Functional Selectivity at the Dopamine D2 Receptor

by

Sean Michael Peterson

Department of Cell Biology
Duke University

Date:_______________________
Approved:

___________________________
Marc G. Caron, Supervisor

___________________________
Scott H. Soderling, Chair

___________________________
Vadim Y. Arshavsky

___________________________
Terrence Peter Kenakin

___________________________
Sudha Shenoy

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy, Master of Science in the Department of
Cell Biology in the Graduate School
of Duke University

2015
ABSTRACT

Functional Selectivity at the Dopamine D2 Receptor

by

Sean M Peterson

Department of Cell Biology
Duke University

Date:_______________________

Approved:

___________________________
Marc G. Caron, Supervisor

___________________________
Scott H. Soderling, Chair

___________________________
Vadim Y. Arshavsky

___________________________
Terrence Peter Kenakin

___________________________
Sudha Shenoy

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

2015
Abstract

The neuromodulator dopamine signals through the dopamine D2 receptor (D2R) to modulate central nervous system functions through diverse signal transduction pathways. D2R is a prominent target for drug treatments in disorders where dopamine function is aberrant, such as schizophrenia. D2R signals through distinct G protein and β-arrestin pathways and drugs that are functionally selective for these pathways could have improved therapeutic potential. How D2R signals through the two pathways is still not well defined, and efforts to elucidate these pathways have been hampered by the lack of adequate tools for assessing the contribution of each pathway independently. To address this, Evolutionary Trace was used to produce D2R mutants with strongly biased interactions for either G protein or β-arrestin. Additionally, various permutations of these mutants were used to identify critical determinants of D2R functional selectivity. D2R interactions with the two major downstream signal transducers were effectively dissociated and G protein signaling accounts for D2R canonical MAP kinase signaling cascade activation. Nevertheless, when expressed in mice, the β-arrestin biased D2R caused a significant potentiation of amphetamine-induced locomotion, while the G protein biased D2R had minimal effects. The mutant receptors generated here provide a new molecular tool set that enable a better definition of the individual roles of G protein and β-arrestin signaling in D2R pharmacology, neurobiology and associated pathologies.
Contents

Abstract ......................................................................................................................................... iv

List of Tables .............................................................................................................................. viii

List of Figures ............................................................................................................................... ix

Acknowledgements ...................................................................................................................xiv

1. Introduction ............................................................................................................................... 1

1.1 The conformational theory of functional selectivity.................................................... 2

1.2 A molecular and mechanistic basis for functional selectivity at D₂R...................... 5

1.2.1 The physical properties of dopamine....................................................................... 5

1.2.2 Lipid membrane domains and interactions with other receptors modulate D₂R function............................................................................................................................... 6

1.2.3 D₂R interacting proteins ........................................................................................... 10

1.2.4 Non-canonical D₂R interacting proteins.................................................................. 18

1.2.5 D₂R possesses a common allosteric modulator binding site, the extracellular vestibule............................................................................................................................... 20

1.2.6 Unexpected sites of allosteric modulation............................................................. 22

1.3 D₂R functional selectivity is a function of cellular organization.............................. 24

1.3.1 Dopamine and D₂R genetic mouse models reveal functional selectivity in vivo ............................................................................................................................... 27

1.3.2 Functional selectivity in D₂R circuitry..................................................................... 29

1.4 The therapeutic implications of D₂R functional selectivity ........................................ 31

2. Functional separation of D₂R using Evolutionary Trace .............................................. 33

2.1 Evolutionary Trace guided mutagenesis of D₂R....................................................... 33
2.2 Evolutionary Action at D2R yields a robust landscape of functional selectivity ...35
3. Ligand, receptor and transducer contributions to D2R functional selectivity ..........42
  3.1 [Gprot]D2R and [βarr]D2R display distinct but expected properties ..................42
  3.2 Operationally defined, receptor dictated functional selectivity .......................45
  3.3 Agonist texture reveals novel modes of functional selectivity ......................49
  3.4 The status of receptor interacting partners in extremely biased mutant D2Rs ......53
4. The physiological relevance of D2R functional selectivity ..................................56
  4.1 MAP kinase signaling in [Gprot]D2R and [βarr]D2R ........................................56
  4.2 [Gprot]D2R and [βarr]D2R are biologically active and display functional differences ..58
5. Application of functional selectivity principles to diverse receptor systems ..........62
  5.1 Evolutionary Action at related but diverse GPCRs ..........................................62
  5.2 A roadmap to universal functional separation .................................................64
  5.3 D2R neuronal subtype expression dictates functional selectivity .................68
6. Discussion ...........................................................................................................71
  6.1 Future Directions ..........................................................................................77
  6.2 Outlook and Impact .....................................................................................82
7. Materials and Methods ........................................................................................84
  7.1 Evolutionary Trace .......................................................................................84
  7.2 Molecular Pharmacology .............................................................................85
    7.2.1 Mutagenesis PCR ......................................................................................85
    7.2.2 Cell culture and transfections .................................................................86
    7.2.3 G protein activity ......................................................................................86
7.2.4 Bioluminescent Resonance Energy Transfer ......................................................... 86
7.2.5 Radioligand binding ................................................................................................. 86
7.2.6 Confocal microscopy .............................................................................................. 87
7.2.7 Receptor internalization assay ................................................................................. 87
7.2.8 MAP kinase transcriptional activity reporter ........................................................ 87
7.2.9 ERK phosphorylation by western blot ................................................................. 87
7.2.10 Alternative G protein signaling ............................................................................ 88
7.3 Neuropsychopharmacology ....................................................................................... 88
7.3.1 Adeno-associated viral expression vectors ............................................................ 88
7.3.2 Adeno-associated virus production ....................................................................... 88
7.3.3 Mouse lines ............................................................................................................ 89
7.3.4 Mouse stereotaxic injection for viral delivery ....................................................... 89
7.3.5 Locomotor activity ................................................................................................. 89
7.3.6 Motor coordination ................................................................................................. 89
7.3.7 HA-staining of virally transduced brains .............................................................. 90
7.4 Data Analysis ............................................................................................................. 90
References ...................................................................................................................... 95
Biography ...................................................................................................................... 115
List of Tables

Table I: Evolutionary Action guided mutagenesis. Each residue predicted from ET is color coded the same in Figure 3 and Figure 4 in the first column. The second column shows the predicted residues in increasing ET harshness (amino acid conservation and side chain chemistry) from left to right. Each single point mutation was generated and tested for G protein and β-arrestin activity. Note that A135 was found to be a critical residue for G protein activation, but not β-arrestin activation and all 19 mutations were made in order to titrate the most β-arrestin biased mutant................................................. 36

Table II: Evolutionary Action predicted functional selectivity phenotype mutations in diverse GPCRs. Receptors presented in Figure 14 are predicted for mutations at the [Gprot]D₂R (L3.42 and Y3.51) and [βarr]D₂R (A3.53 and M3.58) residues. Predicted harshness on phenotype is displayed from left to right. ......................................................................... 64

Table III: Calculated values from Figure 6 and 12.................................................................. 91

Table IV: Receptor manipulations yield operationally defined functional selectivity. Values derived from Figures 7 and 8 to demonstrate the receptor’s contributions to functional selectivity. *p<0.05 when compared to [WT]D₂R for efficacy and potency as determined by Bonferroni post-hoc test after p<0.05 for one-way ANOVA. BINF and ΔΔlog(τ/KA) were calculated according to references in the table............................................. 92

Table V: Ligand contributions to functional selectivity. Calculated from Figures 9 and 10. *p<0.05 when compared to [WT]D₂R for efficacy and potency at each ligand as determined by Bonferroni post-hoc test after p<0.05 by one-way ANOVA................................................. 92

Table VI: Transducer contributions to functional selectivity. Calculated from Figure 11. *p<0.05 when compared to [WT]D₂R or control receptors (β2AR for Gαs or AT1A for Gαq) for efficacy and potency as determined by Bonferroni post-hoc test after p<0.05 for one-way ANOVA......................................................... 94
List of Figures

Figure 1: The functional selectivity of D2R. (A) Interaction with dopamine stimulates G proteins (Gα in green, Gβ in cyan and Gγ in magenta), recruitment of GPCR kinases (GRKs) which phosphorylate the receptor (red spheres) and β-arrestins (yellow) at D2R. Subunit variations that are known to interact with D2R are labelled (seven Gα, three Gβ, and ten Gγ subunits, five major serine/threonine kinases, and two β-arrestins). (B) Aripiprazole is a potent antagonist at the β-arrestin recruitment assay as determined by Bioluminescent Resonance Energy Transfer (BRET) but a weak partial agonist when the same assay is performed with high levels of GRK2 (C). ......................................................... 4

Figure 2: D2R functional selectivity is dependent upon context. (A) D2R interactions and downstream effectors leading to signaling events. (B) D2R physical states are dynamic and determine extent of functional selectivity. (C) Central nervous system architecture of D2R expression and connectivity. Dopaminergic projections (blue arrows) to cortex and striatum modulate excitatory (green arrows) and inhibitory (red arrows) neural circuits. D2R is prominently expressed in dopaminergic, striatal and cortical cells. VTA: ventral tegmental area; SN: substantia nigra; GPI/SNr: internal globus pallidus/substantia nigra reticulate; STN: subthalamic nucleus; GPe: external globus pallidus. ................................ 25

Figure 3: Evolutionary Trace of D2R facilitated by advances in crystallography and algorithm design. (A) TYY mapped onto D3R (159) (PDB ID: 3PBL in red). (B) Four rounds of ET predicted residues for mutagenesis mapped onto D2R. (C) piET (green residues depicted as spheres). (D) Ga-CT proximity (160) (PDB ID: 3DQB residues in yellow, Ga-CT is represented as a green α-helix cartoon), and (E) Gaβγ (161) (PDB ID: 3SN6 α is green and β is blue) interaction with IC2 of D2R (grey spheres). ....................... 34

Figure 4: G protein and β-arrestin activity of ET predicted mutants. Dose response curves for each mutant were normalized to a WT control that was performed the same day and the log(τ/Ka) was calculated. Since all values were ................................................ 38

Figure 5: Generation of functionally selective D2R mutants. (A) Snake-like plot of D2R with each round of mutagenesis color-coded according to Table I and Figure 3. Red spheres: residues derived from TYY, green spheres: predicted from piET algorithm, yellow spheres: predicted from proximity to rhodopsin/transducin α subunit C-terminal fragment co-crystal; grey spheres: identified residues from β2AR/Gαβγ co-crystal in intracellular loop two. Ballesteros-Weinstein numbering identified for each transmembrane domain. The same color scheme was used to highlight the residues on the structure of D3R (159) because D3R is the most closely related GPCR to D2R with an
available crystal structure (81% sequence identity for transmembrane domains). (B), D3R structure represented as a blue ribbon, and ET-identified residues are spheres. (C) The biased mutants all occur within 20 amino acids of the DRY motif on transmembrane domain three (TM3). (D) D3R aligned to β2AR in complex with Gαβγ, green cylinder PDB ID: 3SN6 (161) as well as rhodopsin in complex with the finger-loop domain of visual arrestin (167) (purple cylinder PDB ID: 4PXF). D3R to β2AR alignment yielded an RMSD = 1.8 and D3R alignment to rhodopsin RMSD = 2.7 using pymol MatchAlign command.

Figure 6: Biased D2R mutants derived from Evolutionary Trace. (A) Inhibition of cAMP as determined by GloSensor compared to [WT]D2R positive control and [D80A]D2R negative control. (B) β-arrestin 2 recruitment determined by BRET for the same receptors as in (A). All points are SEM of n=3-7 done in duplicate. Confocal images of (C) [WT]D2R, (D) [Gprot]D2R, (E) [βarr]D2R, and (F) [D80A]D2R expressed in live cells. (G) BMAX (with SEM) determined from n=3 radioligand binding experiments. (H) KD from BMAX determination experiments. (I) DA competition binding experiments to determine Kᵢ. (J) D2R internalization assessed by live cell HA antibody staining of D2R (SEM, n=5 done in triplicate).

Figure 7: Functional selectivity is operationally defined. (A) β-arrestin 2 recruitment comparing [WT]D2R and [IYIV]D2R as determined by bioluminescent resonance energy transfer (BRET). (B) GRK2 overexpression enhances β-arrestin 2 recruitment by BRET for [IYIV]D2R and [WT]D2R, but only slightly for [Gprot]D2R, [βarr]D2R, and [D80A]D2R when compared to Figure 6B. All data are presented with SEM from n=3-4 independent experiments, with statistical significance calculated in Table IV. Quantification of bias between G protein activity and β-arrestin 2 recruitment using (C) a statistical formalism (171) or (D) bias plot mapping under normal (solid lines) and GRK2 overexpression enhanced (broken lines) conditions.

Figure 8: Receptor control of partial agonism at D2R. (A) G protein activity as determined by inhibition of isoproterenol-induced cAMP accumulation of basic and acidic residue substitutions at A135 (A3.53) and (B) β-arrestin 2 recruitment. (C) G protein activity of bulky polar substitution (tyrosine) is roughly 75% (τ/1.5) of [WT]D2R (dotted line) and (D) β-arrestin 2 recruitment is similarly reduced. When combined with one residue from [Gprot]D2R (L125N) or [βarr]D2R (M140D) these mutants display partial biased agonism for their respective retained pathways. (E) G protein and (F) β-arrestin 2 recruitment of an even more reduced (50%; τ/2) parital agonism induced by substitution of A135 for a bulky nonpolar residue (phenylalanine). Similarly combined with L125N
or M140D to generate weak partial biased agonism in response to full agonists. All data presented with SEM from n=3-5 independent experiments.

Figure 9: A unique G protein biased mutant demonstrates agonist texture. (A) Dopamine (DA) and quinpirole equivalently inhibit cAMP production, which is equivalent to [WT]D₂R for [Gprot4PM]D₂R (T69F Y133L Y209N A372S). (B) [Gprot4PM]D₂R has roughly 50% efficacy in response to DA but not quinpirole for β-arrestin 2 recruitment. (C) GRK2 overexpression rescues both DA and quinpirole β-arrestin 2 recruitment activity nearly to [WT]D₂R levels (dotted line, from Figure 7B). (D) GRK2 recruitment as determined by BRET (where GRK2 is tagged with YFP) shows the same ligand discrepancy as β-arrestin 2. All data are presented with SEM from n=3 independent experiments, with statistical significance calculated in Table V.

Figure 10: Agonists and antagonists with diverse pharmacophores elicit predictable responses at [Gprot]D₂R and [βarr]D₂R. The D₂R agonists quinpirole, apomorphine, and NPA were tested for G protein activity (A,C,E, respectively) and β-arrestin 2 recruitment (B,D,F, respectively). For each agonist, [Gprot]D₂R showed a response similar to [WT]D₂R at G protein activation and more similar to [D80A]D₂R for β-arrestin recruitment, while [βarr]D₂R was not active at the G protein pathway but retained activity at the β-arrestin pathway. The antagonists raclopride (G,H) haloperidol (I,J) and partial antagonist aripiprazole (K,L) were able to block DA elicited D₂R activation at the G protein pathway (G,I,K, respectively) for [Gprot]D₂R and [WT]D₂R to the same extent, while [D80A]D₂R and [βarr]D₂R had no effect to inhibit. In contrast, these antagonists block DA elicited β-arrestin 2 recruitment (H,J,L, respectively) for [βarr]D₂R and [WT]D₂R. All data are presented with SEM from n=3-4 independent experiments, with statistical significance calculated in Table V.

Figure 11: Interacting partners and allosteric D₂R determinants of functional selectivity. (A) GRK2 and (B) β-arrestin 1 recruitment as assessed by BRET show a similar profile as β-arrestin 2; [βarr]D₂R recruits normally, while [Gprot]D₂R is severely deficient. (C) Each D₂R construct was expressed in HEK 293T cells and assessed for its ability to stimulate cAMP in response to DA. Stimulation of endogenous receptor by isoproterenol was used as a control response. (D) Gαq-mediated Ca²⁺ flux, as measured by the aequorin luminescence assay, is not stimulated by [WT]D₂R, [Gprot]D₂R, [βarr]D₂R or [D80A]D₂R, compared to AngII induced Ca²⁺ flux induced by transient expression of AT₁A. (E) BMAX was determined by binding, while luciferase-tagged receptors provided a BMAX-independent measure of receptor number. In this assay, the responsiveness to salt is retained for all mutants (except [D80A]D₂R). All data are presented with SEM from n=3-4 independent experiments, with statistical significance calculated in Table VI.
Figure 12: Assessment of MAP Kinase activity at D₂R. (A) SRF and (B) SRE MAP kinase transcriptional promoter mediated expression of luciferase (SEM, n=5-6 done in triplicate). (C) Traditional western blot analysis of ERK (*p<0.05 Newman-Keuls posthoc when compared to [D80A]D₂R or untransfected after one-way ANOVA p<0.05, SEM, n=3-6) with and without β-arrestin 2 overexpression. (D) Representative blot for the data presented in (C). ................................................................. 57

Figure 13: The physiological relevance of D₂R functional selectivity. (A) Viral transgene packaged into AAV, which allowed for Cre-dependent expression of D₂R through a double-floxed open reading frame (DIO). (B) 0.75 µL of virus was injected bilaterally into the dorsal and ventral striatum with each injection site indicated by the red dots, and a total of 3 µL was injected into the striatum of each mouse. CPu: caudate putamen; AcbC: nucleus accumbens, core; AcbSh: nucleus accumbens, shell. (C) Representative staining pattern of the N-terminal HA tagged D₂R shows transduction of a majority of the dorsal striatum and at least 50% of the ventral striatum with variable transduction in the olfactory tubercle. Radioligand binding revealed a two- to four-fold overexpression of each receptor as determined from membranes prepared from striatal dissections from Adora2a-Cre (D) and Adora2a-Cre::β-arrestin 2FLOX/FLOX (E) mice (*p<0.05 Newman-Keuls posthoc when compared to Cre (-) controls after one-way ANOVA p<0.05, SEM, n=4-6). (F) Potentiation of amphetamine-induced locomotion in mice when D₂R is overexpressed (*p<0.05 bonferroni posthoc when compared to [D80A]D₂R after repeated measures two-way ANOVA p<0.05 for receptor expression type SEM, n=11-12, color coded for receptor type). (G) The amphetamine response potentiation of [WT]D₂R and [βarr]D₂R is abolished when β-arrestin 2 is genetically deleted from D₂R-expressing medium spiny neurons (*p<0.05 bonferroni posthoc when compared to [D80A]D₂R after repeated measures two-way ANOVA p<0.05 for receptor expression type SEM, n=8-13). ................................................................................................. 61

Figure 14: Evolutionary Trace at pharmacologically and genetically diverse GPCRs for the generation of functionally selective mutants. hAGTR1 (human angiotensin receptor), hAPLNR (human apelin receptor), hM3 (human muscarinic type 3 receptor), hADRB2 (human β-adrenergic receptor, type 2A), hDRD1 (human dopamine D1 receptor), hHTR1A (human serotonin type 1A receptor), and mDRD2 (mouse D₂R) GPCRs were chosen based on genetic diversity (Tree calculated from % identity), as well as G protein (Gαq, Gαi or Gαs) and β-arrestin coupling (Class A: transient β-arrestin interaction, Class B: robust β-arrestin interaction) profiles. The alignment of the twenty residues surrounding the DRY motif was the basis of the tree presented, and [Gprot]D₂R is color coded as green residues while [βarr]D₂R is red........................................................................................................ 63
Figure 15: Achieving functional selectivity in GPCRs with diverse pharmacological and genetic profiles. (A) Cytosolic influx of Ca\textsuperscript{2+} as determined by the aequorin assay shows AT1AR M3.53G mutation completely abolishes G protein activity, while (B) β-arrestin 2 recruitment as determined by BRET remains intact. For the apelin receptor (APLNR), G\textsubscript{ai} activation as determined by inhibition of isoproterenol induced cAMP increase (C) is intact for Y3.51S but slightly decreased for A3.53S. In contrast, β-arrestin 2 recruitment (D) is intact for A3.53S but not Y3.51S. Finally, for D\textsubscript{1R}, Y3.51S showed partial agonist activity at G\textsubscript{as} mediated cAMP accumulation as measured by GloSensor (E) and A3.53S showed full activity. While at β-arrestin 2 recruitment (B), Y3.51S had no activity while A3.53S showed partial activity, which means that both mutations display bias for the G protein pathway. ......................................................................................................................... 65

Figure 16: Dissection of diverse D\textsubscript{2R}-expressing neuronal subpopulations. D\textsubscript{2R}\textsubscript{FLOX/FLOX}:::Adora2A-Cre induces genetic deletion of D\textsubscript{2R} and reexpression (using the same injection scheme in Figure 13) of each mutant D\textsubscript{2R}. Assessment of (A) basal locomotor activity at the beginning of the night cycle (lights off) and (B) motor coordination with the beam walking assay. (C) D\textsubscript{2R}\textsubscript{FLOX/FLOX}:::ChAT-Cre has D\textsubscript{2R} deleted only from cholinergic interneurons which causes a major deficiency in the amphetamine locomotor response but not the basal/habituation locomotor phase.................................. 68

Figure 17: Potential inducible mouse knock-in construct design. This design relies upon expression of the DOX inducible tTA transcription factor and Flp recombinase from the introns of the endogenous DRD2 gene (polyadenylation and promoters abridged for simplicity). (A) tTA and the WT D\textsubscript{2R} exon are expressed in normal cells (restricted by the endogenous DRD2 expression pattern). (B) Mouse crossed to Cre expressing lines orients Flp recombinase Cerulean expression cassette that is not active until (B) addition of DOX drives Flp::P2A::GFP (cerulean or citrusine), and Flp recombines WT D\textsubscript{2R} exon to βarr D\textsubscript{2R} exon (with cerulean or citrusine serving as reporters of recombination). .................. 82
Acknowledgements

I would like to thank my advisor, Dr. Marc Caron, for guidance and inspiration throughout this project. This work was made possible by my collaborators. Drs. Olivier Lichtenhage and Angela Dawn Wilkins at Baylor who developed the Evolutionary Trace approach. Dr. Caroline Bass at SUNY-Buffalo who generated adeno-associated viruses. My coworkers in the laboratory: Tom Pack who generated pERK and \(^{[\text{h}2]}\)D\(_2\)R data, Kathryn Walder who worked on applying the mutations to other GPCRs, Dr. Daniel Urban who trained me in stereotactic injections and animal behavior, Dr. PJ Nicholls who designed the inducible knock-in construct, and Dr. Nikhil Urs who provided valuable experimental design insight throughout the project.
1. Introduction

The dopamine D2 receptor (D2R) is an inhibitory G protein-coupled receptor (GPCR) that is a common target of all antipsychotics. D2R responds to the binding of a single ligand, dopamine (DA), to transduce pleiotropic signaling patterns. Synthetic D2R ligands have been shown to elicit or blunt a subset of these responses, a property referred to as functional selectivity. The literature describing these phenomena is reviewed and a mechanistic understanding of D2R functional selectivity is proposed based on the receptor’s physical environment in the plasma membrane as well as its interactions with canonical signaling molecules (heterotrimeric G proteins) and non-canonical signaling molecules (β-arrestins and other proteins). This mechanistic understanding of functional selectivity will then be used to explore the role of functional selectivity of D2R in the central nervous system at the cellular and circuitry levels. The therapeutic potential of D2R functional selectivity requires a mechanistic understanding of D2R in physiologically relevant systems in order to be leveraged for the development of improved drugs targeting D2R. This review of the current state of the literature places context for the work presented in Chapters 2-5 and provides a clear rationale for elucidating D2R functional selectivity.
1.1 The conformational theory of functional selectivity

Functional selectivity is a term that describes the phenomenon of ligands eliciting multiple downstream signaling events through one GPCR (1). Functional selectivity was originally observed when the administration of a drug to a system resulted in pleiotropic responses (2-4) and D2R functional selectivity was observed when studying the effects of the atypical antipsychotic aripiprazole (5, 6). The concept has evolved and the molecular details have been elucidated for many prototypical GPCRs, culminating in the structural determination of the serotonin type 2B receptor in complex with different functionally selective ligands (7). The dopamine system is one of the earliest examples of functional selectivity observed and the therapeutic implications are manifold.

Functional selectivity has been theorized to arise from the ability of a protein to adopt multiple heterogeneous conformations. The functional significance of conformational heterogeneity is that each conformation allows for distinct interactions with ligands, G proteins, kinases, and β-arrestins (Figure 1A). The conformational theory was proposed in 1995 and described ligands that would interact preferentially with a specific conformation as a protean agonist (8) because the character of the ligand (agonist or antagonist) would change depending on how the receptor was observed. For example, a cell that expresses a high concentration of GPCR kinases (GRKs) will make
an antagonist (such as aripiprazole, Figure 1B) seem to act as an agonist (Figure 1C) in a 
β-arrestin recruitment assay. The term biased agonism describes a similar phenomenon: 
an agonist that is able to preferentially signal through a specific pathway (9) such as β-
arrestin rather than G proteins.
Figure 1: The functional selectivity of D<sub>2</sub>R. (A) Interaction with dopamine stimulates G proteins (G<sub>α</sub> in green, G<sub>β</sub> in cyan and G<sub>γ</sub> in magenta), recruitment of GPCR kinases (GRKs) which phosphorylate the receptor (red spheres) and β-arrestins (yellow) at D<sub>2</sub>R. Subunit variations that are known to interact with D<sub>2</sub>R are labelled (seven G<sub>α</sub>, three G<sub>β</sub>, and ten G<sub>γ</sub> subunits, five major serine/threonine kinases, and two β-arrestins). (B) Aripiprazole is a potent antagonist at the β-arrestin recruitment assay as determined by
Bioluminescent Resonance Energy Transfer (BRET) but a weak partial agonist when the same assay is performed with high levels of GRK2 (C).

While the distinctions between functional selectivity, protean agonism, and biased agonism are not always clear, the term functional selectivity is a broad definition that encompasses phenomenon observed in crystallography all the way to intact neural circuits. D₂R functional selectivity can arise from predicted mechanisms (such as GRK expression and ligand structure) as well as novel mechanisms (such as receptor dimerization and ligand allosterism). Furthermore, the therapeutic implications of D₂R functional selectivity are apparent in preclinical models of D₂R related diseases such as schizophrenia, Parkinson’s disease, bipolar disorder, attention deficit-hyperactivity disorder, and addiction.

1.2 A molecular and mechanistic basis for functional selectivity at D₂R

A bare-bones model of D₂R signal transduction is presented in Figure 1A. The functional selectivity of D₂R is based on the interaction between D₂R, ligand, and interacting proteins. Described below is a thorough (though not exhaustive) examination of each of these elements as they relate to D₂R.

1.2.1 The physical properties of dopamine

Dopamine (DA) is the endogenous ligand for D₂R and is considered the reference agonist (meaning that the bias of agonists at D₂R are relative to the ability of DA to elicit
DA is synthesized from the amino acid tyrosine and is only one step in the biosynthesis of many small molecule ligands for other GPCRs. Tyrosine is hydroxylated by tyrosine hydroxylase to L-DOPA, which is decarboxylated by aromatic L-amino acid decarboxylase to form DA. DA is further metabolized into norepinephrine and epinephrine, as well as trace amines (all of which are the endogenous ligands for other GPCRs) (10, 11). DA is toxic to cells, as it is a catechol and can be oxidized to release free radicals (12). However, D2R appears to modulate L-DOPA (and DA) free radical cytotoxicity (13) in a pleiotropic manner. DA itself may also have an effect on the plasma membrane and lipid environment of D2R by binding directly to membranes (14).

1.2.2 Lipid membrane domains and interactions with other receptors modulate D2R function

D2R is embedded into the plasma membrane, thus functional selectivity and conformational heterogeneity is dependent on the lipid environment. D2R has been shown to exist in both detergent soluble and insoluble fractions of the plasma membrane and the insoluble fraction was shown to interact with G proteins and β-arrestin (15). Additionally, the N-terminus of D2R has been shown to regulate compartmentalization through lipid microdomains into intracellular vesicles (16). D2R has been shown to preferentially internalize in nonsynaptic membranes of dendrites in vivo (17). Recently, GPCRs have been shown to signal from intracellular vesicles (18) which is another means by which D2R can achieve functional selectivity. The lipid environment
fundamentally affects D2R conformational heterogeneity, however, the other GPCRs present in these membrane domains also affect D2R signaling.

There is considerable evidence for GPCR dimerization as a substrate for D2R functional selectivity (19). D2R can interact with many other GPCRs, which results in diverse functional consequences. There are five DA receptors that belong to two pharmacologically and genetically homologous families: the D1-like family (D1R and D5R) and the D2-like family (D2R, D3R, and D4R). All D2-like family receptors have a large third intracellular (IC3) loop and no C-terminus (the protein ends with the lipidated cysteine of helix 8). There is evidence that D2R can heterodimerize with all of the members of the DA receptor family. Functional selectivity can be a direct consequence of GPCR dimerization. The unique aspect of dimerization mediated functional selectivity is that it is dependent upon which specific GPCRs are interacting with D2R. However, it should be noted that the functional and physiological relevance of the concept of GPCR dimerization is still highly debated (20, 21), with D2R at the center of the physiological relevance debate (22).

D2R homodimers have been shown using cross-linking and mutagenesis (23). The functional consequences of D2R homodimerization was shown using a novel, albeit artificial, system of D2R fused to a Gα subunit (24). This system showed that D2R homodimers are more efficient than monomers at transducing their signal. This
principle may have therapeutic significance, as D\(_2\)R homodimers were shown to be increased in post-mortem tissue from schizophrenia patients (25). Functional selectivity could be achieved by ligands that specifically manipulate the balance of D\(_2\)R dimers and monomers. For instance, SB269652 was recently described as functioning as a D\(_2\)R allosteric modulator for the D\(_2\)R homodimer, but a competitive antagonist for the monomer (26). Functional selectivity was shown for the agonist NPA at D\(_1\)R/D\(_2\)R heterodimers when compared to D\(_2\)R homodimers (27) when G\(_{ai}\) activation was used as the readout.

D\(_2\)R has been shown to heterodimerize with many receptors, including the dopamine D1 receptor (D\(_1\)R). D\(_1\)R, unlike D\(_2\)R, is intronless in its coding sequence, coupled to G\(_{as}\), and possess a traditional GPCR C-terminal tail. In vitro and in vivo it was shown that D\(_1\)R and D\(_2\)R heterodimerize but instead of signaling through inhibitory or stimulatory G proteins, the heterodimer signals through G\(_{aq}\) and activates calcium release when both D\(_1\)R and D\(_2\)R are activated (28). D\(_2\)R is thus functionally selective in this regard, depending on the dimerization state with D\(_1\)R. It has been suggested that GRK2 regulates aspects of this signaling paradigm (29) and that the G protein signal is mediated through G\(_{by}\) subunits (30). This adds another layer of functional selectivity to D\(_2\)R: functional selectivity through D\(_2\)R monomers (i.e. G protein versus \(\beta\)-arrestin) and D\(_2\)R functional selectivity through D\(_1\)R heterodimers (i.e. G\(_{ai}\) versus G\(_{by}\)).
While D$i$R was shown to heterodimerize with D$z$R to generate a new G protein coupling, for the closely related D$z$R it was shown that D$z$R signals through G$αq$ as a monomer and that this signal is inhibited by heterodimerization with D$z$R (31). However, this inhibition is disinhibited when D$z$R is activated. D$z$R also has been shown to heterodimerize with its closely related D2-like family members, D$i$R and D$z$R. The significance of the D$i$R/D$z$R heterodimer is that each receptor is able to transactivate G proteins, which was shown by G protein-receptor fusions (32). The D$z$R/D$z$R dimerization state seems to be sensitive to the alternative splicing that the IC3 of D$z$R undergoes and the variable number of tandem repeats of the IC3 of D$i$R (33). This D$z$R/D$z$R heterodimer was shown to signal through MAP kinases and modulate glutamate release in vivo.

D$z$R has also been shown to dimerize with many diverse GPCRs. The adenosine 2A receptor (A$2A$R) is coexpressed with D$z$R in the striatal medium spiny neurons (MSNs) and has been shown to be in a complex with D$z$R and calmodulin (34). In vitro the functional consequence of the D$z$R/A$2A$R heterodimer is that D$z$R acts as a negative allostERIC modulator of A$2A$R ligand binding (35). In vivo, the D$z$R/A$2A$R dimer works in an antagonistic fashion when the depolarized membrane potential is measured by patch-clamp recordings (36). A small molecule was discovered that modulates the D$z$R/A$2A$R dimer by inducing cointernalization of the receptors (37). Thus, a functionally
selective ligand has been developed that targets the dimerization state of D₂R. Finally, D₂R has also been shown to dimerize with neurotensin receptors (38) as well as the ghrelin receptor (39). Both of these receptors have high therapeutic potential as small molecule targets, and D₂R dimerization with them adds a more specific potential to develop effective therapeutic small molecules.

1.2.3 D₂R interacting proteins

Just as D₂R functional selectivity is dependent on the lipid environment and other receptors present, it is also dependent on the presence and abundance of signaling molecules. Figure 1A depicts the possible interacting partners (including different complements of G protein subunits, kinases and β-arrestins). D₂R’s interaction partners are perhaps the most important determinant of functional selectivity, as Figure 1B and 1C demonstrate, the level of kinase determines the efficacy of aripiprazole and its classification as an antagonist or partial agonist.

D₂R is a cell surface, ligand activated guanine nucleotide exchange factor (GEF) for heterotrimeric G proteins. Heterotrimeric G proteins are comprised of a Gα subunit and Gβγ subunits (referred to as Gβγ because the Gβ and Gγ subunits are always bound to each other) (40). The Gα subunit contains a GTPase domain (that shares homology to small GTPases and is referred to as a ras-like domain) as well as an alpha-helical domain. The GTPase domain hydrolyzes GTP into GDP to go from an active to inactive
conformation (41), and D₂R catalyzes the exchange of GDP for GTP. D₂R binds to the pertussis toxin sensitive family of inhibitory Gα subunits referred to as the Gαi/o family (Gαi1, Gαi2, Gαi3, and Gαo) as well as the pertussis toxin insensitive inhibitory G protein Gαz. The specificity of G protein/D₂R interactions is regulated by the Gα C-terminal tail, as well as the complement of Gβγ subunits. D₂R could possibly be queried with 280 different Gβγ combinations and D₂R binds to the GDP-bound state of the heterotrimer under basal conditions (42). D₂R functional selectivity is largely dependent upon the complement and specificity of G proteins present and the significance of functional selectivity is largely dependent on the functional diversity of the different G proteins.

The three related inhibitory G proteins (Gαi1, Gαi2, and Gαi3) are all able to inhibit cAMP production by binding to adenylate cyclase (AC). The N-terminus is alternatively spliced into two isoforms for Gαi1, and six for Gαi2, while Gαi3 is not alternatively spliced which may dictate specificity between AC subtypes and other effectors. There is also a short C-terminal splice variant of Gαi2 (sGαi2) that regulates intracellular D₂R functions (43). cAMP is a diverse second messenger and inhibition of cAMP production by D₂R activation has long been regarded as D₂R’s canonical signaling paradigm. However, cAMP functions as a pleiotropic second messenger in its own right, thus D₂R can exhibit functional selectivity even within its canonical signaling paradigm. For instance, cAMP is known to activate protein kinase A (PKA) and DARPP-32 in D₂R expressing medium
spiny neurons (MSNs) and this has been shown to regulate a diverse set of functional outputs such as epigenetic changes (44). Furthermore, the role of cAMP was tested directly in D₁R expressing MSNs using pharmacogenetics. The Gαs coupled DREADD was expressed in the D₁R MSNs and activated to cause an increase in cAMP and this activated DARPP-32 and blunted amphetamine sensitization (45). D₁R and its regulation of cAMP encompass a vast body of literature and the history of some of the groundbreaking work by John Kebabian and Paul Greengard is reflected upon by one of the luminaries of DA research, Solomon Snyder in (46). D₁R can modulate cAMP in functionally selective ways, and cAMP can signal in a functionally diverse manner. However, functional selectivity at D₁R also encompasses the ability of D₁R to engage novel interacting signaling partners and generate unexpected responses that can be leveraged for clinically effective pharmaceuticals.

The “other” inhibitory G protein, Gαo, is alternatively spliced in its C-terminus to generate two distinct C-termini (47). These alternative splice variants are referred to as GαoA and GαoB and most likely affect specificity between Gαo and GPCRs (48) as well as Gβγ. Gαo is largely regarded to have unknown effector binding in its GTP-activated state. Despite this mysterious function, Gαo is the most highly expressed Gα subunit, comprising up to 2% of all membrane proteins in the brain (49). Furthermore, it has been asserted that Gαo is responsible for most of the signal transduction downstream of D₁R.
These results are hard to reconcile with the substantial body of work regarding the actions of cAMP, PKA and DARPP-32 in D2R expressing MSNs. Nevertheless it is hard to deny the evidence presented: GTPγS binding in Gαo versus Gαi1/Gαi2 or Gαi1/Gαi3 knockout mice, as well as earlier work that showed Gαo knockout mice showed neurological abnormalities in many DA related phenotypes (51) and a recent study that showed Gαo mutations cause epileptic encephalopathy (52). It is clear that D2R is able to engage both Gαi and Gαo subunits and this yields diverse functional outcomes, however targeting D2R activation of one subunit (i.e. creating a biased agonist for Gαo) has not been successfully done, partly because of the difficulty in assessing Gαo activation and separating it from Gαi signaling as the proteins are closely related.

Gαo’s primary function may be as a molecular clock (via its intrinsic GTPase activity) for Gβγ signaling, a functional relationship that has been well characterized in yeast (53). Gβγ is an important transducer for D2R signaling in the brain that has been shown to couple to diverse signaling pathways. The most well characterized effector for Gβγ is G protein-gated inward rectifier K+ (GIRK) channels because this provides a direct link from D2R activation to plasma membrane excitability. Interestingly, a crystal structure of GIRK/Gβγ was recently solved that showed that there are minimal conformational changes that occur in Gβγ when bound to GDP bound Gα versus GIRK (54). This illustrates a unique principle of D2R/Gβγ functional selectivity: unlike the
receptor, which exists in a conformationally heterogeneous population, $G_{\beta\gamma}$ needs to simply be liberated from $G_\alpha$ to be activated. $G_{\beta\gamma}$ binds to $G_\alpha$ GTPase-activating proteins (GAPs) and regulators of G protein signaling (RGSs) to inhibit GTPase activity thus extending their active time as well as the $G_\alpha$ effector PLC (the normal effector for $G_{\alpha q}$) (55). Although the details of $G_{\beta\gamma}$ have long been elucidated, new signaling paradigms, such as $G_\alpha$ binding two $G_{\beta\gamma}$ subunits (56) and inhibition of DA transporters (57) continue to emerge. Functionally selective ligands at D2R could selectively activate a particular $G_{\alpha\beta\gamma}$ heterotrimer, the consequence of which could be long-term depression (LTD) through GIRK, but not cAMP inhibition through $G_\alpha$. Such structure-activity ligand relationships would require significant technical achievement in screening platforms and even more advances in the molecular details of D2R activation of G proteins before they could be feasible.

Like $G_{\beta\gamma}$’s ability to slow down the GTPase activity of $G_\alpha$, $G_{\beta\gamma}$ participates in another crucial point in D2R signal transduction, it binds to GPCR kinase (GRK) 2 and 3, which recruits the kinases to the activated receptor (58). Conversely, GRK 5 and 6 are lipidated which localizes them to the plasma membrane independent of G protein activation (59). D2R can interact with all four of these proteins and is coexpressed with them (albeit at different ratios, depending on the cell). GRKs phosphorylate the intracellular loops of D2R which mediates the desensitization and internalization of D2R.
(60). The significance of multiple GRKs is that different patterns of phosphorylation (most likely on D2R’s long third intracellular loop) would yield different functional outcomes, such as intracellular trafficking to different compartments or recruitment of different intracellular signaling molecules. This is the barcode hypothesis, which means different phosphorylation barcodes would be scanned by intracellular proteins (61). This is a theory that has been validated in D2R: GRK 2/3 phosphorylation sites were mutated which resulted in no deficiency in β-arrestin recruitment or desensitization but rather a deficiency in receptor recycling (62). Additionally, D2R is also phosphorylated by PKC (63) the consequences of which yield distinct signaling paradigms from GRK2: Gβγ activated GIRK channels are more rapidly desensitized. GRK2 may play a role in D2R signaling in its own right, as it (along with GRK3) possesses an RGS domain and has been shown to regulate D2R independently of its kinase activity (64).

Another significant consequence of D2R phosphorylation is increased affinity for β-arrestins. β-arrestins are multifunctional adaptor proteins that bind to clathrin-coated pits to mediate GPCR internalization (65). Furthermore, β-arrestins have been shown to cause changes in up to 1,500 phosphorylation sites in vitro, and these interactions are mediated by the active state of the receptor (66). β-arrestins are the major transducer of G protein-independent signaling from GPCRs. β-arrestins are phosphosensors encoded by two different genes, named β-arrestin 1 and 2. The β-
arrestin 2 global knock out mouse was used to discover the existence of a D2R/β-arrestin 2/PP2A/Akt signaling complex that results in the inhibition of Akt, which relieves Akt-mediated inhibition of GSK3β signaling (67). This pathway was validated with GSK3β heterozygous mice and mice with GSK3β knocked out specifically in D2R expressing MSNs (68). This D2R mediated inhibition of Akt has also been shown to be crucial in the developing zebrafish brain (69) and implicated in DA related feeding behavior (70). D2R/β-arrestin has also been implicated in MAP kinase (ERK) signaling in vitro (71), and has been shown to significantly be increased in vivo when mice are given amphetamine and methylphenidate (72). Finally, an intronic single nucleotide polymorphism (SNP) in D2R, as well as a synonymous SNP in Akt1 were shown to be associated with schizophrenia phenotypes as well as olanzapine treatment efficacy in patients (73).

Antipsychotics, which have long been known to bind to D2R (74) also all function as β-arrestin 2 antagonists (75). Taken together, these data suggest a clear impetus for functionally selective biased ligands between β-arrestin and G protein signaling pathways.

Antipsychotics, because of their therapeutic relevance, have undergone extensive molecular characterization. The first principle of antipsychotic efficacy is D2R binding (74), and typical antipsychotics are potent D2R antagonists. However, atypical antipsychotics have yielded a more complex pharmacology: two of the best studied
atypical antipsychotics are aripiprazole and clozapine. Clozapine binds D2R weakly, while aripiprazole is a partial agonist. The selectivity of its partial agonism is debatable, as it could be considered a biased partial agonist or a balanced partial agonist, depending on the kinase expression, see Figure 1B and 1C. The study of antipsychotics is where the concept of functional selectivity at D2R originated, and as more advanced techniques have developed, more data has been made available to elucidate the robust and complex activation of D2R. For instance, the phosphorylation pattern downstream of D2R was originally shown to involve Thr 308 of Akt and not Ser 473 (67). However, a recent study showed that haloperidol increased Ser 473 phosphorylation in cultured MSNs (76) and a study in HEK cells showed that D2R activation caused an increase in both Akt phosphorylation sites (77). In another in vivo study, the phosphorylation status of Akt is shown to be decreased 90 minutes after methamphetamine injection, however, in another panel of the same study, Akt is shown to be increased with methamphetamine injection (78). In addition, different brain regions have been shown to have increased Akt phosphorylation in response to amphetamine and methyphenidate. The cortex is increased, while the striatum is decreased in this study (79). The picture gets even more out of focus when targets of Akt are considered. GSK3β heterozygous (80) and D2R-cell type specific knock-out mice (68) show a decreased response to amphetamine (just as β-arrestin 2 knock-out mice do) and a well characterized GSK3β
target, β-catenin was shown to be stabilized in β-arrestin 2 knock-out mice (80) and activated by haloperidol and clozapine (81). However, genetic stabilization of β-catenin did not phenocopy GSK3β heterozygous knock-outs (GSK3β causes the destabilization of β-catenin) (68). These discrepancies illustrate the incredible complexity of pleiotropic signaling at one single GPCR but also demonstrate the utility of understanding functional selectivity in more detail.

1.2.4 Non-canonical D\(_2\)R interacting proteins

Akt/GSK3β is an integrator of cellular signaling and while functional selectivity at D\(_2\)R (e.g. G\(_{βγ}\) activation of PI3K and Akt versus β-arrestin 2 inactivation of Akt via PP2A recruitment) contributes to the contradictory findings, it is also because of non-canonical D\(_2\)R interacting proteins. For example, D\(_2\)R has been shown to interact directly with β-catenin (82) and a small GTPase expressed in the striatum called Rhes was shown to interact with β-arrestins, PP2A, and be crucial for Akt dephosphorylation in Rhes knockout animals (83).

D\(_2\)R has been shown to interact with many other non-canonical signaling proteins, which increases the possibilities for functional selectivity exponentially (a ligand could activate β-arrestin/Rhes/PP2A signaling but block β-arrestin/clathrin internalization). D\(_2\)R directly interacts with and increases the expression of the transient receptor potential channel canonical (TRPC) 1 (84). TRPC channels are expressed in the
brain and are known to modulate intracellular calcium concentration. The D2R/β-arrestin interaction may also play a role in this function, as β-arrestins have been shown to ubiquitinate (85) and desensitize (86) transient receptor potential channel vanilloid (TRPV). This is yet another possibility for D2R functional selectivity, as D2R could both increase TRP function by directly binding and decrease its function through β-arrestin.

Another physiologically important channel, the NMDA receptor, was shown to interact directly with D2R through the NR2B subunit in vivo and D2R modulated NMDA receptor activity, which controls neuronal firing directly (87).

Many D2R interactions have been identified by standard biochemical methods. A yeast two-hybrid screen of the long third intracellular (IC3) loop of D2R yielded a specific interaction with Par-4, a leucine zipper (88). Par-4 was shown to compete for a previously characterized calmodulin binding site in the IC3, and the authors detected behavioral abnormalities in a mouse with a mutated Par-4. Interestingly, the authors verified the D2R/Par-4 interaction and believed that the mobility of D2R on a gel from a Par-4 immunoprecipitation “may suggest a functionally selective interaction of Par-4 with a subspecies of D2DR [sic] in vivo” presumably because of glycosylation, phosphorylation, and dimerization among other posttranslational modifications such as ubiquitination (all factors that contribute to gel mobility shift subspecies of GPCRs).
Similarly, a yeast two-hybrid screen of D₂R’s IC3 also revealed a functional interaction with ZIP, a PKCζ interacting protein (89) that inhibited Gαi signaling in HEK cells.

Many D₂R interactions have also been identified \textit{in vivo}. For instance, the D₂R knockout mice revealed interactions with striatal-enriched protein tyrosine phosphatase (STEP) (90) and Nurr1, a nuclear receptor (91). Both of these interactions appear to be involved in MAP kinase/ERK signaling in D₂R mediated neuronal development. Cbl-interacting protein of 85 kDa (CIN85) mutant mice (92) showed subcellular localization with D₂R. Colocalization using immunolabeling of D₂R and neuronal calcium sensor-1 (NCS-1) (93) showed a direct interaction. The data presented in this section is by no means a comprehensive list of all of the D₂R interacting proteins. However, it illustrates another facet of functional selectivity that can be leveraged for therapeutic ligand development. At this stage such development would be premature, while the work presented here has extensive characterization of the D₂R interactions, it pales in comparison to the decades of research on G protein and β-arrestin interactions with D₂R.

\textbf{1.2.5 D₂R possesses a common allosteric modulator binding site, the extracellular vestibule}

Facile manipulation of functional selectivity could be achieved through allosteric modulation. Allosteric modulation of GPCR signaling has been the focus of many studies because of the therapeutic potential of such a ligand. Recently, cocaine was shown to function as a positive allosteric modulator \textit{in vivo} at D₂R using behavioral,
microdialysis, and GTPγS binding experiments (94). It is not unreasonable to assume that an allosteric modulator could preferentially potentiate or inhibit a particular DₐR signaling pathway, for example, DA binding could be modulated by an allosteric ligand to favor G protein activation over β-arrestin signaling or Gₐi over Gₐo. This very assumption has been proven for the activation of the M1 DREADD, because an allosteric modulator behaved as a negative allosteric modulator for Ca²⁺ mobilization, while having a neutral effect on MAP kinase signaling (95). A negative allosteric modulator at D₂R was shown to inhibit both G protein and β-arrestin 2 recruitment (96) while this does not show functional selectivity at D₂R, it demonstrates that D₂R has the capacity to be allosterically modulated in a biased fashion.

The recently crystallized muscarinic 2 receptor (M₂R) proved the existence of the binding of allosteric modulators to the extracellular vestibule, a binding site 1.5 nm proximal to the orthosteric binding site (97). Furthermore, molecular dynamics studies have computationally modeled the mechanism of positive and negative allosteric modulation at M₂R (98). To illustrate the significance of this extracellular vestibule, a rigorous structure-activity relationship (SAR) study was done at D₂R with cariprazine on functional selectivity (cAMP and MAP kinase signaling) (99). The authors speculate that one mechanism of achieving functional selectivity is through bitopic ligand interactions (i.e. orthosteric and allosteric binding in one ligand).
Allosterism through the extracellular vestibule allosteric site is an exciting avenue for achieving therapeutically relevant functionally selective D₂R ligands, as structural studies allow for precise modulation of activity and SAR studies allow for precise ligand design (100). In fact, D₂R functionally selective ligands have been created and screened for preclinical efficacy (101, 102). The studies mentioned here are the beginning of an exciting era for D₂R functionally selective ligands.

1.2.6 Unexpected sites of allosteric modulation

The extracellular vestibule is proximal to the orthosteric site and allows for the creation of bitopic ligands, however GPCRs have been shown to possess other sites of allosteric modulation. The A₂A R was crystallized to 1.8 angstroms, which was a high enough resolution to solve the location of sodium binding (103). Sodium has long been known to be required for proper D₂R radioligand binding (104) and also important in opiate receptor (105) and alpha adrenergic receptor (106) radioligand binding. A crucial and highly conserved residue, Asp 80 (2.50) coordinates sodium binding and was shown in D₂R to abolish G protein signaling while maintaining ligand binding affinity for antagonists (107) when mutated to an Ala, a property that was also shown in the α-adrenergic 2A receptor (108) with sodium mimetic mutations at the same residue. Sodium has also been suggested to be crucial to D₂R dimer formation (109). Recent molecular dynamics simulations have shown that the sodium binds from the
extracellular space (110), where sodium concentrations are high. Recently sodium ingestion was studied in mice lacking sour and bitter receptors (which are GPCRs) and it was shown that sodium, which is normally aversive at high concentrations, is not aversive when the sour and bitter receptors are knocked out (111). This is a remarkable result because it demonstrates the direct molecular effect sodium has on GPCRs, as bitter and sour receptors normally activate aversive responses, and high concentrations of sodium are able to elicit the same response. Functional selectivity due to sodium regulation has been shown directly in the δ-opiod receptor: mutation of Asp 2.50 to an Ala caused a G protein biased ligand to become a β-arrestin biased ligand (112). Similar to sodium, many other uncommon allosteric modulators have been characterized for D₂R. L-prolyl-L-leucyl-gycinamide (PLG) was shown to enhance agonist and G protein coupling for D₂R (113) but have no effects on antagonists (114). While a molecule related to PLG, called POAPA, enhanced the β-arrestin expression and function at D₂R (115). Additionally, zinc has been shown to modulate antagonist binding to D₂R (116). Sodium and other allosteric modulators are another important contributor to the heterogeneous conformational landscape of D₂R and allow for a better understanding of functional selectivity.
1.3 D₂R functional selectivity is a function of cellular organization

Every molecular and mechanistic contribution to D₂R functional selectivity mentioned in this chapter is dependent upon the cell, the tissue, and the organism. Kenakin proposes that in signal transduction the ligand and the signaling proteins are signaling vectors (Figure 2A) that work upon a conduit, the receptor (117). The presence and concentration of these vectors depends on the cellular expression as well as ligand availability, and the efficiency of signaling depends on the expression of the receptor. For this reason, functional selectivity must always be considered in the context of an organism. The actions of D₂R in the central nervous system are the basis for many therapeutics, including treatments for attention deficit-hyperactivity disorder, Parkinson’s disease, schizophrenia, bipolar disorder, and addiction among many other diseases.
Figure 2: D₂R functional selectivity is dependent upon context. (A) D₂R interactions and downstream effectors leading to signaling events. (B) D₂R physical states are dynamic and determine extent of functional selectivity. (C) Central nervous system architecture of D₂R expression and connectivity. Dopaminergic projections (blue arrows) to cortex and striatum modulate excitatory (green arrows) and inhibitory (red arrows) neural circuits. D₂R is prominently expressed in dopaminergic, striatal and cortical cells. VTA: ventral tegmental area; SN: substantia nigra; GPi/SNr: internal globus
D_{2}R is expressed in the basal ganglia in a subpopulation of medium spiny neurons (MSNs) along with many other GPCRs. MSNs form the basis of the direct and indirect circuitry (where direct MSNs express D_{1}Rs and indirect MSNs express D_{2}Rs) and all drugs of abuse cause a robust increase in dopamine in the nucleus accumbens, a structure of the basal ganglia. In fact, the striatum (comprised of the ventral nucleus accumbens and the dorsal caudate putamen) receive 90% of their inputs, which are excitatory, from the cortex, the layer of the brain that has evolved the most in humans. The cortex is involved in information processing, and the striatum, through D_{2}R and D_{1}R expressing MSNs executes cortical commands to control locomotion, cognition and reward. MSNs release the inhibitory neurotransmitter GABA and connect to the midbrain. The midbrain in turn projects and releases dopamine (DA) to the dorsal striatum (through the substantia nigra, SN, a subset of cells that decay in Parkinson’s disease) and the ventral striatum (through the ventral tegmental area, VTA). D_{2}R is highly expressed in the MSNs, the SN and VTA projection neurons, some cortical neurons, and interneurons in the striatum.
1.3.1 Dopamine and D₂R genetic mouse models reveal functional selectivity in vivo

The role of D₂R in vivo was most directly dissected with genetic models. The critical enzyme for tyrosine metabolism into DA is tyrosine hydroxylase (TH) and when TH was knocked out (and restored in adrenergic cells) DA could no longer be synthesized (118). These mice showed severe deficiencies in locomotor activity, feeding, and consuming water and all died within 30 days without treatment with L-DOPA, the enzymatic product of TH. Interestingly, rescue of TH to only the dorsolateral (119) or ventromedial (120) striatum rescued many of the feeding, cognitive, and locomotor deficits suggesting functional plasticity in the DA circuitry. In contrast, when the DA transporter (DAT) is knocked out, extracellular DA is increased and mice display increased locomotion (121) and D₂R is downregulated (122).

The function of D₂R signaling is most easily assessed in D₂R knockout mice, which have severe locomotion deficits (123) and are deficient in reward learning (124). D₂R knockout mice are also deficient in cocaine induced locomotion, and have an ablated MSN c-fos activation in response to cocaine (125). This result may seem unexpected, as c-fos is normally upregulated in D₁R expressing MSNs in response to cocaine (126). However, D₂R is able to exert control over D₁R MSNs because of its expression in presynaptic SN and VTA projection neurons. D₂R is alternatively spliced in the third intracellular loop into a long isoform, called D₂L and a short isoform called
D2S (127) and D2S is more abundantly expressed presynaptically, while D2L is more abundantly expressed postsynaptically (128). The presynaptic D2R directly inhibits the release of DA as well as the production of DA through TH phosphorylation (129) thus presynaptic D2R can affect D1R MSNs by controlling dopaminergic tone in the striatum. More recently, D2R has been deleted specifically presynaptically (130) and postsynaptically (131) and it was shown that postsynaptic D2R is necessary for cocaine induced locomotion, while loss of presynaptic D2R resulted in supersensitivity to cocaine. The postsynaptic D2R population was also shown to be sufficient for locomotion, as viral reexpression in the nucleus accumbens rescued the amphetamine response in D2R knockout mice (132) and overexpression of D2R in the nucleus accumbens caused an increase in basal locomotion (133). The cellular organization and splicing of D2R is yet another layer of functional selectivity that affords D2R the ability to transduce multiple, complex, and sometimes contradictory signaling events in response to a single stimulus. Cocaine and amphetamine are used to probe the behavioral responses in these genetically modified mice because they act to increase synaptic DA and have relevance to human disease (134). While behavioral outcomes are not as easily interpreted as biochemical assays, the relevance of D2R splicing yielding functional selectivity has been shown biochemically (135). D2R functions within a complex circuit...
in the CNS, and the therapeutic implications of D₂R functional selectivity is that D₂R’s control over that circuit can be manipulated specifically

1.3.2 Functional selectivity in D₂R circuitry

The most important property of D₂R in CNS circuitry is that it does not signal in a vacuum. As discussed earlier, presynaptic D₁R’s inhibition of DA release directly affects D₁R expressing MSNs. However, activation of both D₁R and D₂R expressing MSNs (referred to as the direct and indirect pathway, respectively) is necessary for normal D₂R circuit modulation (136) and behavioral outcomes (137). In addition to the well-established direct and indirect pathway relationship, endocannabinoids induce long-term depression (eCB-LTD) in D₂R expressing MSNs selectively (138) and the induction of this eCB-LTD requires D₂R activation and induces parkinsonian motor deficits. Additionally, A₂AR signaling modulates this circuit, along with RGS4, a regulator of Gα subunits in MSNs (139). The limbic circuit, from the midbrain to the striatum and the cortex is modulated by D₂R, and D₂R itself is modulated by long term changes in circuitry. For instance, chronic haloperidol (an antipsychotic and potent D₂R antagonist) causes an upregulation of striatal D₂R but not cortical D₂R (140) while the atypical antipsychotic (and D₂R antagonist) sulpiride did not increase striatal D₂R expression (141) suggesting that the modulation of D₂R circuitry is a consequence of haloperidol’s more dramatic antipsychotic efficacy and higher capacity for extra
pyramidal side effects. Behavioral changes can also alter D2R circuitry directly, for example enhanced DA synaptic strength has been shown in response to cue-reward learning (142).

The role of D2R MSNs was dissected directly using optogenetic activation and DREADD inactivation of the neurons which showed that they induce freezing behavior in the dorsal striatum (143) and restrain addictive behavior (cocaine self-administration) in the ventral striatum (144). In addition to the important role of D2R in MSNs, D2R is also expressed in the small population of cholinergic interneurons in the striatum, cells which have been shown to cause DA release themselves (145) and override the normal nucleus accumbens response to cocaine to block cocaine conditioning (146). The VTA projects to the striatum and cortex and individual projections have been shown to control aversion and also reward (147). The firing rate of the VTA projections is important for DA transmission, as tonic DA firing maintains dopaminergic tone, while phasic burst firing can cause a rapid increase in DA and the differences between these circuits has been shown to be anatomical and genetic (some projections do not express the autoinhibitory D2R) (148). The phasic firing of the VTA neurons was shown to be sufficient for some behavioral adaptations implicated in addiction (149) while the precise anatomical circuitry of the VTA has only begun to be mapped and manipulated (150). The role of the projection neuron circuitry firing was also implicated in depression
phenotypes (151) and susceptibility to depression (152). While it is more difficult to directly assess the role of D2R functional selectivity when the circuitry is assessed, it is obvious that DA and D2R expressing cells play fundamental roles in many behaviors and human diseases.

**1.4 The therapeutic implications of D2R functional selectivity**

D2R functional selectivity has long been observed, and D2R is a central target for many psychiatric disorders. The molecular mechanisms underlying D2R functional selectivity, including dimerization, G protein and β-arrestin selectivity, and more recent hypotheses such as intracellular receptor signaling provide a unique opportunity to isolate D2R signaling pathways. New classes of antipsychotics and antidepressants could be developed that manipulate these molecular properties. D2R has long been a protein target with enormous therapeutic potential, and understanding its signal transduction properties only increases the capacity for the development of more effective pharmaceuticals with less occurrences of side effects.

The work presented in the next chapters represents elucidation of the functional selectivity of D2R in a systematic and robust manner. Novel mutant D2R were generated using the Evolutionary Trace (ET) approach (Chapter 2) and these mutants were extensively characterized (Chapter 3). Two mutants displayed extreme bias between G protein and β-arrestin pathways and these mutants retained the major functions of D2R.
The two biased mutants were then used to unravel MAP kinase signaling \textit{in vitro} and amphetamine induced locomotion \textit{in vivo} (Chapter 4). Finally, the mutagenesis approach was applied to diverse GPCRs as well as more complex \textit{in vivo} systems (Chapter 5). This work provides a clear impetus for more detailed functional selectivity studies of D2R for the development of therapies with predictable outcomes.
2. Functional separation of D₂R using Evolutionary Trace

Understanding the contributions of functional selectivity at D₂R in intact biological systems is a challenge that cannot be elucidated in heterologous systems alone. In order to develop tools where this challenge can begin to be addressed, the Evolutionary Trace (ET) approach (153) was used to engineer D₂R mutants that selectively interact with either G proteins or β-arrestins, designated \([\text{Gprot}]D₂R\) and \([\text{βarr}]D₂R\), respectively.

2.1 Evolutionary Trace guided mutagenesis of D₂R

The ET method identifies amino acids that determine the function of a protein and map its functional sites when a structure is available (154, 155). ET algorithms exploit protein orthologs and paralogs to correlate sequence variations with phylogenetic divergences and determine whether substitutions at a particular residue are likely to produce a functional change in the protein (156). The predictive power of ET is further enhanced when specific crystal structures (157) and more sophisticated models of the evolution of structure and function can be applied (155, 158). Here, a combination of these approaches (co-crystals of receptors and signaling molecules as well as novel algorithms, Figure 3) was used to identify residues in D₂R that could be mutated to achieve functional selectivity. The residues that were identified as being potentially critical for functional selectivity are mapped onto the crystal structure of D₂R.
(Figure 3B; PDB ID: 3PBL) (159), which shows the physical proximity of each residue to each other as well as to the cytosolic side of the receptor.

Figure 3: Evolutionary Trace of D$_2$R facilitated by advances in crystallography and algorithm design. (A) TYY mapped onto D$_3$R (159) (PDB ID: 3PBL in red). (B) Four rounds of ET predicted residues for mutagenesis mapped onto D$_3$R. (C) piET (green residues depicted as spheres). (D) G$\alpha$-CT proximity (160) (PDB ID: 3DQB residues in yellow, G$\alpha$-CT is represented as a green $\alpha$-helix cartoon), and (E) G$_{\text{Gq/11y}}$ (161) (PDB ID: 3SN6 $\alpha$ is green and $\beta$ is blue) interaction with IC2 of D$_3$R (grey spheres).

The $\beta$2 adrenergic receptor ($\beta$2AR) was originally mutated based on the crystal structure of rhodopsin (162) to yield a $\beta$-arrestin biased $\beta$2AR termed $\beta$2AR-TYY. These residues were mutated in D$\alpha$R (Figure 3A) however, robust separation between G
protein and β-arrestin pathways was not achieved. Therefore, four rounds of ET-guided residue determination (mapped to D3R) (159); in Figure 3B) were carried out with the development of the more complex ET algorithm piET (Figure 3C) and as more detailed crystal structures were solved including the C-terminus of Gαt in complex with rhodopsin (Figure 3D) (160) and β2AR in complex with Gαβγ (Figure 3E) (161).

2.2 Evolutionary Action at D3R yields a robust landscape of functional selectivity

In order to achieve specific and robust separation of G protein- and β-arrestin-dependent interactions, Evolutionary Action (EA) (163) was used to predict residue changes. EA models the evolutionary relationship between genotype and phenotype as a smooth process upon which a mutation causes a small perturbation. Explicitly, if γ is the genotype sequence and φ the fitness phenotype, EA postulates an evolutionary function f between them exists, such that:

\[ f(γ) = φ \]  

and f is differentiable so that the Evolutionary Action point mutation \( Δγ \) on fitness as:

\[ f'(γ) \cdot Δγ = Δφ \]  

In practice, f remains unknown but its derivative (or gradient) \( f' \) is given by ET, and \( Δγ \) is given by substitution odds. The Evolutionary Action equation (2) is thus generally solvable for coding mutation of proteins and quantifies the effect of mutations over multiple scales, spanning molecular, clinical and population genetics effects (163-
Here, for each residue identified in Figure 3A-B, mutations were predicted and scored by EA according to how likely they would produce a phenotype (Table I). Each point mutation was tested for G protein activity by cAMP inhibition and β-arrestin 2 recruitment by Bioluminescent Resonance Energy Transfer (BRET) (75), fidelity of plasma membrane trafficking as well as lack of constitutive activity. These mutants were binned into four categories: 1) β-arrestin biased, 2) G protein biased, 3) deficient at both pathways, or 4) unaffected at either pathway (Figure 4A). Residues that retained the desired phenotype were further combined into double (Figure 4B), triple (Figure 4C), quadruple, and quintuple (Figure 4D) point mutations. This initial characterization yielded a robust landscape of unique functionally selective mutants.

**Table I: Evolutionary Action guided mutagenesis.** Each residue predicted from ET is color coded the same in Figure 3 and Figure 4 in the first column. The second column shows the predicted residues in increasing ET harshness (amino acid conservation and side chain chemistry) from left to right. Each single point mutation was generated and tested for G protein and β-arrestin activity. Note that A135 was found to be a critical residue for G protein activation, but not β-arrestin activation and all 19 mutations were made in order to titrate the most β-arrestin biased mutant.
<table>
<thead>
<tr>
<th></th>
<th>T69</th>
<th>Y133</th>
<th>Y209</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>V</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>S</td>
<td>F</td>
</tr>
<tr>
<td>A135</td>
<td>C</td>
<td>S</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>F</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>Q</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>K</td>
<td>D</td>
</tr>
<tr>
<td>A372</td>
<td>S</td>
<td>Y</td>
<td>W</td>
</tr>
<tr>
<td>L376</td>
<td>G</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>N431</td>
<td>A</td>
<td>M</td>
<td>L</td>
</tr>
<tr>
<td>L72</td>
<td>V</td>
<td>N</td>
<td>D</td>
</tr>
<tr>
<td>I73</td>
<td>T</td>
<td>Q</td>
<td>D</td>
</tr>
<tr>
<td>L76</td>
<td>V</td>
<td>N</td>
<td>D</td>
</tr>
<tr>
<td>L125</td>
<td>V</td>
<td>N</td>
<td>D</td>
</tr>
<tr>
<td>I128</td>
<td>T</td>
<td>S</td>
<td>D</td>
</tr>
<tr>
<td>S129</td>
<td>G</td>
<td>Y</td>
<td>I</td>
</tr>
<tr>
<td>V379</td>
<td>L</td>
<td>D</td>
<td>K</td>
</tr>
<tr>
<td>I426</td>
<td>F</td>
<td>Q</td>
<td>G</td>
</tr>
<tr>
<td>T428</td>
<td>N</td>
<td>L</td>
<td>W</td>
</tr>
<tr>
<td>P139</td>
<td>A</td>
<td>S</td>
<td>W</td>
</tr>
<tr>
<td>M140</td>
<td>L</td>
<td>V</td>
<td>D</td>
</tr>
<tr>
<td>Y142</td>
<td>F</td>
<td>H</td>
<td>E</td>
</tr>
<tr>
<td>T144</td>
<td>A</td>
<td>N</td>
<td>W</td>
</tr>
</tbody>
</table>
Figure 4: G protein and β-arrestin activity of ET predicted mutants. Dose response curves for each mutant were normalized to a WT control that was performed the same day and the log(τ/Kₐ) was calculated. Since all values were
normalized to WT, $\tau=1$ and $K_A=1$ for WT, thus 0 is depicted as yellow, and -3 (100 log fold shift in $\tau/K_A$) is depicted as black in the heat maps. (A) G protein and $\beta$-arrestin activity of single point mutations generated mutants that were misfolded, mutants that were biased toward G protein or $\beta$-arrestin, or mutants that had little to no effect on any tested function. (B) Mutations that had no effect on the desired pathway (red lines for $\beta$-arrestin and green for G protein) were combined into double point mutations and (C) triple and (D) quadruple and one quintuple. The mutations that were further characterized in Figure 6 are highlighted in boxes.

The four rounds of ET guided mutagenesis are depicted in a traditional snake-like plot of D2R in Figure 5A with residues highlighted with the same color scheme as Figure 3 and Table I. The two mutants that showed the greatest functional separation are designated $^{[\text{Gprot}]}\text{D}_2\text{R}$ (L125N Y133L) and $^{[\text{βarr}]}\text{D}_2\text{R}$ (A135R M140D). Each of these mutations occurs within 20 amino acids of the DRY motif of TM3 (Figure 5C). Interestingly, $^{[\text{Gprot}]}\text{D}_2\text{R}$ mutations are more distal from the recently resolved co-crystals of receptors and G proteins or arrestin fragments, while $^{[\text{βarr}]}\text{D}_2\text{R}$ mutations are more proximal (Figure 5D). The $^{[\text{Gprot}]}\text{D}_2\text{R}$ mutant is derived from the more sophisticated ET algorithms (TYY and piET, Figure 3A and 3C), while $^{[\text{βarr}]}\text{D}_2\text{R}$ arose from residues identified in the more specific crystal structures of receptors and G proteins (Figure 3D and 3E)(160, 161).
Figure 5: Generation of functionally selective D_{2}R mutants. (A) Snake-like plot of D_{2}R with each round of mutagenesis color-coded according to Table I and Figure 3. Red spheres: residues derived from TYT, green spheres: predicted from piET algorithm, yellow spheres: predicted from proximity to rhodopsin/transducin α subunit C-terminal fragment co-crystal; grey spheres: identified residues from β2AR/G_{αβγ} co-crystal in intracellular loop two.
Ballesteros-Weinstein numbering identified for each transmembrane domain. The same color scheme was used to highlight the residues on the structure of D₃R (159) because D₃R is the most closely related GPCR to D₂R with an available crystal structure (81% sequence identity for transmembrane domains). (B), D₃R structure represented as a blue ribbon, and ET-identified residues are spheres. (C) The biased mutants all occur within 20 amino acids of the DRY motif on transmembrane domain three (TM3). (D) D₃R aligned to β2AR in complex with Gαβγ, green cylinder PDB ID: 3SN6) (161) as well as rhodopsin in complex with the finger-loop domain of visual arrestin (167) (purple cylinder PDB ID: 4PXF). D₃R to β2AR alignment yielded an RMSD = 1.8 and D₃R alignment to rhodopsin RMSD = 2.7 using pymol MatchAlign command.
3. Ligand, receptor and transducer contributions to D$_2$R functional selectivity

While molecular and conformational theories have framed the development of biased agonist based therapies, the molecular details of functional selectivity remain elusive. D$_2$R’s prominence as a pharmaceutical target for many disorders makes it a good receptor candidate for precise and robust dissection of functional selectivity.

Here, the contributions of ligand, receptor and transducer to functional selectivity are rigorously assessed. Novel mutants are characterized and assessed for their unique functional selectivity properties that provide insight into the quality of D$_2$R functional selectivity. These data demonstrate that functional selectivity is dynamic and malleable, and that leveraging specific D$_2$R pathways is possible.

3.1 $[G_{prot}]$D$_2$R and $[\beta_{arr}]$D$_2$R display distinct but expected properties

Each receptor mutant (Table I) was profiled for G protein and β-arrestin activity. In addition, a negative control point mutation, $[D80A]$D$_2$R (107) was included in all experiments because this mutant has been shown to bind ligands and traffic to the plasma membrane but is deficient in signaling. As shown in Figure 6A, $[G_{prot}]$D$_2$R retained full efficacy and potency at cAMP inhibition compared to $[WT]$D$_2$R, while $[\beta_{arr}]$D$_2$R and $[D80A]$D$_2$R are severely deficient. In contrast, β-arrestin 2 recruitment is retained and even enhanced in $[\beta_{arr}]$D$_2$R while both efficacy and potency are either lost or markedly reduced in $[D80A]$D$_2$R and $[G_{prot}]$D$_2$R as determined by BRET (Figure 6B).
Figure 6: Biased D₂R mutants derived from Evolutionary Trace. (A) Inhibition of cAMP as determined by GloSensor compared to [WT]D₂R positive control and [D80A]D₂R negative control. (B) β-arrestin 2 recruitment determined by BRET for the same receptors as in (A). All points are SEM of n=3-7 done in duplicate. Confocal images of (C) [WT]D₂R, (D) [Gprot]D₂R, (E) [βarr]D₂R, and (F) [D80A]D₂R expressed in live cells. (G) BMAX (with SEM) determined from n=3 radioligand binding experiments. (H) Kᵢ from BMAX determination experiments. (I) DA competition binding experiments to determine Ki. (J) D₂R internalization assessed by live cell HA antibody staining of D₂R (SEM, n=5 done in triplicate).

Point mutations in GPCRs, especially ones that affect signaling, are notorious for inducing unstable proteins (168). To address this concern, the membrane localization of [WT]D₂R (Figure 6C) in live cells was compared to [Gprot]D₂R (Figure 6D), [βarr]D₂R (Figure 6E), and [D80A]D₂R (Figure 6F). None of the receptors studied showed major trafficking...
deficits. However, in order to quantitatively assess the expression of these mutants, traditional radioligand determinations of $B_{MAX}$ (Figure 6G) and $K_D$ (Figure 6H) were performed. When transiently transfected into HEK 293T cells all mutated receptors expressed between 1-1.5 pmol/mg protein and their levels were 30-50% lower than the WT $D_2$R under the same conditions (Figure 6G). However, the $K_D$ for the antagonist raclopride was virtually identical (Figure 6H) and the $K_I$ for DA was also unchanged (Figure 6I). Receptor internalization, as assessed by cell surface ELISA on live cells (169) demonstrates predictable internalization patterns: $[^{β}π]D_2$R and WT $D_2$R internalize to the same degree (30%) as previously reported (62) while $[^{Gprot}]D_2$R and $[^{D80A}]D_2$R are severely deficient (Figure 6J).

The separation in apparent affinity for the endogenous ligand dopamine for cAMP inhibition, $β$-arrestin 2 recruitment and internalization is 100-1000 fold between the two engineered receptors while the $K_D$ of raclopride and $K_I$ of DA remains unchanged. Similarly, the response of each receptor mutant would be greater than 90% distinct even at the highest physiological levels of dopamine (100 µM in Figure 6A, 6B, and 6J). Additionally, the slight differences in expression levels of the various $D_2$R mutants does not seem to affect their coupling potencies as increasing the amounts of transfected $D_2$Rs has the same effects for WT $D_2$R and $[^{Gprot}]D_2$R. Thus, these ET-derived mutants display a robust but selective disruption in $D_2$R function.
3.2 Operationally defined, receptor dictated functional selectivity

The bias of a receptor can be simply viewed as being dependent upon its propensity to engage one signaling mechanism over the other. A well characterized biased D₂R was previously engineered (71, 170) by mutating a motif unique to D2-like receptors (IYIV) to four alanines ([IYIV]D₂R). As seen in Figure 7A, [IYIV]D₂R induces a complete loss of β-arrestin 2 recruitment, along with the previously observed (170) ~50% loss of G protein activity. Thus, [IYIV]D₂R is a G protein-biased mutant D₂R under these conditions. However, when GRK2 is overexpressed (Figure 7B), β-arrestin 2 recruitment potency is enhanced at [WT]D₂R and [IYIV]D₂R, and only slightly potentiated for the previously characterized [Gprot]D₂R and [βarr]D₂R when compared to endogenous levels of GRK2 (Figure 6B). Quantifying the bias between [Gprot]D₂R and [IYIV]D₂R using a statistical formalism (171) (Figure 7C), a bias plot (172) (Figure 7D) and ΔΔlog(τ/Kₐ) calculations (Table IV) reveal the quality of G protein bias. [Gprot]D₂R and [IYIV]D₂R display similar degrees of bias using each method because while G protein activity is slightly perturbed at [IYIV]D₂R, at [Gprot]D₂R the G protein activity is completely intact, while a small amount of β-arrestin activity remains. However, when GRK2 is overexpressed [Gprot]D₂R does not shift appreciably whereas [IYIV]D₂R gains significantly in both efficacy and potency, this is revealed by the greater shift observed using the bias statistical formalism (Figure 7C). In contrast, the bias plot reveals that [IYIV]D₂R is inefficiently biased toward G protein activity, and ΔΔlog(τ/Kₐ) values reveal that [IYIV]D₂R bias is dependent on GRK2 levels.
The GRK2 overexpression assay demonstrates the receptor capacity for β-arrestin recruitment under the most favorable conditions, and shows that \[\text{YIV}\]D₂R displays partial agonism at both G protein and β-arrestin activities. In contrast, \[\text{Gprot}\]D₂R retains its original biased signaling profile: full agonism at the G protein pathway and weak partial agonism at the β-arrestin pathway.

**Figure 7:** Functional selectivity is operationally defined. (A) β-arrestin 2 recruitment comparing \([\text{WT}]\)D₂R and \([\text{YIV}]\)D₂R as determined by bioluminescent resonance energy transfer (BRET). (B) GRK2 overexpression enhances β-arrestin 2 recruitment by BRET for \([\text{YIV}]\)D₂R and \([\text{WT}]\)D₂R, but only slightly for \([\text{Gprot}]\)D₂R, \([\text{βarr}]\)D₂R, and \([\text{D80A}]\)D₂R when compared to Figure 6B. All data are presented with SEM from \(n=3-4\) independent experiments, with statistical significance calculated in Table IV. Quantification of bias between G protein activity and β-arrestin 2 recruitment using (C) a statistical formalism (171) or (D) bias plot mapping under normal (solid lines) and GRK2 overexpression enhanced (broken lines) conditions.
The controlled perturbation of G protein and β-arrestin pathways allows for a more detailed examination of partial biased agonism. Partial biased agonism is an important property displayed by many GPCR ligands, and mutants that avoid the operational inconsistency displayed between \[^{IVIV}D_2\]R and \[^{Gprot}D_2\]R would prove valuable for further understanding of functional selectivity. The conserved residue A3.53 (A135) was mutated into all 19 possible amino acids (Table I, Figure 4) because it conferred remarkable functional selectivity properties. Basic residues lost ~50% of their G protein activity, while still retaining complete, and slightly more potent β-arrestin activity (Figures 8A and 8B, respectively). In contrast, acidic substitutions ablated activity at both pathways. Furthermore, substitution with a bulky polar residue (tyrosine) yielded a balanced reduction in both G protein and β-arrestin 2 activity to roughly 75% (Figure 8C and 8D, \[^{1.5}D_2\]R) and substitution with a bulky nonpolar residue (phenylalanine) yielded a balanced 50% reduction (Figure 8E and 8F, \[^{2}D_2\]R). These unique partial agonist mutants were combined with one residue substitution from \[^{Gprot}D_2\]R (L125N) or \[^{βarr}D_2\]R (M140D) to confer partial biased agonism for each pathway.
Figure 8: Receptor control of partial agonism at D₃R. (A) G protein activity as determined by inhibition of isoproterenol-induced cAMP accumulation of basic and acidic residue substitutions at A135 (A3.53) and (B) β-arrestin 2 recruitment. (C) G protein activity of bulky polar substitution (tyrosine) is roughly 75% (τ/1.5) of [WT]D₃R (dotted line) and (D) β-arrestin 2 recruitment is similarly reduced. When combined with one residue from [Gprot]D₃R (L125N) or [βarr]D₃R (M140D) these mutants display partial biased agonism for their respective retained pathways. (E) G protein and (F) β-arrestin 2 recruitment of an even more reduced (50%; τ/2) parital agonism induced by substitution of A135 for a bulky nonpolar residue (phenylalanine). Similarly combined with L125N or M140D to generate weak partial biased agonism in response to full agonists. All data presented with SEM from n=3-5 independent experiments.
3.3 Agonist texture reveals novel modes of functional selectivity

A unique G protein-biased mutant (termed $[\text{Gprot4PM}]D_2R$) displayed remarkable retention of G protein activity and loss of β-arrestin 2 recruitment when the reference agonist quinpirole was used to probe activity (Figure 9A and 9B, respectively). In contrast, when the endogenous ligand DA was used, the G protein activity was unchanged, but the β-arrestin activity was ~50% of $[\text{WT}]D_2R$ (Figure 9A and 9B, Table V). To again assess the β-arrestin recruitment capacity, GRK2 overexpression rescued $[\text{Gprot4PM}]D_2R$ for both DA and quinpirole to almost the same extent of $[\text{WT}]D_2R$ (Figure 9C, Table V). However, the recruitment of GRK2 by $[\text{Gprot4PM}]D_2R$ showed the same agonist selectivity between DA and quinpirole (Figure 9D) as observed with β-arrestin 2. These data demonstrate the concept of agonist texture (173), which is the concept that full agonists induce distinct receptor activation states and conformations.
Figure 9: A unique G protein biased mutant demonstrates agonist texture. (A) Dopamine (DA) and quinpirole equivalently inhibit cAMP production, which is equivalent to [WT]D2R for [Gprot4PM]D2R (T69F Y133L Y209N A372S). (B) [Gprot4PM]D2R has roughly 50% efficacy in response to DA but not quinpirole for β-arrestin 2 recruitment. (C) GRK2 overexpression rescues both DA and quinpirole β-arrestin 2 recruitment activity nearly to [WT]D2R levels (dotted line, from Figure 7B). (D) GRK2 recruitment as determined by BRET (where GRK2 is tagged with YFP) shows the same ligand discrepancy as β-arrestin 2. All data are presented with SEM from n=3 independent experiments, with statistical significance calculated in Table V.

[Gprot]D2R and [βarr]D2R display unprecedented separation of signal in response to DA (Figure 6) and diverse D2R agonists and antagonists were used to probe the extent of agonist texture between the mutants at G protein and β-arrestin 2 activation. Each agonist tested at cAMP inhibition was effectively equivalent for [WT]D2R and [Gprot]D2R while being severely disrupted for [βarr]D2R and [D80A]D2R (Figure 10A, 10C
and 10E, Table V). In contrast, [βarr]D2R and [WT]D2R were active at β-arrestin 2 recruitment and deficient with [Gprot]D2R and [D80A]D2R (Figure 10B, 10D and 10F, Table V). Similarly, the well characterized antagonist raclopride, typical antipsychotic haloperidol, and atypical antipsychotic aripiprazole inhibited DA-induced cAMP reduction for [Gprot]D2R and [WT]D2R (Figure 10G, 10I, and 10K, Table V), and β-arrestin 2 recruitment for [βarr]D2R and [WT]D2R (Figure 10H, 10J and 10L, Table V). These diverse D2R ligands behave as expected for each assay and provide evidence that the complex activation states of [Gprot]D2R and [βarr]D2R remain intact, as opposed to [Gprot4PM]D2R, which has lost responsiveness to quinpirole at β-arrestin 2 recruitment selectively.
Figure 10: Agonists and antagonists with diverse pharmacophores elicit predictable responses at \( [G_{\text{prot}}]D_2R \) and \( [\beta_{\text{arr}}]D_2R \). The D\(_2\)R agonists quinpirole, apomorphine, and NPA were tested for G protein activity (A,C,E, respectively) and \( \beta \)-arrestin 2 recruitment (B,D,F, respectively). For each agonist, \( [G_{\text{prot}}]D_2R \) showed a response similar to \( [\text{WT}]D_2R \) at G protein activation and more similar to \( [D80A]D_2R \) for \( \beta \)-arrestin recruitment, while \( [\beta_{\text{arr}}]D_2R \) was not active at the G protein pathway but retained activity at the \( \beta \)-arrestin pathway. The antagonists raclopride (G,H) haloperidol (I,J) and partial antagonist aripiprazole (K,L) were able to block DA elicited D\(_2\)R activation at the G protein pathway (G,I,K, respectively) for \( [G_{\text{prot}}]D_2R \) and \( [\text{WT}]D_2R \) to the same extent, while \( [D80A]D_2R \) and \( [\beta_{\text{arr}}]D_2R \) had no effect to inhibit. In contrast, these antagonists block DA elicited \( \beta \)-arrestin 2 recruitment (H,J,L, respectively) for \( [\beta_{\text{arr}}]D_2R \) and \( [\text{WT}]D_2R \). All data are presented with SEM from \( n=3-4 \) independent experiments, with statistical significance calculated in Table V.

3.4 The status of receptor interacting partners in extremely biased mutant D\(_2\)Rs

The final determinant of functional selectivity: receptor interacting proteins and allosteric modulators, were assessed at \( [G_{\text{prot}}]D_2R \) and \( [\beta_{\text{arr}}]D_2R \) while being compared to positive and negative controls (\( [\text{WT}]D_2R \) and \( [D80A]D_2R \), respectively). The interaction of each receptor with other components of the desensitization machinery mirrored the recruitment of \( \beta \)-arrestin 2. GRK2 (Figure 11A) and \( \beta \)-arrestin 1 (Figure 11B) showed a similar slight potentiation and loss of efficacy at \( [\beta_{\text{arr}}]D_2R \) when compared to \( [\text{WT}]D_2R \), while \( [G_{\text{prot}}]D_2R \) was severely deficient (Table VI). To test whether the mutagenesis induced loss of function at G protein and \( \beta \)-arrestin achieved with \( [G_{\text{prot}}]D_2R \) and \( [\beta_{\text{arr}}]D_2R \) could potentially have induced aberrant activation of normally inactive receptor interacting proteins, major GPCR signaling avenues (\( G_{\alpha_S} \) and \( G_{\alpha_Q} \)) were assessed and neither were activated by any D\(_2\)R mutant (Figure 11C and 11D, respectively, Table VI).
Figure 11: Interacting partners and allosteric D_2R determinants of functional selectivity. (A) GRK2 and (B) β-arrestin 1 recruitment as assessed by BRET show a similar profile as β-arrestin 2: [βarr]D_2R recruits normally, while [Gprot]D_2R is severely deficient. (C) Each D_2R construct was expressed in HEK 293T cells and assessed for its ability to stimulate cAMP in response to DA. Stimulation of endogenous receptor by isoproterenol was used as a control response. (D) G_{aq} mediated Ca^{2+} flux, as measured by the aequorin luminescence assay, is not stimulated by [WT]D_2R, [Gprot]D_2R, [βarr]D_2R or [D80A]D_2R, compared to AngII induced Ca^{2+} flux induced by transient expression of AT_{1A}R. (E) B_{MAX} was determined by binding, while luciferase-tagged receptors provided a B_{MAX}-independent measure of receptor number. In this assay, the responsiveness to salt is retained for all mutants (except [D80A]D_2R). All data are presented with SEM from n=3-4 independent experiments, with statistical significance calculated in Table VI.
The effect of the GPCR allosteric modulator sodium has been previously shown to inhibit dopamine binding to D₂R (107) and has been proposed to function as an efficacy switch for receptor bias (174). When sodium is removed from binding buffer this results in a 50% reduction in $B_{MAX}$. To assess sodium dynamics in D₂R, radioligand binding was used to determine $B_{MAX}$ with and without sodium on the Renilla luciferase tagged D₂R constructs (75). The relative receptor amounts were normalized to luminescence output to provide an independent readout of receptor number. This assay yielded a 50% reduction in $[\text{WT}] \text{D}_2\text{R}$ and $[\text{βarr}] \text{D}_2\text{R}$ apparent $B_{MAX}$ (Figure 11E) and $[\text{Gprot}] \text{D}_2\text{R}$ also showed a sodium dependent reduction, although $[\text{Gprot}] \text{D}_2\text{R}$ expresses lower than $[\text{WT}] \text{D}_2\text{R}$ as previously described (Figure 6). $[\text{D80A}] \text{D}_2\text{R}$ $B_{MAX}$ did not change with sodium, validating the experiment as $[\text{D80A}] \text{D}_2\text{R}$ is mutated at the presumed site of sodium interaction and has been previously shown to not bind sodium (107).
4. The physiological relevance of D$_2$R functional selectivity

The relationship between GPCR G protein- and β-arrestin-dependent signaling is complex. G protein-mediated signaling is rapid and transient and engagement of β-arrestin inhibits the G protein pathways. In addition, formation of the GPCR/β-arrestin complex normally depends upon phosphorylation of the receptor (61). Moreover, G proteins and β-arrestins can engage the same pathway, but with distinct cellular consequences, as detailed in Chapter 1. As discussed, the physiological relevance of functional selectivity is profound for therapeutic applications, yet remains largely undefined. Thus, the $[^{Gprot}]$D$_2$R and $[^{βarr}]$D$_2$R mutants were applied to complex D$_2$R signaling systems to unravel functional selectivity.

4.1 MAP kinase signaling in $[^{Gprot}]$D$_2$R and $[^{βarr}]$D$_2$R

One well-documented example of G protein and β-arrestin control of a common signaling pathway in D$_2$R is the MAP kinase cascade (175). To address this relationship, two related transcriptional reporters for MAP kinase signaling were transfected along with the mutated D$_2$Rs in HEK 293T cells. As shown in Figure 12A and 12B a dose-dependent DA activation of MAP kinase transcription was observed in $[^{WT}]$D$_2$R and $[^{Gprot}]$D$_2$R but absent in $[^{βarr}]$D$_2$R and $^{[D80A]}$D$_2$R. Probing ERK phosphorylation through western blot analysis revealed activation by $[^{βarr}]$D$_2$R when compared to $^{[D80A]}$D$_2$R or untransfected cells only when β-arrestin 2 is overexpressed (Figure 12C and 12D). In contrast, $[^{Gprot}]$D$_2$R activated ERK phosphorylation regardless of β-arrestin expression,
when compared to $^{[D80A]}D_2R$. This indicates that $D_2R$ activates canonical MAP kinase activity through G proteins, while β-arrestin may produce non-canonical ERK activity under conditions of enhanced β-arrestin or kinase expression, as observed in other GPCR systems (176).

**Figure 12: Assessment of MAP Kinase activity at $D_2R$.** (A) SRF and (B) SRE MAP kinase transcriptional promoter mediated expression of luciferase (SEM, n=5-6 done in triplicate). (C) Traditional western blot analysis of ERK (*p<0.05 Newman-Keuls posthoc when compared to $^{[D80A]}D_2R$ or untransfected after one-way ANOVA p<0.05, SEM, n=3-6) with and without β-arrestin 2 overexpression. (D) Representative blot for the data presented in (C).
4.2 \([Gprot]D_2R\) and \([βarr]D_2R\) are biologically active and display functional differences

The biological activity and functional properties of \([Gprot]D_2R\) and \([βarr]D_2R\) were tested with a virally mediated in vivo overexpression approach. Adeno-associated viral (AAV) vectors containing a double-floxed inverted open reading frame (DIO) of each HA-tagged D\(_2\)R transgene driven by the housekeeping gene EF1\(_α\) promoter (177) were synthesized (Figure 13A). Each D\(_2\)R construct was injected bilaterally into the dorsal striatum (caudate putamen) and the ventral striatum (nucleus accumbens) (Figure 13B). Extent of viral transduction was assessed by staining for the HA epitope tag on the N-terminus of D\(_2\)Rs (Figure 13C). Neuronal specificity of expression was achieved using the Adora2a-Cre mouse line, which selectively expresses Cre in D\(_2\)R-expressing medium spiny neurons (MSNs), but not presynaptic DA projection cells (178), and these Adora2a-Cre mice were also crossed to a mouse strain with β-arrestin 2 floxed to allow for specific deletion of β-arrestin 2 in indirect pathway MSNs. \([WT]D_2R\), \([Gprot]D_2R\), \([βarr]D_2R\) or \([D80A]D_2R\) yielded a two- to four-fold increase in striatal D\(_2\)R expression as measured by radioligand binding (Figure 13D, expression in Adora2a-Cre and Figure 13E, expression when β-arrestin 2 is genetically deleted). Overexpression of the \([WT]D_2R\) lead to a ~1.5 fold potentiation in the amphetamine induced locomotor response (Figure 13F). \([βarr]D_2R\) overexpression lead to a similar potentiation while the \([Gprot]D_2R\) overexpression was much less effective. However, in order to demonstrate construct validity, the same experimental design was carried out in Adora2a-Cre:β-arrestin 2\(^{FLOX/FLOX}\) mice. As
shown in Figure 13G, while overexpression of [Gprot]D2R produced a slight increase in the amphetamine response, the robust increase previously observed with both [WT]D2R and [βarr]D2R was completely absent. Note the differences in baseline responses to amphetamine between Figure 13F and 13G as these mice are on a different background. This suggests that the enhanced amphetamine response of [βarr]D2R is dependent upon β-arrestin 2. These findings demonstrate that the functionally selective engineered D2R mutants are biologically active in vivo and mediate distinct functions.
Figure 13: The physiological relevance of D2R functional selectivity. (A) Viral transgene packaged into AAV, which allowed for Cre-dependent expression of D2R through a double-floxed inverted open reading frame (DIO). (B) 0.75 µL of virus was injected bilaterally into the dorsal and ventral striatum with each injection site indicated by the red dots, and a total of 3 µL was injected into the striatum of each mouse. CPu: caudate putamen; AcbC: nucleus accumbens, core; AcbSh: nucleus accumbens, shell. (C) Representative staining pattern of the N-terminal HA tagged D2R shows transduction of a majority of the dorsal striatum and at least 50% of the ventral striatum with variable transduction in the olfactory tubercle. Radioligand binding revealed a two- to four-fold overexpression of each receptor as determined from membranes prepared from striatal dissections from Adora2a-Cre (D) and Adora2a-Cre::β-arrestin 2LOX/FLOX (E) mice (*p<0.05 Newman-Keuls posthoc when compared to Cre (-) controls after one-way ANOVA p<0.05, SEM, n=4-6). (F) Potentiation of amphetamine-induced locomotion in mice when D2R is overexpressed (*p<0.05 bonferroni posthoc when compared to [D80A]D2R after repeated measures two-way ANOVA p<0.05 for receptor expression type SEM, n=11-12, color coded for receptor type). (G) The amphetamine response potentiation of [WT]D2R and [βarr]D2R is abolished when β-arrestin 2 is genetically deleted from D2R-expressing medium spiny neurons (*p<0.05 bonferroni posthoc when compared to [D80A]D2R after repeated measures two-way ANOVA p<0.05 for receptor expression type SEM, n=8-13).
5. Application of functional selectivity principles to diverse receptor systems

Functional selectivity allows for the design of more effective therapeutics at previously identified receptor targets. D₂R serves as one of the prototypical GPCRs to exhibit functional selectivity. However, many principles of functional selectivity and D₂R contextual activation remain to be fully elucidated. The approaches presented in previous chapters will be applied here to begin to address functional selectivity in diverse receptors types and more complex D₂R neuronal systems.

5.1 Evolutionary Action at related but diverse GPCRs

Evolutionary Trace (ET) is a powerful method to predict residues that will generate phenotypes when guided by the robust predictive value of Evolutionary Action. \([\text{Gprot}]\text{D}_2\text{R}\) and \([\beta\text{arr}]\text{D}_2\text{R}\) were generated through iterative rounds of mutagenesis screening for specific phenotypes (retention and loss of G protein or β-arrestin signaling). Therefore, the residues determined to confer functional selectivity to D₂R could be used to generate functionally selective mutants in other GPCRs, because ET predicted residues are conserved across all known sequences of Rhodopsin-like GPCRs. In order to test the feasibility of this approach, GPCRs with diverse genetic and signaling profiles were selected (Figure 14).
Figure 14: Evolutionary Trace at pharmacologically and genetically diverse GPCRs for the generation of functionally selective mutants. hAGTR1 (human angiotensin receptor), hAPLNR (human apelin receptor), hM3 (human muscarinic type 3 receptor), hADRB2 (human β-adrenergic receptor, type 2A), hDRD1 (human dopamine D1 receptor), hHTR1A (human serotonin type 1A receptor), and mDRD2 (mouse D2R) GPCRs were chosen based on genetic diversity (Tree calculated from % identity), as well as G protein (Gαq, Gαi or Gαs) and β-arrestin coupling (Class A: transient β-arrestin interaction, Class B: robust β-arrestin interaction) profiles. The alignment of the twenty residues surrounding the DRY motif was the basis of the tree presented, and [Gprot]D2R is color coded as green residues while [βarr]D2R is red.

The analysis of these diverse receptors shows that L3.43 and Y3.51 do not vary, while A3.53 only varies with the M3R (hM3) to serine while M3.58 varies greatly among these GPCRs. While each particular residue was chosen based on the residues identified from the generation of [Gprot]D2R and [βarr]D2R, the same substitutions would not necessarily result in the same profile of functional selectivity. This is due to two important features in the D2R mutagenesis program: 1) the impact of mutations on phenotype (harshness) was predicted based on conservation relative to D2R, and 2) the degree of harshness was determined by retention of G protein or β-arrestin activity at D2R. Thus, Evolutionary Action was once again used to determine those residues that would most likely produce a phenotype, and different substitutions with varying

<table>
<thead>
<tr>
<th>Tree</th>
<th>Receptor</th>
<th>G protein</th>
<th>β-arrestin</th>
<th>Residues</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAGTR1</td>
<td>Gaq</td>
<td>Class B</td>
<td>119-134</td>
<td>LTCLSIDRYLAIVHPM</td>
<td></td>
</tr>
<tr>
<td>hAPLNR</td>
<td>Gaq</td>
<td>Class B</td>
<td>120-135</td>
<td>LTGLSFDRYLAIVRPV</td>
<td></td>
</tr>
<tr>
<td>hM3</td>
<td>Gaq</td>
<td>Class A</td>
<td>159-174</td>
<td>LVLISFDRYFSSITRP</td>
<td></td>
</tr>
<tr>
<td>hADRB2</td>
<td>Gas</td>
<td>Class A</td>
<td>124-139</td>
<td>LCIAVDRYFAITSPF</td>
<td></td>
</tr>
<tr>
<td>hDRD1</td>
<td>Gas</td>
<td>Class A</td>
<td>114-129</td>
<td>LCVISVDRYWAISSPF</td>
<td></td>
</tr>
<tr>
<td>hHTR1A</td>
<td>Gai</td>
<td>Class A</td>
<td>127-142</td>
<td>LCAIALDRYWAITDPI</td>
<td></td>
</tr>
<tr>
<td>mDRD2</td>
<td>Gai</td>
<td>Class A</td>
<td>125-140</td>
<td>LCAISIDRTAVAMP</td>
<td></td>
</tr>
</tbody>
</table>

63
degrees of predicted harshness were calculated (Table II). The EA predictions yielded
the same predicted substitutions for each receptor at L3.43 and Y3.51 because of the
remarkable conservation of these residues across GPCRs.

Table II: Evolutionary Action predicted functional selectivity phenotype
mutations in diverse GPCRs. Receptors presented in Figure 14 are predicted for
mutations at the [Gprot]D2R (L3.42 and Y3.51) and [βarr]D2R (A3.53 and M3.58) residues.
Predicted harshness on phenotype is displayed from left to right.

<table>
<thead>
<tr>
<th>Residue (WT)</th>
<th>Receptor</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3.43</td>
<td>hAGTR1</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>hAPLNR</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>hM3</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>hADRB2</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>hDRD1</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>hHTR1A</td>
<td>W</td>
</tr>
<tr>
<td>Y3.51</td>
<td>hAGTR1</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hAPLNR</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hM3</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hADRB2</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hDRD1</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hHTR1A</td>
<td>M</td>
</tr>
<tr>
<td>A3.53</td>
<td>hAGTR1</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>hAPLNR</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>hM3</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>hADRB2</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>hDRD1</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>hHTR1A</td>
<td>S</td>
</tr>
<tr>
<td>M3.58</td>
<td>hAGTR1</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>hAPLNR</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hM3</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>hADRB2</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>hDRD1</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>hHTR1A</td>
<td>F</td>
</tr>
</tbody>
</table>

5.2 A roadmap to universal functional separation

Mutating the residues from [Gprot]D2R and [βarr]D2R in other GPCRs represents a
means by which functionally selective mutant receptors that retain most of the normal
functions of the wild type receptors might be generated. Some of the mutations presented in Table II were generated and tested for their G protein and β-arrestin activities (Figure 15).

**Figure 15:** Achieving functional selectivity in GPCRs with diverse pharmacological and genetic profiles. (A) Cytosolic influx of Ca\(^{2+}\) as determined by the aequorin assay shows AT1AR M3.53G mutation completely abolishes G protein activity, while (B) β-arrestin 2 recruitment as determined by BRET remains intact. For the apelin...
receptor (APLNR), Gαi activation as determined by inhibition of isoproterenol induced cAMP increase (C) is intact for Y3.51S but slightly decreased for A3.53S. In contrast, β-arrestin 2 recruitment (D) is intact for A3.53S but not Y3.51S. Finally, for D2R, Y3.51S showed partial agonist activity at Gαs mediated cAMP accumulation as measured by GloSensor (E) and A3.53S showed full activity. While at β-arrestin 2 recruitment (B), Y3.51S had no activity while A3.53S showed partial activity, which means that both mutations display bias for the G protein pathway.

Many of the mutations proposed in Table II were generated and tested. However, many of these mutations did not produce relevant signaling profiles (loss of activity at both pathways) or were not profiled at each signaling pathway. Nevertheless, the data presented in Figure 15 shows striking patterns of bias achieved. First, β-arrestin bias was achieved at AT1AR with high pharmacological fidelity from only a single harsh point mutation (M3.58G, Figure 15A and 15B). As shown in Table I, the residue change in βarrD2R (M3.58D) was also predicted to produce a harsh phenotype. However, this single point mutation did not completely abolish G protein signaling (Figure 4). Surprisingly, this same residue change was used to generated a biased ghrelin receptor (179). For the apelin receptor, some residues that produced bias at D2R produced the same quality of bias, albeit with different changes. The mutation at Y3.51 to the mild predicted phenotype Ser (as opposed to Lys in GprotD2R) caused a ~50% reduction in β-arrestin recruitment (Figure 15D) and no appreciable shift in G protein activity (Figure 15C). In contrast, A3.53 when mutated to the weak predicted phenotype Ser (as opposed to Arg in βarrD2R) yielded a slight decrease in G protein activity (Figure 15C) and no change in β-arrestin 2 recruitment (Figure 15D). Thus, these mutations produce weak G
protein and β-arrestin bias at the apelin receptor, respectively. Finally, at D1R bias was achieved in unexpected ways. The Y3.51S mutation generated a ~50% decrease at G protein activity (Figure 15E) and an almost complete ablation of β-arrestin activity (Figure 15F). In contrast, A3.53S (a critical residue in generating both βarrD2R and τ2/D2R) yielded no change in G protein activity (Figure 15E) and a ~50% reduction in β-arrestin activity (Figure 15F), which surprisingly indicates that this mutation is also G protein biased. However, the quality of the G protein bias is very different, a phenomenon that is explored in Chapter 3 with the comparison of [IYIV]D2R and [Gprot]D2R in Figure 7 as well as the generation of partial biased D2R mutants in Figure 8.

Figure 15 demonstrates the feasibility of generating biased GPCRs in diverse systems, and Table II presents a starting point that is likely to generate fruitful data without expending vast resources. In order to achieve robust separation of signal at other GPCRs more complex experimental manipulations would be necessary. This could include strategies such as generating all 19 permutations at a particular likely candidate residue or combining mutations in unexpected combinations (such as D1R A3.53S with Y3.51S, a combination that should produce G protein bias but could not be predicted from ET/EA or the D2R data sets). More complete data sets produced for even more diverse GPCRs will allow for deciphering universal patterns of functional activity and be incredibly powerful in understanding and manipulating GPCR signal transduction.
5.3 $D_2R$ neuronal subtype expression dictates functional selectivity

The overexpression strategy presented in Chapter 5 yielded direct evidence that the activities of both $[\text{Gprot}]D_2R$ and $[\beta\text{arr}]D_2R$ are physiologically relevant in indirect pathway medium spiny neurons. However, as presented in Figure 2, the pleiotropic actions of $D_2R$ can be attributed to both the biochemical diversity and heterogeneous neuronal expression in the central nervous system. Therefore, the actions of $D_2R$ were assessed using the $D_2R^{\text{FLOX/FLOX}}$ mouse which allows for cell-type specific deletion of $D_2R$ and viral reexpression of biased $D_2R$ mutants.

![Figure 16: Dissection of diverse $D_2R$-expressing neuronal subpopulations.](image)

$D_2R^{\text{FLOX/FLOX}}::\text{Adora2A-Cre}$ induces genetic deletion of $D_2R$ and reexpression (using the same injection scheme in Figure 13) of each mutant $D_2R$. Assessment of (A) basal locomotor activity at the beginning of the night cycle (lights off) and (B) motor coordination with the beam walking assay. (C) $D_2R^{\text{FLOX/FLOX}}::\text{ChAT-Cre}$ has $D_2R$ deleted only from cholinergic interneurons which causes a major deficiency in the amphetamine locomotor response but not the basal/habituation locomotor phase.

The $D_2R^{\text{FLOX/FLOX}}$ mouse was crossed to the same Adora2A-Cre presented in Chapter 4, and underwent the same viral injection surgery in order to achieve $D_2R$ genetic replacement (deletion since birth and reexpression in adulthood in indirect
Deletion of D\(_2\)R from these indirect pathway neurons has previously been shown to cause loss of basal locomotion and motor coordination (131). The reexpression of [WT]D\(_2\)R rescued both basal locomotor activity (Figure 16A) as well as motor coordination (Figure 16B) when compared to [D80A]D\(_2\)R. Surprisingly, the biased mutants showed striking separation of function, especially when compared to the amphetamine response seen in the overexpression strategy (Figure 13). In contrast to those observations, [Gprot]D\(_2\)R mirrored the [WT]D\(_2\)R for basal locomotion, while [βarr]D\(_2\)R was not different from [D80A]D\(_2\)R (Figure 16A). Additionally, neither receptor fully rescued motor coordination but neither receptor was completely inactive at rescuing motor coordination (Figure 16B). Taken together, these data demonstrate an unpredicted role for G proteins in D\(_2\)R’s control of locomotion in the indirect pathway and point toward a component effect for G proteins and β-arrestins in the control of coordination in these same neurons.

Finally, as discussed previously, D\(_2\)R functions as a postsynaptic receptor in GABAergic MSNs, cholinergic interneurons as well as cortical pyramidal and interneurons (180). Therefore, the D\(_2\)R\(^{FLOX/FLOX}::\text{ChAT-Cre}\) mouse was generated to delete D\(_2\)R from the population of cholinergic interneurons (which are predominantly in the striatum). This deletion caused a remarkable effect that is unique from any other D\(_2\)R genetic model: a reduction in the amphetamine response (Figure 16C) without an appreciable effect on basal locomotion. This is a distinct and unappreciated role for D\(_2\)R-
expressing interneurons that has profound implications for functional selectivity based D2R pharmacotherapies.
6. Discussion

DA is an important regulator of both CNS and peripheral physiological homeostasis. Disruptions in the function of DA have been associated with schizophrenia, depression, mania, attention deficit disorders, drug abuse and Parkinson’s disease in the CNS and hypertension and prolactinemia in the periphery (181). DA exerts its function through two major GPCRs: D2R and D1R (as well as the D1R-like D5R and D2R-like D3R and D4R receptors which are expressed at lower levels). The results described here provide a functional template to begin to investigate the pharmacological, biochemical and neuronally selective actions of D2R and how to apply these principals to other receptors. Precise molecular control was achieved by engineering D2Rs specifically designed to interact with either G proteins or β-arrestins and these receptors can be reconstituted in cell culture or in specific neuronal populations in vivo.

Over the last several years, state of the art optogenetic (182) and pharmacogenetic (183) approaches have been developed to map brain pathways and cellular functions of neuronal populations. However, these approaches are not amenable to the elucidation of molecular mechanisms because they are not designed to manipulate the specific biochemical mechanisms of an endogenous ligand through its cognate receptor. Additionally, optogenetic or pharmacogenetic control of intracellular signaling cascades, such as G proteins (184) or ERK (185), do not allow for the interrogation of
endogenous ligand dynamic changes or the impact of therapeutics to the system.

Understanding the biology of D2R will require determinants such as the contextual influence of phasic and tonic DA release (149) and the monitoring of therapeutics, such as antipsychotics. Although less widely applicable, functionally selective mutant GPCRs are a desirable alternative as they resolve many of the limitations of the more general approaches.

Previously, biochemical studies in mice carrying complete deletion of β-arrestin 2 (67) or cell type specific genetic deletion of GSK3β (68) have provided evidence for the importance of the β-arrestin 2-mediated D2R signaling pathway in the actions of DA. However, evidence from such studies is limited by the fact that β-arrestin 2 interacts with multiple GPCRs and GSK3β is a signaling hub downstream of multiple signaling networks, including both G proteins (186) and β-arrestin 2 (67). The current in vivo approach more specifically targets D2R and these pathway specific mutant D2Rs were developed to begin to elucidate the contribution of individual pathways to physiological and pharmacological DA responses. Biased mutants in other GPCRs like the β2-adrenergic (T69F Y133G Y209A) and angiotensin 1A (DRY 131-133 AAY) receptors (162, 187) generated unstable proteins when engineered into D2R. Additionally, several D2R mutants have also been generated and shown to affect β-arrestin and GPCR kinase interactions (64, 71), post-endocytic trafficking (62), desensitization (63), and resensitization (188). While these various D2R mutants have informed various aspects of
function and regulation of D2Rs, consideration of some of these mutants for the work described here did not fulfill all necessary inclusion criteria.

GPCR bias is operationally defined by the degree of engagement of a given signaling pathway. It is clear that [IYIV]D2R and [Gprot]D2R both share G protein bias, however the quality of bias differs, depending on their operational definition. For example, in some cases it may be beneficial to retain all of the G protein activity, while in others it may be crucial to abolish all β-arrestin recruitment at the sacrifice of some G protein activity. In fact, [IYIV]D2R was mutated to contain fewer alanine substitutions which resulted in full G protein activity and partial β-arrestin activity (170). Nevertheless, [IYIV]D2R and [Gprot]D2R were used to dissect D2R-mediated ERK phosphorylation (Figure 12) and (71) and both mutants yielded a similar conclusion: that D2R-mediated ERK phosphorylation is largely G protein mediated.

Operational consistency allows for meaningful conclusions about D2R signaling pathways to be drawn. The partial agonist activity of aripiprazole has raised the possibility that complete blockade of D2R is not necessary to impart antipsychotic efficacy (189). This partial agonism allows for effective targeting of G protein or β-arrestin pathways (102). However, the causal relationship of partial agonism and biased partial agonism (as opposed to partial antagonism) has not been explored. The [τ/2]D2R mutants described here are tools that allow for operationally defined agonism that is consistent, robust and specific. Coupled with viral expression technologies and
neuronal-specific Cre expressing lines, it will now be possible to test various hypotheses of D₃R partial agonism in intact biological systems and preclinical disease models.

While receptor manipulation is desirable to demonstrate causal relationships, biased ligands provide valuable insight and are a more reasonable avenue toward therapy development. However, precise details and principles governing ligand action remain elusive. Here, the phenomenon of agonist texture (173) is demonstrated with [Gprot4PM]D₃R. As previously observed, full agonists can stabilize different receptor activation states that have functional consequences (173). Therefore, functional selectivity could occur from the loss of function at one pathway or it could be thought of as a gain of new receptor activity. This phenomenon represents a valuable conceptual framework for a fundamental property of receptor activation that is relevant to functional selectivity.

Intracellular signal transduction proteins are key elements in dictating bias. Their interactions with receptors dictate agonist efficacy (117) and targeting their activation with biased agonists is an avenue by which already validated receptor targets can be leveraged for improved therapies. Here, the related desensitization allosteric modulators (β-arrestin 1, β-arrestin 2, and GRK2, Figures 6B, 11A and 11B) were shown to fall into a common activation family using [βarr]D₃R. This indicates that more detailed (but less well characterized) elements of D₃R’s β-arrestin signaling arm remain intact for [βarr]D₃R, such as barcoded phosphorylation patterns (61) and other posttranslational modifications
Additionally, related allosteric modulators (different G proteins, Figure 11C and 11D) remained inactive at each mutant receptor, which indicates that none of the mutants have a gross abnormal gain of function.

Interactions with small molecule allosteric modulators are also exciting avenues by which functional selectivity can be modulated. Sodium, an intracellular GPCR allosteric modulator, remains intact in both $[^{Gprot}]D_2R$ and $[^{βarr}]D_2R$, indicating that both G protein and β-arrestin activation require dynamic sodium regulation. However, allosteric biased ligands may confer functional selectivity by exploiting the recently solved extracellular vestibule (97) to generate noncompetitive negative or positive allosteric modulators (191) or bitopic ligands (192).

The pharmacological fidelity (trafficking, ligand binding and signal transduction) of $[^{Gprot}]D_2R$ and $[^{βarr}]D_2R$ revealed robust and specific engagement of each pathway. Through sequential iterations (Figure 4) each mutation converged on transmembrane domain three (TM3), an alpha helix critical for the transmission of conformational changes from ligand binding to signaling molecules (193). These changes in signal transduction allowed for the elucidation of complex signaling paradigms. MAP kinase cascades have previously been shown to be activated downstream of G proteins and β-arrestins (25, 26) but as shown here, $[^{Gprot}]D_2R$ is responsible for a major component of the ERK signaling cascade with the normal complement of kinases and β-arrestins present in HEK 293T cells. However, overexpression of β-arrestin 2 revealed the ability
of $[^{\beta\text{arr}}]D_2R$ to couple to ERK. It is interesting to note that while $[^{G\text{prot}}]D_2R$ did not significantly enhance the transcriptional activity, there was a small potentiation in pERK observed when compared to $[^{\text{WT}}]D_2R$. Taken together, these data indicate that receptor transducer elements, such as MAP kinases, may also exhibit functional selectivity (in this case the activating phosphorylation induced by $\beta$-arrestin is functionally distinct from the G protein mediated transcriptional activity).

Finally, to assess the \textit{in vivo} function of the engineered receptors a neuronally selective overexpression approach was used, which admittedly carries the caveat of assessing function in the presence of the normal signaling of endogenous receptors. Despite this limitation, expression of mutant D$_2$Rs in D$_2$R+MSNs (indirect pathway, Adora2A+) was able to revealed marked differences in their ability to affect responses to the psychotropic drug amphetamine. Interestingly, $[^{\beta\text{arr}}]D_2R$ was more effective at enhancing the amphetamine response than $[^{G\text{prot}}]D_2R$. Although the extent of the separation was surprising, it is consistent with previous genetic manipulation studies, which have predicted an important role for the D$_2$/$\beta$-arrestin 2 pathway \textit{in vivo} as genetic deletion of $\beta$-arrestin 2 has been shown to decrease the locomotor response to amphetamine (67, 123) Interestingly, $[^{G\text{prot}}]D_2R$ only slightly potentiated the amphetamine response and this trend was enhanced by genetic deletion of $\beta$-arrestin 2 in D$_2$R expressing MSNs. In contrast, $[^{\text{WT}}]D_2R$ and $[^{\beta\text{arr}}]D_2R$ lost their potentiation of the amphetamine response when $\beta$-arrestin 2 was deleted. These data demonstrate the
complexity of even basic GPCR signaling events, and should allow for insights into the
pleiotropic actions of the endogenous neurotransmitter.

In summary, functionally selective or biased signaling engineered GPCRs can display \textit{in vivo} biological activity and mediate distinct pharmacological responses. The robust separation of signal achieved with \([G\text{prot}]D_2R\) and \([\beta\text{arr}]D_2R\) will allow for direct elucidation of more complex functional selectivity principles when applied to diverse D\(_2\)R systems. These mutants differ from \([\text{WT}]D_2R\) by only two amino acids and yet have specific D\(_2\)R functions disrupted. Functional selectivity has considerable therapeutic potential but the molecular details have been obscured by the complexity of receptor activation. Furthermore, some signaling events can only be understood in the context of the \textit{in vivo} architecture (131). \([G\text{prot}]D_2R\) and \([\beta\text{arr}]D_2R\) are unique tools that should allow for a better understanding of the molecular, cellular and physiological actions of dopamine, as well as provide a template for the development of small molecules with therapeutic predictive value.

\textbf{6.1 Future Directions}

The overall goal of this dissertation was to disassemble and reassemble D\(_2\)R’s pleiotropic functions. This goal was necessitated by the unique challenges presented by GPCR signal transduction: a small molecule can permeate an entire organism yet still have specific actions. Thus, the concept of functional selectivity is a framework by which the nature of receptors can begin to be understood. While the present work
demonstrates how D₂R signals as a pleiotropic conduit of extracellular messages, there are many challenges that remain. Undertaking these challenges is crucial in developing a better understanding of the mechanisms of D₂R biology which allows for the design of more effective therapies.

GPCRs are the target of a majority of pharmaceuticals (194) therefore, the understanding of their unique capacity for pleiotropic signaling pathways through G proteins and β-arrestins represents an avenue by which novel pharmaceuticals could be developed for existing targets. Evolutionary Trace is an algorithm that is dependent upon sequence conservation and because GPCRs comprise 5% of the human genome (and more for other mammals) there is a rich set of sequences and now structures available to build precise predictions. In Chapters 3 and 5 the data set developed in Figure 4 was used to predict defined signaling mutants in D₂R and other GPCRs. These data show that more robust and specific mutants can be generated for virtually any GPCR, depending on the biological question. Furthermore, because these sequences are related by ET predictions they are comprised of only minor variations. For example [Gprot4PM]D₂R is comprised of mutations of residues that were used to generate the β2AR-TYY mutant, and [Gprot]D₂R, [βarr]D₂R, [τ/1.5]D₂R and [τ/2]D₂R are all variations of the same four amino acids (L3.43, Y4.51, A3.53, and M3.58). This feature of the mutations is important for disrupting specific actions in complex systems. For instance, if the effect of the strength of partial agonism at D₂R was tested by knocking in the [τ/1.5]D₂R and [τ/2]D₂R,
any phenotype could be attributed to the difference of a single hydroxyl group, the
difference between Tyr ($^{1.5}$D$_2$R) and Phe ($^{1.2}$D$_2$R).

Such rich translations from *in silico* and *in vitro* predictions to complex
physiological systems warrant a more rigorous mechanistic analysis of how these
residue changes induce bias. Some of the pioneering structural work in GPCRs have
allowed for incredible detail for G protein (161) and β-arrestin (167) coupling. While D$_2$R
has yet to be crystallized, the biased mutants are excellent candidates for understanding
how the receptor transmits G protein and β-arrestin activities. An alternative approach
would be to translate D$_2$R mutations to β2AR or rhodopsin and utilize the robust
crystallography protocols already developed for these mutants. This structural insight
could lead to more specific molecular dynamics simulations (98, 195) which in turn
could yield better predictions for more specific mutations, as the impact of changes
could be simulated.

Preclinical models of psychiatric and neurological disorders have offered
predictive and causal relationships for treatments in humans. The dopaminergic system
has provided a rich translation because the fundamental architecture of the basal ganglia
is similar between mice and humans, and the movement phenotypes are robust and
easily assessed. The viral overexpression (Chapter 4.2) and reexpression (Chapter 5.3)
strategies was designed to be iterative to adapt to any unforeseen caveats (such as
unexpected signaling profiles of the mutants in neurons as opposed to HEK cells).
However, this versatility is cumbersome when applied to more complex behavioral assays of the dopaminergic system, such as social interaction, anxiety, or assessment of positive and negative responses to stimuli. The complexity of D2R’s genetic and neuronal architecture make any simple solution difficult to reconcile. For instance, ectopic, Cre-dependent expression of D2R cDNA would lose splicing and receptor levels would be inherently inaccurate because of the use of exogenous promoters, while a knock-in approach would lose cell type specificity.

A knock-in would provide a top-down approach for studying these complex behavioral states. However, as demonstrated with the D2RFLOX/FLOX::ChAT-Cre data (Figure 16C) a unique response is generated that is distinct from the D2R-KO mice (131) as well as models of D2R deletion from indirect pathway neurons (123). Deletion of D2R from the interneurons only affects amphetamine-induced locomotion and not basal locomotion (whereas D2R-KO affects both responses). It should be noted that a viral reexpression strategy was not feasible with these mice, as ChAT-Cre is expressed in all cholinergic interneurons, but all cholinergic interneurons do not express D2R. This strategy produced ectopic D2R expression and generated uninterpretable data, most likely due to D2R expression in the caudal areas of the striatum and adjacent structures such as the amygdala. Therefore, an intersectional approach (such as D2RFlop::ChAT-Cre::D2RFLOX/FLOX injected with a Flp and Cre dependent viral construct expressing mutant D2R) would be necessary to dissect functional selectivity in interneuron control.
of amphetamine induced locomotion. This approach is feasible, albeit cumbersome and
time consuming.

All of these considerations have led to the development of a complex knock-in
strategy in which multiple recombination events are used to knock-in the mutant D2R
exons into the endogenous locus. Such an approach would ideally provide: 1) cell type
specific activation using previously characterized BAC-driven Cre lines (178) 2)
retention of D2R splicing because the endogenous locus and introns are intact, and 3)
time regulated activation using DOX/tTA transcriptional control (196) of expression of a
Flp recombinase (197). A strategy that satisfies all of these considerations is presented in
Figure 17. While it could be argued that this particular strategy is overly complex, the
approach is designed to allow for facile generation of mice (only two transgenes and one
injection of DOX is required) as well as the design of experiments that utilize pairwise
comparisons (before and after DOX treatment in one individual mouse). This important
feature allows for powerful conclusions to be drawn, for instance a test could be
designed in which the mouse is trained to receive a reward or interact with a social
partner and the effect of the β-arrestin signaling pathway could then be examined after
DOX treatment. However, with the complex design there are many points of failure,
such as inappropriate/inefficient splicing. To address this, differential fluorophores
monitor which receptor isoform is present, and radioligand binding experiments
performed on a mouse crossed to a ubiquitous Cre would ensure normal D:\R expression.

Figure 17: Potential inducible mouse knock-in construct design. This design relies upon expression of the DOX inducible tTA transcription factor and Flp recombinase from the introns of the endogenous DRD2 gene (polyadenylation and promoters abridged for simplicity). (A) tTA and the [WT]D\R exon are expressed in normal cells (restricted by the endogenous DRD2 expression pattern). (B) Mouse crossed to Cre expressing lines orients Flp recombinase Cerulean expression cassette that is not active until (B) addition of DOX drives Flp::P2A::GFP (cerulean or citrine), and Flp recombines [WT]D\R exon to [βarr]D\R exon (with cerulean or citrine serving as reporters of recombination).

6.2 Outlook and Impact

Functional selectivity is a complex process derived from the receptor’s function as a conduit of extracellular messages to redistribute the intracellular signaling components through the law of mass action. Here, this redistribution was manipulated, rigorously assessed, and applied to diverse functional systems. While many questions remain regarding the physiological impact of functional selectivity, many of the previous conundrums have been solved with this work. When this project began, it was not clear that any GPCR could retain full agonist activity at G protein activation while losing all of its β-arrestin activity (and of course, vice versa) through mutagenesis, biased ligands, or cellular manipulations. In preliminary experiments, the
administration of pertussis toxin completely abolished G protein activity, while also reducing β-arrestin activity by close to 50% (presumably due to inactivity of GRK2/3). The work presented here was a tremendous effort to generate clear evidence and demonstrate causal relationships for functional selectivity at D₂R. While much work remains to be done before these data can be applied to the design of novel therapeutics, it is clear that biased ligands based on functional selectivity principles have the potential to yield a more effective standard of care for dopamine related disorders.
7. Materials and Methods

7.1 Evolutionary Trace

Multiple rounds of ET-guided mutagenesis were conducted on D2R. Each round took advantage of enhancements to the Evolutionary Trace method and GPCR crystallography. The previously reported (162) β-adrenergic 2A receptor “TYY” served as a starting point for D2R mutagenesis. β2AR-TYY was previously shown to signal through β-arrestins but not G proteins. The first round targeted these homologous positions in D2R (T69, Y133, and Y209). Based on the initial results of “TYY” mutations, new targets were added based on ET importance and structural location. Substitutions for targeted positions were based on homology in the multiple sequence alignment. In order for D2R to be functional, mutations to cognate amino acids found in other GPCRs at the equivalent sequence position were selected.

Due to the variation in the GPCR loop regions, the transmembrane domains and loops were analyzed separately. The multiple sequence alignment of the transmembrane region was made up of 2512 Class A GPCRs. These sequences were gathered from GPCRDB, aligned, and filtered for the 195 gapless seven transmembrane helix residues. We used the updated pair interaction ET algorithm (piET) (155), which achieves greater accuracy by taking into account the residue contacts seen in a structure; here, the crystal structure of rhodopsin in complex with the C-terminus peptide of the endogenous G protein (160). The residues targeted for mutation were selected based on their
evolutionarily importance (top 5%) and proximity to the C-terminus peptide (within 12 Angstroms, the residues in DRY and NPXXY motifs being ignored). The Evolutionary Action algorithm (163) was used to identify substitutions with varying harshness.

An analysis specific to D2R was used to identify the key ET residues in the second intracellular loop region. The crystal structure of β2AR in complex with Gαs (161) was also used to narrow down to the crucial residues for G protein activation. The multiple sequence alignment for D2R entire sequence (including loops) was made of 66 homologues extracted from a BLAST analysis of the NCBI Reference Sequence database where we filtered based on protein length (90% of the query protein) and sequence identity (>60%). This was to ensure we use the most relevant information for ET analysis. Substitutions for targeted positions were also identified with the Evolutionary Action algorithm.

7.2 Molecular Pharmacology

7.2.1 Mutagenesis PCR

The Agilent Technologies (Santa Clara, CA) QuikChange mutagenesis kit was used to carry out all mutagenesis according to manufacturer’s instructions. Primers were designed as instructed, with the minimum amount of nucleotide changes required to achieve a mutation. Multiple point mutations were created by using the same primers for single point mutations on already mutated constructs. All constructs were confirmed to have no coding errors by sequencing.
7.2.2 Cell culture and transfections

HEK-293T (ATCC, Manassas, VA) cells were cultured and transfected as previously reported (75).

7.2.3 G protein activity

D₃R’s ability to inhibit cAMP production was carried out as previously described (102) using the Promega (Madison, WI) GloSensor assay with minor modifications. D₃R was expressed at a mass of 1 µg of DNA (except where indicated) and the GloSensor construct was transiently transfected along with D₃R at a mass of 5 µg of DNA. The luminescence was quantified with the Mithras LB940 instrument with no wavelength filter between the cells and the photomultiplier.

7.2.4 Bioluminescent Resonance Energy Transfer

BRET was performed as previously described (75) with some minor modifications. GRK2-YFP or β-arrestin 1-YFP replaced β-arrestin 2-YFP, where indicated. Untagged GRK2 was overexpressed at a ratio of 2-fold higher than receptor, while β-arrestin 2-YFP was always kept at the maximum allowable expression.

7.2.5 Radioligand binding

[3H]-raclopride (Promega, Waltman, MA) binding was carried out as previously described (68). When sodium was removed, the salt was not replaced with any other ion in the buffer. Rluc counts were conducted on the same membrane preparations, the
same day that the ligand binding was carried out, using the same RLuc counting protocol as (75).

### 7.2.6 Confocal microscopy

HEK 293T cells were transiently transfected with YFP C-terminally tagged to D₂R and plated onto 5% fibronectin treated glass bottom culture dishes. The cells were cultured and imaged as previously described (198) with minor modifications. Cells were cultured for 24 hours post transfection and images were captured on live cells.

### 7.2.7 Receptor internalization assay

All D₂R constructs have an N-terminal triple HA-tag, and internalization was performed as previously described (169). Percent internalization was determined from unstimulated receptor expressing cells.

### 7.2.8 MAP kinase transcriptional activity reporter

The SRF and SRE transcriptional reporter was assessed as previously described (179) with minor modifications. The cells were transfected using the calcium phosphate method described here, with 1 µg of D₂R DNA and 5 µg of SRF or SRE reporter DNA. Finally, the cells were incubated in serum-free DMEM overnight rather than using serum replacement medium.

### 7.2.9 ERK phosphorylation by western blot

HEK 293T cells were transiently co-transfected with 2.5 µg of mutant D₂Rs or pcDNA (for untransfected control) and 1 µg of either pcDNA or β-arrestin 2-YFP (the
same BRET construct). After 24 hrs, cells were starved in serum-free DMEM, and 24 hrs later, cells were stimulated with 1 μM quinpirole for 10 minutes. Then cells were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). Lysates were blotted for p-ERK (Cell Signaling Technology, #9106) and total ERK (Cell Signaling Technology, #9102).

7.2.10 Alternative G protein signaling

$G_{\alpha s}$ activation was determined using the GloSensor assay described above. However, cAMP production was not induced by stimulation of endogenous β2AR. $G_{\alpha q}$ was measured using the aequorin assay, as previously described (179).

7.3 Neuropsychopharmacology

7.3.1 Adeno-associated viral expression vectors

The triple HA-D3R constructs had NheI and Ascl sites cloned onto the 5’ and 3’ end of D3R (respectively). This construct was ligated into the NheI and Ascl sites of the pAAV-EF1a-DIO EYFP construct provided by the laboratory of Dr. Karl Deisseroth. The YFP was replaced with D3R.

7.3.2 Adeno-associated virus production

The constructs were packaged into pseudotyped AAV 2/10 using the triple-transfection technique, as previously described (199), which produces helper free virus. The titer was determined to be approximately $1 \times 10^{13}$ vector genome copies/ml for each virus.
7.3.3 Mouse lines

All mouse studies were conducted in accordance with the National Institutes of Health Guidelines for Animal Care and Use and with an approved animal protocol from the Duke University Animal Care and Use Committee.

7.3.4 Mouse stereotaxic injection for viral delivery

Mice were anesthetized under 1-2% isoflurane and the coordinates from bregma for striatal viral deliver were AP = +1.1 mm; ML = +/- 1.7 mm; and DV = -2.9 mm for dorsal and -4.0 mm for ventral striatum. 0.75 µL of virus was injected into each site, for a total volume of 3 µL/mouse. The mice were allowed to recover for 2-4 weeks and then behavioral experiments were performed.

7.3.5 Locomotor activity

The Accuscan activity monitor (Omnitech Electronics, Inc. Columbus, OH) was used to assess locomotor activity, as previously described (68).

7.3.6 Motor coordination

Motor coordination was assessed via the beam walking assay. Briefly, a 3 cm circumference wooden dowel is suspended approximately 1 meter from the ground with open sides but closed edges and roof. The mice were video recorded for 3 minutes and scored for crossings on 5 cm hashes on the beam. The mice were allowed to fall onto a cushioning below and were quickly reinstated to the beam. Number of falls were also recorded but not significant as it did not frequently occur. The data presented were for
mice assayed after basal locomotor and amphetamine locomotor assays in the same room.

### 7.3.7 HA-staining of virally transduced brains

Transduced brains were fixed by perfusion of the mouse and left overnight in 4% PFA in PBS. 30 µM sections were collected by vibratome sectioning and stained by free-floating in 0.3% triton-X in PBS supplemented with 2% NGS and 3% BSA. The HA-antibody (3724, Cell Signaling) was diluted 1:1,000 and allowed to incubate overnight.

### 7.4 Data Analysis

Dose response curves were fit to the nonlinear regression curve $y=Bottom + (Top-Bottom)/(1+10^\{(LogEC_{50}-X)\})$ for agonist curves and $y=Bottom + (Top-Bottom)/(1+10^\{(X-LogIC_{50})\})$ for antagonists in Graphpad Prism 5. Statistical tests were performed (described in Figure and Table legends) in Graphpad Prism 5. Binding curves were fit to $Y=B_{max}*X/(K_d+X)$. Bias quantification was carried out as previously described in (171) and (172). For bias plots (Figures 8D) the points were calculated from normalized (to WT D2R) responses to the two assays and fit to a quadratic equation with the constraint that $B_0=0$. Each bias quantification used the same G protein activity when compared to endogenous and GRK2 overexpression data sets at β-arrestin. All values calculated in Tables III, IV, V, and VI were normalized to WT D2R (or control receptors in Table VI) for each individual assay for efficacy but not potency. Tables III-VI presented
below present all of the calculations from dose response curves as well as statistical analyses performed.

**Table III: Calculated values from Figure 6 and 12**

<table>
<thead>
<tr>
<th></th>
<th>WT D₂R</th>
<th>[Gpro]D₂R</th>
<th>[βarr]D₂R</th>
<th>D₈₀A D₂R</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP Eₘₐₓ (% of WT)</td>
<td>100 +/- 6.9</td>
<td>103 +/- 7.1</td>
<td>49 +/- 15</td>
<td>28 +/- 16</td>
</tr>
<tr>
<td>cAMP EC₅₀ (nM)</td>
<td>6.6 +/- 1.6</td>
<td>8.4 +/- 1.5</td>
<td>7,000 +/- 3,000</td>
<td>18,000 +/- 12,000</td>
</tr>
<tr>
<td>β-arrestin Eₘₐₓ (% of WT)</td>
<td>104 +/- 4.0</td>
<td>15 +/- 7.0</td>
<td>88 +/- 3.3</td>
<td>-3.0 +/- 5.4</td>
</tr>
<tr>
<td>β-arrestin EC₅₀ (nM)</td>
<td>41 +/- 1.0</td>
<td>360 +/- 92</td>
<td>6.2 +/- 0.1</td>
<td>39 +/- 32</td>
</tr>
<tr>
<td>Kᵢ of DA (nM)</td>
<td>810 +/- 10</td>
<td>260 +/- 10</td>
<td>310 +/- 10</td>
<td>1,100 +/- 100</td>
</tr>
<tr>
<td>Internalization % of surface D₂R</td>
<td>29 +/- 2.5</td>
<td>8.4 +/- 3.1</td>
<td>26 +/- 3.1</td>
<td>3.4 +/- 2.7</td>
</tr>
<tr>
<td>Internalization EC₅₀ (nM)</td>
<td>510 +/- 10</td>
<td>47 +/- 16</td>
<td>830 +/- 10</td>
<td>410 +/- 40</td>
</tr>
<tr>
<td>SRF Eₘₐₓ (% of FBS)</td>
<td>7.8 +/- 1.0</td>
<td>5.8 +/- 0.9</td>
<td>-0.2 +/- 0.2</td>
<td>Not Converged</td>
</tr>
<tr>
<td>SRF EC₅₀ (nM)</td>
<td>90 +/- 2.0</td>
<td>32 +/- 2.0</td>
<td>250 +/- 200</td>
<td>Not Converged</td>
</tr>
<tr>
<td>SRE Eₘₐₓ (% of FBS)</td>
<td>5.6 +/- 1.8</td>
<td>4.2 +/- 1.4</td>
<td>Not Converged</td>
<td>1.3 +/- 0.8</td>
</tr>
<tr>
<td>SRE EC₅₀ (nM)</td>
<td>35 +/- 5.0</td>
<td>8.0 +/- 12</td>
<td>Not Converged</td>
<td>0.09 +/- 0.03</td>
</tr>
</tbody>
</table>
Table IV: Receptor manipulations yield operationally defined functional selectivity. Values derived from Figures 7 and 8 to demonstrate the receptor’s contributions to functional selectivity. *p<0.05 when compared to [WT]D2R for efficacy and potency as determined by Bonferroni post-hoc test after p<0.05 for one-way ANOVA.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Assay (ligand)</th>
<th>EC50 (nM)</th>
<th>EMAX (% [WT]D2R)</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>[IYIV]D2R</td>
<td>β-arrestin 2 (DA)</td>
<td>0.2 +/- 0.3*</td>
<td>13 +/- 3*</td>
<td>1A</td>
</tr>
<tr>
<td>[Gpro]D2R</td>
<td>β-arrestin 2+GRK2 (DA)</td>
<td>15 +/- 2*</td>
<td>36 +/- 3*</td>
<td>1B</td>
</tr>
<tr>
<td></td>
<td>β-arrestin 2+GRK2 (DA)</td>
<td>5.3 +/- 1.8</td>
<td>44 +/- 5*</td>
<td>1B</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculation Method</th>
<th>Bias Factor</th>
<th>Bias Factor with GRK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>[IYIV]D2R</td>
<td>0.9 ((B_{INF}))</td>
<td>0.3 ((B_{INF}))</td>
</tr>
<tr>
<td>[Gpro]D2R</td>
<td>0.7 ((B_{INF}))</td>
<td>0.4 ((B_{INF}))</td>
</tr>
<tr>
<td>[IYIV]D2R</td>
<td>4.2 ((\Delta\Delta\log(\tau/K_A)))</td>
<td>0.5 ((\Delta\Delta\log(\tau/K_A)))</td>
</tr>
<tr>
<td>[Gpro]D2R</td>
<td>1.3 ((\Delta\Delta\log(\tau/K_A)))</td>
<td>0.5 ((\Delta\Delta\log(\tau/K_A)))</td>
</tr>
</tbody>
</table>

Table V: Ligand contributions to functional selectivity. Calculated from Figures 9 and 10. *p<0.05 when compared to [WT]D2R for efficacy and potency at each ligand as determined by Bonferroni post-hoc test after p<0.05 by one-way ANOVA.

<table>
<thead>
<tr>
<th>Assay (ligand)</th>
<th>EC50 (nM)</th>
<th>EMAX (% [WT]D2R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[τ/1.5]D2R</td>
<td>cAMP (DA)</td>
<td>7 +/- 2</td>
</tr>
<tr>
<td></td>
<td>β-arrestin 2 (DA)</td>
<td>33 +/- 1</td>
</tr>
<tr>
<td>[Gpro(τ/1.5)]D2R</td>
<td>cAMP (DA)</td>
<td>6 +/- 1</td>
</tr>
<tr>
<td>[βarr(τ/1.5)]D2R</td>
<td>β-arrestin 2 (DA)</td>
<td>120 +/- 10*</td>
</tr>
<tr>
<td>[c1.5]D2R</td>
<td>cAMP (DA)</td>
<td>110 +/- 2*</td>
</tr>
<tr>
<td>[Gpro(c1.5)]D2R</td>
<td>β-arrestin 2 (DA)</td>
<td>59 +/- 2*</td>
</tr>
<tr>
<td>[βarr(c1.5)]D2R</td>
<td>cAMP (DA)</td>
<td>18 +/- 1*</td>
</tr>
<tr>
<td>[c2]D2R</td>
<td>β-arrestin 2 (DA)</td>
<td>55 +/- 2*</td>
</tr>
<tr>
<td>[Gpro(c2)]D2R</td>
<td>cAMP (DA)</td>
<td>16 +/- 2*</td>
</tr>
<tr>
<td>[βarr(c2)]D2R</td>
<td>β-arrestin 2 (DA)</td>
<td>440 +/- 2500*</td>
</tr>
<tr>
<td>[βarr(c2)]D2R</td>
<td>cAMP (DA)</td>
<td>580 +/- 3*</td>
</tr>
<tr>
<td></td>
<td>β-arrestin 2 (DA)</td>
<td>69 +/- 3*</td>
</tr>
<tr>
<td>Mutant</td>
<td>Ligand (Assay)</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;/IC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>DA (cAMP)</td>
<td>17 +/- 2*</td>
</tr>
<tr>
<td></td>
<td>Quinpirole (cAMP)</td>
<td>29 +/- 2</td>
</tr>
<tr>
<td></td>
<td>DA (β-arrestin 2)</td>
<td>450 +/- 20*</td>
</tr>
<tr>
<td></td>
<td>Quinpirole (β-arrestin 2)</td>
<td>Ambiguous Fit</td>
</tr>
<tr>
<td></td>
<td>DA (β-arrestin 2 + GRK2)</td>
<td>20 +/- 1*</td>
</tr>
<tr>
<td></td>
<td>Quinpirole (β-arrestin2+GRK2)</td>
<td>68 +/- 1*</td>
</tr>
<tr>
<td>[WT]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Quinpirole (GRK2)</td>
<td>110 +/- 2*</td>
</tr>
<tr>
<td></td>
<td>Apomorphine (cAMP)</td>
<td>130 +/- 2*</td>
</tr>
<tr>
<td>[Gproet]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>NPA (cAMP)</td>
<td>450 +/- 20*</td>
</tr>
<tr>
<td></td>
<td>Quinpirole (β-arrestin 2)</td>
<td>17 +/- 2*</td>
</tr>
<tr>
<td>[Gproet]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (cAMP)</td>
<td>5.6 +/- 1*</td>
</tr>
<tr>
<td>[βarrestin]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (cAMP)</td>
<td>10,000 +/- 20*</td>
</tr>
<tr>
<td></td>
<td>NPA (cAMP)</td>
<td>1,300 +/- 229*</td>
</tr>
<tr>
<td>[D80A]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (cAMP)</td>
<td>510 +/- 400*</td>
</tr>
<tr>
<td></td>
<td>NPA (cAMP)</td>
<td>650 +/- 320*</td>
</tr>
<tr>
<td>[WT]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Quinpirole (β-arrestin 2)</td>
<td>66 +/- 1</td>
</tr>
<tr>
<td></td>
<td>Apomorphine (β-arrestin 2)</td>
<td>10 +/- 1</td>
</tr>
<tr>
<td>[Gproet]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (β-arrestin 2)</td>
<td>160 +/- 28</td>
</tr>
<tr>
<td></td>
<td>NPA (β-arrestin 2)</td>
<td>1,700 +/- 200*</td>
</tr>
<tr>
<td>[βarrestin]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (β-arrestin 2)</td>
<td>11 +/- 1</td>
</tr>
<tr>
<td></td>
<td>NPA (β-arrestin 2)</td>
<td>2.0 +/- 0.2</td>
</tr>
<tr>
<td>[D80A]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Apomorphine (β-arrestin 2)</td>
<td>0.3 +/- 0.1</td>
</tr>
<tr>
<td></td>
<td>NPA (β-arrestin 2)</td>
<td>0.1 +/- 0.8</td>
</tr>
<tr>
<td>[WT]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Raclopride (cAMP)</td>
<td>23 +/- 1</td>
</tr>
<tr>
<td></td>
<td>Haloperidol (cAMP)</td>
<td>54 +/- 1</td>
</tr>
<tr>
<td>[Gproet]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Aripiprazole (cAMP)</td>
<td>280 +/- 23</td>
</tr>
<tr>
<td></td>
<td>Haloperidol (cAMP)</td>
<td>19 +/- 1</td>
</tr>
<tr>
<td></td>
<td>Aripiprazole (cAMP)</td>
<td>39 +/- 1</td>
</tr>
<tr>
<td>[βarrestin]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Aripiprazole (cAMP)</td>
<td>16 +/- 3*</td>
</tr>
<tr>
<td></td>
<td>Raclopride (cAMP)</td>
<td>Not Converged</td>
</tr>
<tr>
<td></td>
<td>Haloperidol (cAMP)</td>
<td>2.3 +/- 2.6*</td>
</tr>
<tr>
<td>[D80A]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Aripiprazole (cAMP)</td>
<td>Not Converged</td>
</tr>
<tr>
<td></td>
<td>Raclopride (cAMP)</td>
<td>Ambiguous Fit</td>
</tr>
<tr>
<td>[WT]D&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>Raclopride (β-arrestin 2)</td>
<td>1.7 +/- 1.2</td>
</tr>
<tr>
<td></td>
<td>Haloperidol (β-arrestin 2)</td>
<td>4.0 +/- 1.2</td>
</tr>
<tr>
<td></td>
<td>Aripiprazole (β-arrestin 2)</td>
<td>77 +/- 2</td>
</tr>
</tbody>
</table>
Table VI: Transducer contributions to functional selectivity. Calculated from Figure 11. *p<0.05 when compared to [WT]D2R or control receptors (β2AR for Gαs or AT1A for Gαq) for efficacy and potency as determined by Bonferroni post-hoc test after p<0.05 for one-way ANOVA.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Assay (Ligand)</th>
<th>EC₅₀ (nM)</th>
<th>E₉⁰ (% Control)</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>[WT]D₂R</td>
<td>GRK2 recruitment (DA)</td>
<td>4.6 +/- 1.5</td>
<td>100 +/- 4</td>
<td>5A</td>
</tr>
<tr>
<td>[Gprot]D₂R</td>
<td></td>
<td>6 +/- 2</td>
<td>40 +/- 4*</td>
<td>5A</td>
</tr>
<tr>
<td>[βarr]D₂R</td>
<td></td>
<td>0.3 +/- 0.2</td>
<td>94 +/- 4</td>
<td>5A</td>
</tr>
<tr>
<td>[D80A]D₂R</td>
<td></td>
<td>6,600 +/- 250*</td>
<td>14 +/- 10*</td>
<td>5A</td>
</tr>
<tr>
<td>[WT]D₂R</td>
<td>β-arrestin 1 recruitment (DA)</td>
<td>46 +/- 1</td>
<td>100 +/- 2</td>
<td>5B</td>
</tr>
<tr>
<td>[Gprot]D₂R</td>
<td></td>
<td>280 +/- 10*</td>
<td>17 +/- 2*</td>
<td>5B</td>
</tr>
<tr>
<td>[βarr]D₂R</td>
<td></td>
<td>15 +/- 1*</td>
<td>70 +/- 2*</td>
<td>5B</td>
</tr>
<tr>
<td>[D80A]D₂R</td>
<td></td>
<td>0.1 +/- 1.5*</td>
<td>3 +/- 3*</td>
<td>5B</td>
</tr>
<tr>
<td>Endogenous</td>
<td>Gαs (isoproterenol)</td>
<td>29 +/- 1</td>
<td>100 +/- 2</td>
<td>5C</td>
</tr>
<tr>
<td>[WT]D₂R</td>
<td>(DA)</td>
<td>8,800 +/- 950*</td>
<td>1 +/- 1*</td>
<td>5C</td>
</tr>
<tr>
<td>[Gprot]D₂R</td>
<td></td>
<td></td>
<td>Ambiguous Fit</td>
<td>5C</td>
</tr>
<tr>
<td>[βarr]D₂R</td>
<td></td>
<td>1.8e8 +/- 1.5e7*</td>
<td>5 +/- 7*</td>
<td>5C</td>
</tr>
<tr>
<td>[D80A]D₂R</td>
<td></td>
<td>1.4e8 +/- 8.9e7*</td>
<td>4 +/- 6*</td>
<td>5C</td>
</tr>
<tr>
<td>AT₁A R</td>
<td>Gαq (ANGII)</td>
<td>150 +/- 12</td>
<td>94 +/- 3</td>
<td>5D</td>
</tr>
<tr>
<td>[WT]D₂R</td>
<td>(DA)</td>
<td>210 +/- 40</td>
<td>1 +/- 1*</td>
<td>5D</td>
</tr>
<tr>
<td>[Gprot]D₂R</td>
<td></td>
<td>270 +/- 34</td>
<td>1 +/- 1*</td>
<td>5D</td>
</tr>
<tr>
<td>[βarr]D₂R</td>
<td></td>
<td>77 +/- 52</td>
<td>1 +/- 1*</td>
<td>5D</td>
</tr>
<tr>
<td>[D80A]D₂R</td>
<td></td>
<td>Not Converged</td>
<td></td>
<td>5D</td>
</tr>
</tbody>
</table>
References


186. Mannoury la Cour C, Salles MJ, Pasteau V, & Millan MJ (2011) Signaling pathways leading to phosphorylation of Akt and GSK-3beta by activation of
cloned human and rat cerebral D(2) and D(3) receptors. *Molecular pharmacology* 79(1):91-105.


Biography

I was born on August 23, 1983 to John and Virginia Peterson in Seward, Alaska. I attended the University of South Florida in Tampa, Florida from 2002-2006 and received my Bachelor’s of Science degree in Biology. I worked as a technician at the University of North Carolina, Chapel Hill in the laboratory of Dr. Bryan Roth from 2006-2008 where I was first exposed to GPCR and dopamine receptor research. I was accepted into the Cell and Molecular Biology training program at Duke in 2008 and joined the Cell Biology department under the mentorship of Dr. Marc Caron in the summer of 2009. In Dr. Caron’s laboratory I published “Elucidation of G-protein and β-arrestin functional selectivity at the dopamine D2 receptor” in 2015 in PNAS and a chapter in the textbook “Primer on the Autonomic Nervous System” titled “Dopamine Receptors” in 2012. I will be pursuing a postdoctoral fellowship in Dr. John Kuriyan’s laboratory at the University of California, Berkeley to study how the kinase domain of EGFR is activated by ligands using structural and molecular dynamics techniques, as well as the techniques presented in this dissertation.