pharmacologically has been extremely difficult with most agonists having similar effects on both receptors. Furthermore, relatively little, compared to the D1R, is known about the D5R and its role *in vivo* since most previous agonists were grouped into the D1R-like category. Finally, while β-arrestins are required for D1R agonist-induced endocytosis in HEK293 cells, validating these results in other cell types may be beneficial. As mentioned

above, caveolin-mediated endocytosis may play a role in D1R endocytosis in COS-7 cells. Thus, investigating D1R endocytosis in β-arrestin1/2 knockout COS-7 cells may aid in determining if β-arrestins play a universal role in endocytosis or if caveolin may be important depending on the cell type. However, it may be more relevant to investigate if β-arrestin is essential for D1R endocytosis in a neuronal cell type. To achieve this goal, a neuronal cell line such as SH-SY5Y or primary striatal neurons could be used. However, the limitation is that neuronal cell lines are difficult to transfect and siRNA strategies cannot knockout β-arrestin expression.

Another potential avenue to continue this work is to take the non-catechol agonists into *in vivo* studies. While several G protein biased agonists have entered clinical trials and are showing promise for treating Parkinson's disease, balanced non-catechol agonists have not been defined until very recently. Studying the different effects of balanced and biased agonists was incredibly difficult previously as the catechol agonists have very poor pharmacokinetics while the non-catechols have good pharmacokinetics. Thus, studies that compared catechol and non-catechol agonists always have a major caveat: Is the observed difference due to differences in pharmacokinetics or D1R agonist activity? Both the balanced and G protein biased non-catechol agonists exhibit good *in vivo* pharmacokinetics (*86, 161*). The G protein biased agonists can aid in determining the G protein signaling specific contributions to D1R behaviors. For examples, it appears that voluntary movement is primarily initiated through the G protein pathway due to the efficacy of the G protein biased non-catechol agonists. However, other D1R behaviors such as attention, reward processing, and motivation have not been evaluated using the G protein biased and balanced agonists. Pathway specific contributions to signaling is an exciting new area of research for the non-catechol D1R agonists. In addition, it is not currently known if G protein biased agonists are actually better than balanced agonists. While it is hypothesized that G protein biased agonists will be superior to balanced agonists due to decreased tolerance and *in vivo* tachyphylaxis, this has not been directly tested in animal models. Researchers now have the

appropriate tools to answer this question. The balanced and G protein biased non-catechol agonists would ideally be compared across an array of behaviors and disease models to determine if G protein biased agonists are indeed, therapeutically superior to balanced agonists. For instance, the balanced and G protein biased agonists should be tested in Parkinson's disease models such as the 6-OHDA unilateral lesion model and cognitive deficit models such as ketamine-induced working memory deficits. These studies should be designed to include measures of efficacy (increased rotational behavior, improved working memory) and also test the potential for tolerance after repeated and chronic dosing. Carefully designing these *in vivo* studies will answer the question of whether G protein biased agonists are therapeutically superior to balanced agonists.

Additional clinical trials are also needed to further test the therapeutic benefit of the G protein biased non-catechol agonists in diseases other than Parkinson's. As mentioned above, the D1R has fundamental roles in cognition including working memory and attention as well as reward processing and mood. The major focus of clinical trials has been on Parkinson's disease, which is of course an important target for D1R agonists. In addition, there was a Phase Ib study conducted in patients with stable schizophrenia, but no cognitive or reward processing effects were observed, likely due to limitations of the study design being geared towards safety and tolerability. Repurposing the non-catechol agonists for diseases other than Parkinson's disease requires further clinical trials, but the non-catechol agonists may alleviate symptoms currently untreatable such as deficits in working memory in patients with schizophrenia.

5.5 Conclusions

The non-catechol agonists are an incredible advancement after 40 years of research targeting the D1R. Furthermore, these results highlight a novel mechanism of action for non-catechol D1R agonists that are G protein biased in which there is normal G protein signaling but the agonists do not induce receptor endocytosis. Additionally, the research contained in this thesis

demonstrates an essential role for β-arrestins in D1R agonist-induced endocytosis. We characterized catechol and non-catechol agonists showing novel β-arrestin biased signaling for one catechol agonist and identifying promising balanced and G protein biased non-catechol agonists. Interestingly, the G protein biased non-catechol agonists do not induce endocytosis while balanced agonists do. Together these studies provide a logical progression from β-arrestins role in agonist-induced endocytosis through the lack of endocytosis observed with the G protein biased agonists. It is logical that G protein biased agonists do not induce endocytosis when they do not recruit β-arrestin, which is required for agonist-induced endocytosis.

References

1. M. F. Bear, B. W. Connors, M. A. Paradiso, *Neuroscience: Exploring the brain*. (Lippincott Williams and Wilkins, USA, ed. Third, 2007).
2. E. R. Kandel, J. H. Schwartz, T. M. Jessell, S. A. Siegelbaum, A. J. Hudspeth, *Principles of Neural Science*. (McGraw-Hill Companies, USA, ed. Fifth, 2013).
3. A. Robert, J. R. Howe, How AMPA Receptor Desensitization Depends on Receptor Occupancy. *The Journal of Neuroscience* **23**, 847 (2003).
4. D. M. MacLean, R. J. Durham, V. Jayaraman, Mapping the Conformational Landscape of Glutamate Receptors Using Single Molecule FRET. *Trends in neurosciences* **42**, 128-139 (2019).
5. A. Robert, N. Armstrong, J. E. Gouaux, J. R. Howe, AMPA receptor binding cleft mutations that alter affinity, efficacy, and recovery from desensitization. *J Neurosci* **25**, 3752-3762 (2005).
6. M. J. Lohse, J. L. Benovic, J. Codina, M. G. Caron, R. J. Lefkowitz, beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**, 1547-1550 (1990).
7. J. S. Smith, S. Rajagopal, The beta-Arrestins: Multifunctional Regulators of G Protein-coupled Receptors. *The Journal of biological chemistry* **291**, 8969-8977 (2016).
8. C. A. Moore, S. K. Milano, J. L. Benovic, Regulation of receptor trafficking by GRKs and arrestins. *Annu Rev Physiol* **69**, 451-482 (2007).
9. X. Tian, D. S. Kang, J. L. Benovic, β-arrestins and G protein-coupled receptor trafficking. *Handb Exp Pharmacol* **219**, 173-186 (2014).
10. R. R. Gainetdinov, R. T. Premont, L. M. Bohn, R. J. Lefkowitz, M. G. Caron, Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* **27**, 107-144 (2004).
11. M. C. Lagerstrom, H. B. Schioth, Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* **7**, 339-357 (2008).
12. T. Warne, P. C. Edwards, A. S. Dore, A. G. W. Leslie, C. G. Tate, Molecular basis for high-affinity agonist binding in GPCRs. *Science* **364**, 775-778 (2019).
13. B. Alberts *et al.*, *Molecular Biology of the Cell*. (Garland Science, United Kindom, ed. Fifth, 2008).
14. P. Zhao, S. G. B. Furness, The nature of efficacy at G protein-coupled receptors. *Biochem Pharmacol* **170**, 113647 (2019).
15. M. P. Strathmann, M. I. Simon, G alpha 12 and G alpha 13 subunits define a fourth class of G protein alpha subunits. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 5582-5586 (1991).
16. V. Syrovatkina, K. O. Alegre, R. Dey, X.-Y. Huang, Regulation, Signaling, and Physiological Functions of G-Proteins. *J Mol Biol* **428**, 3850-3868 (2016).
17. S. M. Khan *et al.*, The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. *Pharmacological reviews* **65**, 545-577 (2013).
18. A. Inoue *et al.*, Illuminating G-Protein-Coupling Selectivity of GPCRs. *Cell* **177**, 1933-1947.e1925 (2019).
19. U. Wilden, S. W. Hall, H. Kühn, Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci U S A* **83**, 1174-1178 (1986).
20. H. Attramadal *et al.*, Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *The Journal of biological chemistry* **267**, 17882-17890 (1992).
21. J. G. Krupnick, V. V. Gurevich, J. L. Benovic, Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. *The Journal of biological chemistry* **272**, 18125-18131 (1997).
22. R. Sterne-Marr *et al.*, Polypeptide variants of beta-arrestin and arrestin3. *The Journal of biological chemistry* **268**, 15640-15648 (1993).

23. E. V. Gurevich, V. V. Gurevich, Arrestins: ubiquitous regulators of cellular signaling pathways. *Genome Biol* **7**, 236-236 (2006).
24. O. J. Kim *et al.*, The role of phosphorylation in D1 dopamine receptor desensitization: evidence for a novel mechanism of arrestin association. *The Journal of biological chemistry* **279**, 7999-8010 (2004).
25. B. Konig *et al.*, Three cytoplasmic loops of rhodopsin interact with transducin. *Proc Natl Acad Sci U S A* **86**, 6878-6882 (1989).
26. E. A. Dratz *et al.*, NMR structure of a receptor-bound G-protein peptide. *Nature* **363**, 276-281 (1993).
27. B. Konig, M. Gratzel, Site of dopamine D1 receptor binding to Gs protein mapped with synthetic peptides. *Biochim Biophys Acta* **1223**, 261-266 (1994).
28. Y. Kang *et al.*, Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* **523**, 561-567 (2015).
29. S. G. Rasmussen *et al.*, Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555 (2011).
30. U. Wilden, Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding. *Biochemistry* **34**, 1446-1454 (1995).
31. C. Krasel, M. Bunemann, K. Lorenz, M. J. Lohse, Beta-arrestin binding to the beta2-adrenergic receptor requires both receptor phosphorylation and receptor activation. *The Journal of biological chemistry* **280**, 9528-9535 (2005).
32. D. Jiang, D. R. Sibley, Regulation of D(1) dopamine receptors with mutations of protein kinase phosphorylation sites: attenuation of the rate of agonist-induced desensitization. *Mol Pharmacol* **56**, 675-683 (1999).
33. M. Tiberi, S. R. Nash, L. Bertrand, R. J. Lefkowitz, M. G. Caron, Differential regulation of dopamine D1A receptor responsiveness by various G protein-coupled receptor kinases. *The Journal of biological chemistry* **271**, 3771-3778 (1996).
34. A. Jackson, R. M. Iwasiow, Z. Y. Chaar, M. F. Nantel, M. Tiberi, Homologous regulation of the heptahelical D1A receptor responsiveness: specific cytoplasmic tail regions mediate dopamine-induced phosphorylation, desensitization and endocytosis. *J Neurochem* **82**, 683-697 (2002).
35. M. Lamey *et al.*, Distinct residues in the carboxyl tail mediate agonist-induced desensitization and internalization of the human dopamine D1 receptor. *The Journal of biological chemistry* **277**, 9415-9421 (2002).
36. S. S. Ferguson *et al.*, Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271**, 363-366 (1996).
37. S. Ahn, H. Wei, T. R. Garrison, R. J. Lefkowitz, Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *The Journal of biological chemistry* **279**, 7807-7811 (2004).
38. T. A. Kohout, F. S. Lin, S. J. Perry, D. A. Conner, R. J. Lefkowitz, beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci U S A* **98**, 1601-1606 (2001).
39. O. B. Goodman *et al.*, Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* **383**, 447-450 (1996).
40. S. A. Laporte, W. E. Miller, K. M. Kim, M. G. Caron, beta-Arrestin/AP-2 interaction in G protein-coupled receptor internalization: identification of a beta-arrestin binging site in beta 2-adaptin. *The Journal of biological chemistry* **277**, 9247-9254 (2002).

41. S. A. Laporte, R. H. Oakley, J. A. Holt, L. S. Barak, M. G. Caron, The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. *The Journal of biological chemistry* **275**, 23120-23126 (2000).
42. Y. M. Kim, J. L. Benovic, Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. *The Journal of biological chemistry* **277**, 30760-30768 (2002).
43. Y. Zhuo, S. A. Vishnivetskiy, X. Zhan, V. V. Gurevich, C. S. Klug, Identification of receptor binding-induced conformational changes in non-visual arrestins. *The Journal of biological chemistry* **289**, 20991-21002 (2014).
44. V. V. Gurevich, E. V. Gurevich, GPCR Signaling Regulation: The Role of GRKs and Arrestins. *Frontiers in Pharmacology* **10**, (2019).
45. M. L. Mohan, N. T. Vasudevan, M. K. Gupta, E. E. Martelli, S. V. Naga Prasad, G-protein coupled receptor resensitization-appreciating the balancing act of receptor function. *Curr Mol Pharmacol*, (2012).
46. A. Kliewer, R. K. Reinscheid, S. Schulz, Emerging Paradigms of G Protein-Coupled Receptor Dephosphorylation. *Trends in Pharmacological Sciences* **38**, 621-636 (2017).
47. L. M. Luttrell *et al.*, Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**, 655-661 (1999).
48. P. H. McDonald *et al.*, Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574-1577 (2000).
49. L. M. Luttrell *et al.*, Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* **98**, 2449-2454 (2001).
50. S. K. Shenoy *et al.*, beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *The Journal of biological chemistry* **281**, 1261-1273 (2006).
51. K. Xiao *et al.*, Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc Natl Acad Sci U S A* **104**, 12011-12016 (2007).
52. A. Tohgo, K. L. Pierce, E. W. Choy, R. J. Lefkowitz, L. M. Luttrell, beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *The Journal of biological chemistry* **277**, 9429-9436 (2002).
53. D. Gesty-Palmer *et al.*, Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *The Journal of biological chemistry* **281**, 10856-10864 (2006).
54. M. Grundmann *et al.*, Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun* **9**, 341 (2018).
55. E. Alvarez-Curto *et al.*, Targeted Elimination of G Proteins and Arrestins Defines Their Specific Contributions to Both Intensity and Duration of G Protein-coupled Receptor Signaling. *The Journal of biological chemistry* **291**, 27147-27159 (2016).
56. M. O'Hayre *et al.*, Genetic evidence that beta-arrestins are dispensable for the initiation of beta2-adrenergic receptor signaling to ERK. *Science signaling* **10**, (2017).
57. L. M. Luttrell *et al.*, Manifold roles of beta-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. *Science signaling* **11**, (2018).
58. R. Santos *et al.*, A comprehensive map of molecular drug targets. *Nat Rev Drug Discov* **16**, 19-34 (2017).
59. A. S. Hauser, M. M. Attwood, M. Rask-Andersen, H. B. Schioth, D. E. Gloriam, Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov* **16**, 829-842 (2017).
60. A. S. Hauser *et al.*, Pharmacogenomics of GPCR Drug Targets. *Cell* **172**, 41-54.e19 (2018).

61. M. A. Webber, S. R. Marder, Better pharmacotherapy for schizophrenia: what does the future hold? *Curr Psychiatry Rep* **10**, 352-358 (2008).
62. P. Seeman, Targeting the dopamine D2 receptor in schizophrenia. *Expert Opin Ther Targets* **10**, 515-531 (2006).
63. J. Bhattacharjee, H. G. El-Sayeh, Aripiprazole versus typical antipsychotic drugs for schizophrenia. *Cochrane Database Syst Rev*, Cd006617 (2008).
64. U. D. Renner, R. Oertel, W. Kirch, Pharmacokinetics and pharmacodynamics in clinical use of scopolamine. *Ther Drug Monit* **27**, 655-665 (2005).
65. S. Rajagopal, K. Rajagopal, R. J. Lefkowitz, Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nature reviews. Drug discovery* **9**, 373-386 (2010).
66. J. S. Smith, R. J. Lefkowitz, S. Rajagopal, Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* **17**, 243-260 (2018).
67. T. Kenakin, Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther* **336**, 296-302 (2011).
68. S. Rajagopal *et al.*, Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A* **107**, 628-632 (2010).
69. B. K. Atwood, J. Lopez, J. Wager-Miller, K. Mackie, A. Straiker, Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* **12**, 14 (2011).
70. E. Bychkov, L. Zurkovsky, M. B. Garret, M. R. Ahmed, E. V. Gurevich, Distinct cellular and subcellular distributions of G protein-coupled receptor kinase and arrestin isoforms in the striatum. *PLoS One* **7**, e48912 (2012).
71. S. J. Rose *et al.*, Engineered D2R Variants Reveal the Balanced and Biased Contributions of G-Protein and beta-Arrestin to Dopamine-Dependent Functions. *Neuropsychopharmacology* **43**, 1164-1173 (2018).
72. K. M. Raehal, J. K. Walker, L. M. Bohn, Morphine side effects in beta-arrestin 2 knockout mice. *J Pharmacol Exp Ther* **314**, 1195-1201 (2005).
73. L. M. Bohn *et al.*, Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* **286**, 2495-2498 (1999).
74. T. W. Grim *et al.*, A G protein signaling-biased agonist at the mu-opioid receptor reverses morphine tolerance while preventing morphine withdrawal. *Neuropsychopharmacology*, (2019).
75. C. L. Schmid *et al.*, Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. *Cell* **171**, 1165-1175 e1113 (2017).
76. J. M. Beaulieu, R. R. Gainetdinov, The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacological reviews* **63**, 182-217 (2011).
77. P. Greengard, The Neurobiology of Slow Synaptic Transmission. *Science* **294**, 1024 (2001).
78. S. Naqvi, K. J. Martin, J. S. Arthur, CREB phosphorylation at Ser133 regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling. *Biochem J* **458**, 469-479 (2014).
79. M. W. Szymanski, J. R. Richards, in *StatPearls*. (StatPearls Publishing StatPearls Publishing LLC., Treasure Island (FL), 2019).
80. G. S. Dichter, C. A. Damiano, J. A. Allen, Reward circuitry dysfunction in psychiatric and neurodevelopmental disorders and genetic syndromes: animal models and clinical findings. *Journal of Neurodevelopmental Disorders* **4**, 19-19 (2012).
81. S. D. Iversen, L. L. Iversen, Dopamine: 50 years in perspective. *Trends in neurosciences* **30**, 188-193 (2007).

82. A. F. Arnsten, The neurobiology of thought: the groundbreaking discoveries of Patricia Goldman-Rakic 1937-2003. *Cerebral cortex* **23**, 2269-2281 (2013).
83. D. J. Nutt, A. Lingford-Hughes, D. Erritzoe, P. R. Stokes, The dopamine theory of addiction: 40 years of highs and lows. *Nature reviews. Neuroscience* **16**, 305-312 (2015).
84. R. W. Buchanan, R. Freedman, D. C. Javitt, A. Abi-Dargham, J. A. Lieberman, Recent advances in the development of novel pharmacological agents for the treatment of cognitive impairments in schizophrenia. *Schizophr Bull* **33**, 1120-1130 (2007).
85. R. R. Girgis *et al.*, A proof-of-concept, randomized controlled trial of DAR-0100A, a dopamine-1 receptor agonist, for cognitive enhancement in schizophrenia. *J Psychopharmacol* **30**, 428-435 (2016).
86. D. L. Gray *et al.*, Impaired β-arrestin recruitment and reduced desensitization by non-catechol agonists of the D1 dopamine receptor. *Nature Communications* **9**, 674 (2018).
87. A. F. Arnsten, R. R. Girgis, D. L. Gray, R. B. Mailman, Novel Dopamine Therapeutics for Cognitive Deficits in Schizophrenia. *Biological psychiatry* **81**, 67-77 (2017).
88. T. Sawaguchi, P. S. Goldman-Rakic, D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science* **251**, 947-950 (1991).
89. P. S. Goldman-Rakic, S. A. Castner, T. H. Svensson, L. J. Siever, G. V. Williams, Targeting the dopamine D1 receptor in schizophrenia: insights for cognitive dysfunction. *Psychopharmacology (Berl)* **174**, 3-16 (2004).
90. J. D. Berke, What does dopamine mean? *Nat Neurosci* **21**, 787-793 (2018).
91. S. A. Castner, G. V. Williams, P. S. Goldman-Rakic, Reversal of antipsychotic-induced working memory deficits by short-term dopamine D1 receptor stimulation. *Science* **287**, 2020-2022 (2000).
92. Y. Chudasama, T. W. Robbins, Functions of frontostriatal systems in cognition: comparative neuropsychopharmacological studies in rats, monkeys and humans. *Biol Psychol* **73**, 19-38 (2006).
93. Y. Chudasama, T. W. Robbins, Dopaminergic modulation of visual attention and working memory in the rodent prefrontal cortex. *Neuropsychopharmacology* **29**, 1628-1636 (2004).
94. H. Fischer *et al.*, Simulating neurocognitive aging: effects of a dopaminergic antagonist on brain activity during working memory. *Biological psychiatry* **67**, 575-580 (2010).
95. A. Abi-Dargham *et al.*, Prefrontal dopamine D1 receptors and working memory in schizophrenia. *J Neurosci* **22**, 3708-3719 (2002).
96. W. A. Corrigall, K. M. Coen, K. L. Adamson, Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. *Brain Research* **653**, 278-284 (1994).
97. W. A. Corrigall, K. B. Franklin, K. M. Coen, P. B. Clarke, The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology (Berl)* **107**, 285-289 (1992).
98. R. C. Pierce, V. Kumaresan, The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience & Biobehavioral Reviews* **30**, 215-238 (2006).
99. D. R. Grattan, 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-prolactin axis. *J Endocrinol* **226**, T101-122 (2015).
100. K. Hyland, Neurochemistry and defects of biogenic amine neurotransmitter metabolism. *J Inherit Metab Dis* **22**, 353-363 (1999).
101. R. G. Pendleton, L. Samler, C. Kaiser, P. T. Ridley, Studies on renal dopamine receptors with a new agonist. *European journal of pharmacology* **51**, 19-28 (1978).
102. P. E. Setler, H. M. Sarau, C. L. Zirkle, H. L. Saunders, The central effects of a novel dopamine agonist. *European journal of pharmacology* **50**, 419-430 (1978).

103. R. Mailman, X. Huang, D. E. Nichols, Parkinson's disease and D1 dopamine receptors. *Curr Opin Investig Drugs* **2**, 1582-1591 (2001).
104. J. Hyttel, SCH 23390 - the first selective dopamine D-1 antagonist. *European journal of pharmacology* **91**, 153-154 (1983).
105. D. E. Felsing, M. K. Jain, J. A. Allen, Advances in Dopamine D1 Receptor Ligands for Neurotherapeutics. *Curr Top Med Chem* **19**, 1365-1380 (2019).
106. T. W. Lovenberg *et al.*, Dihydrexidine, a novel selective high potency full dopamine D-1 receptor agonist. *European journal of pharmacology* **166**, 111-113 (1989).
107. D. M. Mottola, W. K. Brewster, L. L. Cook, D. E. Nichols, R. B. Mailman, Dihydrexidine, a novel full efficacy D1 dopamine receptor agonist. *J Pharmacol Exp Ther* **262**, 383-393 (1992).
108. J. R. Taylor *et al.*, Dihydrexidine, a full dopamine D1 agonist, reduces MPTP-ioduced parkinsonism in monkeys. *European journal of pharmacology* **199**, 389-391 (1991).
109. P. J. Blanchet *et al.*, Effects of the full dopamine D1 receptor agonist dihydrexidine in Parkinson's disease. *Clin Neuropharmacol* **21**, 339-343 (1998).
110. O. Rascol *et al.*, ABT-431, a D1 receptor agonist prodrug, has efficacy in Parkinson's disease. *Ann Neurol* **45**, 736-741 (1999).
111. A. F. Arnsten, J. X. Cai, B. L. Murphy, P. S. Goldman-Rakic, Dopamine D1 receptor mechanisms in the cognitive performance of young adult and aged monkeys. *Psychopharmacology (Berl)* **116**, 143-151 (1994).
112. D. R. Rosell *et al.*, Effects of the D1 dopamine receptor agonist dihydrexidine (DAR-0100A) on working memory in schizotypal personality disorder. *Neuropsychopharmacology* **40**, 446-453 (2015).
113. J. W. Kebabian *et al.*, A-77636: a potent and selective dopamine D1 receptor agonist with antiparkinsonian activity in marmosets. *European journal of pharmacology* **229**, 203-209 (1992).
114. M. P. DeNinno *et al.*, A68930: a potent agonist selective for the dopamine D1 receptor. *European journal of pharmacology* **199**, 209-219 (1991).
115. P. Blanchet, P. J. Bedard, D. R. Britton, J. W. Kebabian, Differential effect of selective D-1 and D-2 dopamine receptor agonists on levodopa-induced dyskinesia in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- exposed monkeys. *J Pharmacol Exp Ther* **267**, 275-279 (1993).
116. J. X. Cai, A. F. T. Arnsten, Dose-Dependent Effects of the Dopamine D1 Receptor Agonists A77636 or SKF81297 On Spatial Working Memory in Aged Monkeys. *Journal of Pharmacology and Experimental Therapeutics* **283**, 183-189 (1997).
117. K. E. Asin, D. Wirtshafter, Effects of repeated dopamine D1 receptor stimulation on rotation and c-fos expression. *European journal of pharmacology* **235**, 167-168 (1993).
118. P. J. Blanchet, R. Grondin, P. J. Bedard, K. Shiosaki, D. R. Britton, Dopamine D1 receptor desensitization profile in MPTP-lesioned primates. *European journal of pharmacology* **309**, 13-20 (1996).
119. J. E. Davoren *et al.*, Discovery and Lead Optimization of Atropisomer D1 Agonists with Reduced Desensitization. *J Med Chem* **61**, 11384-11397 (2018).
120. J. W. A. Coe, John Arthur; Davoren, Jennifer Elizabeth; Dounay, Amy Beth; Efremov, Ivan Viktorovich; Gray, David Lawrence Firman; Guilmette, Edward Raymond; Harris, Anthony Richard; Helal, Christopher John; Henderson, Jaclyn Louise; Mente, Scot Richard; Nason II, Deane Milford; O'Neil, Steven Victor; Subramanyam, Chakrapani; Xu, Wenjian. (2014), chap. WO2014072881A1.
121. M. A. D. Brodney, Jennifer Elizabeth; Dounay, Amy Beth; Efremov, Ivan Viktorovich; Gray, David Lawrence Firman; Green, Michael Eric; Henderson, Jaclyn Louise; Lee, Chewah; Mente, Scot Richard; O'Neil, Steven Victor; Rogers, Bruce Nelsen; Zhang, Lei. (2014), chap. WO2014207601A1.

122. J. E. D. Davoren, A. B.; Efremov, I. V.; Gray, D. L. F.; Lee, C.; Mente, S. R.; O'Neil, S. V.; Rogers, B. N.; Subramanyam, C.; Zhang, L. . (2015), chap. WO2015162518A1.
123. E. Arce *et al.*, A novel approach to evaluate the pharmacodynamics of a selective dopamine D1/D5 receptor partial agonist (PF-06412562) in patients with stable schizophrenia. *J Psychopharmacol* **33**, 1237-1247 (2019).
124. D. R. Gray, R.; Werth, J.; Zhang, Y.; Versavel, M.; Duvvuri, S., Efficacy, Safety and Tolerability of Tavapadon in Subjects With Early Stage Parkinson's Disease [abstract]. *Movement Disorders* **34**, (2019).
125. S. Papapetropoulos, W. Liu, S. Duvvuri, K. Thayer, D. L. Gray, Evaluation of D1/D5 Partial Agonist PF-06412562 in Parkinson's Disease following Oral Administration. *Neurodegener Dis* **18**, 262-269 (2018).
126. U. S. Sohur *et al.*, Phase 1 Parkinson's Disease Studies Show the Dopamine D1/D5 Agonist PF-06649751 is Safe and Well Tolerated. *Neurol Ther* **7**, 307-319 (2018).
127. A. Christopoulos, T. Kenakin, G protein-coupled receptor allosterism and complexing. *Pharmacological reviews* **54**, 323-374 (2002).
128. D. Wootten, A. Christopoulos, P. M. Sexton, Emerging paradigms in GPCR allostery: implications for drug discovery. *Nat Rev Drug Discov* **12**, 630-644 (2013).
129. E. A. Wold, J. Chen, K. A. Cunningham, J. Zhou, Allosteric Modulation of Class A GPCRs: Targets, Agents, and Emerging Concepts. *J Med Chem* **62**, 88-127 (2019).
130. A. Hall, L. Provins, A. Valade, Novel Strategies To Activate the Dopamine D1 Receptor: Recent Advances in Orthosteric Agonism and Positive Allosteric Modulation. *J Med Chem* **62**, 128-140 (2019).
131. P. Wang *et al.*, Synthesis and Pharmacological Evaluation of Noncatechol G Protein Biased and Unbiased Dopamine D1 Receptor Agonists. *ACS Med Chem Lett* **10**, 792-799 (2019).
132. M. L. Martini *et al.*, Defining Structure-Functional Selectivity Relationships (SFSR) for a Class of Non-Catechol Dopamine D1 Receptor Agonists. *J Med Chem* **62**, 3753-3772 (2019).
133. W. K. Kroeze *et al.*, PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat Struct Mol Biol* **22**, 362-369 (2015).
134. M. C. Lagerström, H. B. Schiöth, Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* **7**, 339-357 (2008).
135. S. Marion, R. H. Oakley, K. M. Kim, M. G. Caron, L. S. Barak, A beta-arrestin binding determinant common to the second intracellular loops of rhodopsin family G protein-coupled receptors. *The Journal of biological chemistry* **281**, 2932-2938 (2006).
136. H. Yin *et al.*, Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nature Biotechnology* **32**, 551 (2014).
137. Patrick D. Hsu, Eric S. Lander, F. Zhang, Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell* **157**, 1262-1278 (2014).
138. T. Cermak *et al.*, Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* **39**, e82 (2011).
139. F. A. Ran *et al.*, Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281-2308 (2013).
140. Y. Fu *et al.*, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**, 822-826 (2013).
141. Y. Lin *et al.*, CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Res* **42**, 7473-7485 (2014).
142. R. Peng, G. Lin, J. Li, Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS J* **283**, 1218-1231 (2016).

143. M. L. Martin-Negrier, G. Charron, B. Bloch, Receptor recycling mediates plasma membrane recovery of dopamine D1 receptors in dendrites and axons after agonist-induced endocytosis in primary cultures of striatal neurons. *Synapse* **60**, 194-204 (2006).
144. S. J. Kotowski, F. W. Hopf, T. Seif, A. Bonci, M. von Zastrow, Endocytosis promotes rapid dopaminergic signaling. *Neuron* **71**, 278-290 (2011).
145. R. G. Vickery, M. von Zastrow, Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol* **144**, 31-43 (1999).
146. M. M. Kong *et al.*, Regulation of D1 dopamine receptor trafficking and signaling by caveolin-1. *Mol Pharmacol* **72**, 1157-1170 (2007).
147. P. Yu *et al.*, D1 dopamine receptor signaling involves caveolin-2 in HEK-293 cells. *Kidney Int* **66**, 2167-2180 (2004).
148. M. M. Kong, V. Verma, B. F. O'Dowd, S. R. George, The role of palmitoylation in directing dopamine D1 receptor internalization through selective endocytic routes. *Biochem Biophys Res Commun* **405**, 445-449 (2011).
149. A. Bhatnagar *et al.*, The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT2A) serotonin receptors reveals differential sorting of arrestins and 5-HT2A receptors during endocytosis. *The Journal of biological chemistry* **276**, 8269-8277 (2001).
150. N. R. Hanley, J. G. Hensler, Mechanisms of ligand-induced desensitization of the 5-hydroxytryptamine(2A) receptor. *J Pharmacol Exp Ther* **300**, 468-477 (2002).
151. J. A. Gray *et al.*, Cell-type specific effects of endocytosis inhibitors on 5-hydroxytryptamine(2A) receptor desensitization and resensitization reveal an arrestin-, GRK2-, and GRK5-independent mode of regulation in human embryonic kidney 293 cells. *Mol Pharmacol* **60**, 1020-1030 (2001).
152. E. Simard *et al.*, β-Arrestin regulation of myosin light chain phosphorylation promotes AT1aR-mediated cell contraction and migration. *PloS one* **8**, e80532-e80532 (2013).
153. X.-R. Ren *et al.*, Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 1448-1453 (2005).
154. J. Kim, S. Ahn, K. Rajagopal, R. J. Lefkowitz, Independent beta-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *The Journal of biological chemistry* **284**, 11953-11962 (2009).
155. L. S. Barak, S. S. Ferguson, J. Zhang, M. G. Caron, A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *The Journal of biological chemistry* **272**, 27497-27500 (1997).
156. C. L. Schmid, K. M. Raehal, L. M. Bohn, Agonist-directed signaling of the serotonin 2A receptor depends on beta-arrestin-2 interactions in vivo. *Proc Natl Acad Sci U S A* **105**, 1079-1084 (2008).
157. W. J. Giardina, M. Williams, Adrogolide HCl (ABT-431; DAS-431), a prodrug of the dopamine D1 receptor agonist, A-86929: preclinical pharmacology and clinical data. *CNS Drug Rev* **7**, 305-316 (2001).
158. J. L. Waddington, Therapeutic potential of selective D-1 dopamine receptor agonists and antagonists in psychiatry and neurology. *General pharmacology* **19**, 55-60 (1988).
159. R. B. Mailman, GPCR functional selectivity has therapeutic impact. *Trends in pharmacological sciences* **28**, 390-396 (2007).
160. L. Tan, W. Yan, J. D. McCorvy, J. Cheng, Biased Ligands of G Protein-Coupled Receptors (GPCRs): Structure-Functional Selectivity Relationships (SFSRs) and Therapeutic Potential. *J Med Chem* **61**, 9841-9878 (2018).

161. M. L. Martini *et al.*, Designing Functionally Selective Noncatechol Dopamine D1 Receptor Agonists with Potent In Vivo Antiparkinsonian Activity. *ACS Chem Neurosci* **10**, 4160-4182 (2019).
162. J. L. Conroy, R. B. Free, D. R. Sibley, Identification of G protein-biased agonists that fail to recruit beta-arrestin or promote internalization of the D1 dopamine receptor. *ACS Chem Neurosci* **6**, 681-692 (2015).
163. J. A. Allen *et al.*, Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proc Natl Acad Sci U S A* **108**, 18488-18493 (2011).
164. M. F. Pedersen *et al.*, Biased agonism of clinically approved mu-opioid receptor agonists and TRV130 is not controlled by binding and signaling kinetics. *Neuropharmacology*, 107718 (2019).
165. N. M. Urs *et al.*, Targeting beta-arrestin2 in the treatment of L-DOPA-induced dyskinesia in Parkinson's disease. *Proc Natl Acad Sci U S A* **112**, E2517-2526 (2015).
166. I. Urits *et al.*, The Utilization of Mu-Opioid Receptor Biased Agonists: Oliceridine, an Opioid Analgesic with Reduced Adverse Effects. *Curr Pain Headache Rep* **23**, 31 (2019).
167. E. R. Viscusi *et al.*, APOLLO-1: a randomized placebo and active-controlled phase III study investigating oliceridine (TRV130), a G protein-biased ligand at the micro-opioid receptor, for management of moderate-to-severe acute pain following bunionectomy. *J Pain Res* **12**, 927-943 (2019).
168. J. A. Allen, R. A. Halverson-Tamboli, M. M. Rasenick, Lipid raft microdomains and neurotransmitter signalling. *Nature reviews. Neuroscience* **8**, 128-140 (2007).
169. Cerevel, Cerevel Therapeutics Announces Positive Results from a Phase 2 Study of Tavapadon in Patients with Early-stage Parkinson's Disease., (2019).
170. M. Haney, A. S. Ward, R. W. Foltin, M. W. Fischman, Effects of ecopipam, a selective dopamine D1 antagonist, on smoked cocaine self-administration by humans. *Psychopharmacology (Berl)* **155**, 330-337 (2001).
171. A. Astrup *et al.*, Randomized Controlled Trials of the D1/D5 Antagonist Ecopipam for Weight Loss in Obese Subjects. *Obesity* **15**, 1717-1731 (2007).
172. A. Dagher *et al.*, Reduced dopamine D1 receptor binding in the ventral striatum of cigarette smokers. *Synapse* **42**, 48-53 (2001).
173. A. H. Ashok, Y. Mizuno, N. D. Volkow, O. D. Howes, Association of Stimulant Use With Dopaminergic Alterations in Users of Cocaine, Amphetamine, or Methamphetamine: A Systematic Review and Meta-analysis. *JAMA Psychiatry* **74**, 511-519 (2017).