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This work examines the contribution of transmembrane segments two and three to the activation state of the D2 dopamine receptor by using ligand probes which are highly sensitive to substitutions at specific amino acid positions within this microdomain. Specifically, D2 receptors were modified by substitution of one to three specific amino acids with the corresponding amino acids of the D4 receptor to enhance the binding of D4 selective 1,4-disubstituted aromatic piperidines/piperazines. The ability of these ligands to elicit G protein mediated inhibition of cyclic adenosine monophosphate was then tested. Modification of all three amino acid residues was found to modify ligand function at the D2 receptor to match the function elicited at the D4 receptor. Additionally, the modification of specific ligand interactions with the D2-V2.61F receptor in the presence of sodium provides evidence for transmembrane segment repositioning in the inactive state of G protein coupled receptors.

PROBING THE ROLE OF TRANSMEMBRANE SEGMENTS TWO AND THREE IN THE ACTIVATION STATE OF THE D2 DOPAMINE RECEPTOR

THESIS

Presented to the Graduate Council of the Graduate School of Biomedical Sciences

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By

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CHAPTER I

INTRODUCTION

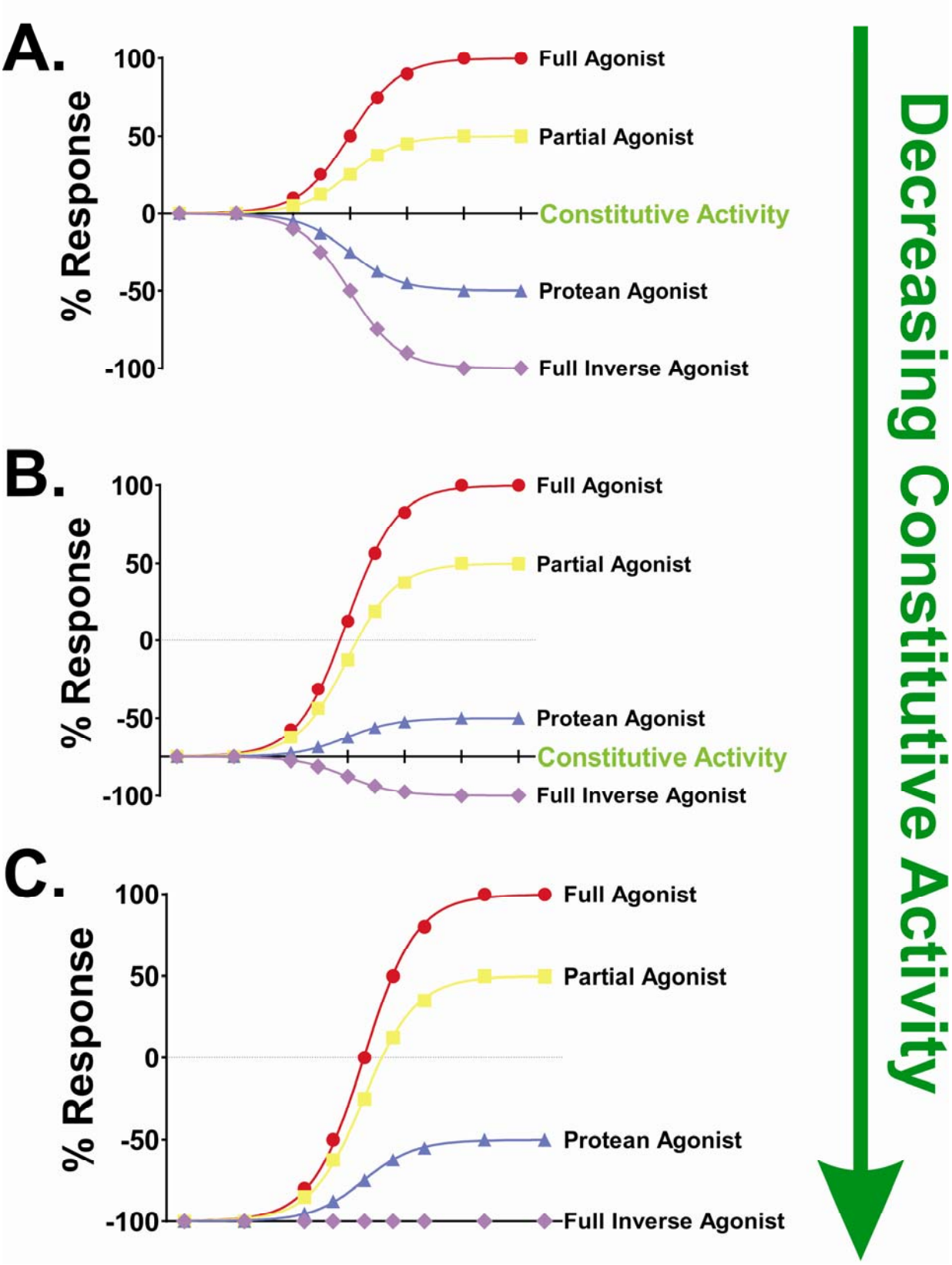
Heterotrimeric G protein-coupled receptors

Heterotrimeric G protein-coupled receptors (GPCRs) are a superfamily of signaling molecules responsible for translating extracellular stimuli into intracellular signals (Fredriksson and Schioth 2005). Endogenous stimuli, including lipids, proteins and photons, can either block or potentiate GPCR signaling (Bockaert and Pin 1999). Depending on the physiological need, effects of these stimuli are either localized or widely distributed. Localized cell-cell communication is simply the mechanism used for communication between adjacent cells using stimuli and intracellular signaling. For example, in a synapse a stimulus sent from a presynaptic cell through the intercellular space is received by membrane bound receptors on the postsynaptic cell. The duration, availability, and intercellular location of the molecules comprising the stimulus are restricted through signal degradation by enzymes such as monoamine oxidase or reuptake by presynaptic transporters with high specificity for the stimulus. Postsynaptic membrane bound receptors translate the stimulus into an intracellular signal through the use of secondary messenger pathways. These signals either stimulate or inhibit the cellular communication to the next cell of the circuit. Stimuli with wide distributions, such as hormones, are released into the bloodstream and affect all cells expressing the receptor specific to the stimulus. Pharmacological remedies are generally molecular stimuli with a wide distribution

pattern; therefore, compartmental localization and receptor specificity are necessary to limit adverse reactions to these agents. The diversity and wide distribution of GPCRs, along with their paramount roles in a wide variety of physiological and cognitive processes, makes them ideal targets for pharmaceuticals. Currently, half of all the prescribed pharmacological remedies, including several of the most popular drugs, primarily target GPCRs to elicit therapeutic effects (Drews 2000; Flower 1999). Additionally, the principle mechanisms behind many drugs of abuse are the result of indirect or direct modulation of GPCR function (Nakagawa and Kaneko 2008).

Intracellular signaling through a GPCR is accomplished by recruiting heterotrimeric G proteins or other effector molecules to the active state of the receptor. The probability of a membrane bound receptor being in a state of high or low activity depends on receptor type, location, and the presence or absence of ligands docked at the orthosteric, or primary, binding site. If the probability is high in lieu of bound agonist, the receptor has high constitutive activity and will generate a large baseline intracellular signal. If the probability is low, the opposite is true. Agonists bound to the orthosteric site of a receptor stabilize the active conformation of that receptor and therefore promote intracellular signaling. These are divided into full and partial agonists, which delineate agonists that elicit a maximal response from those that elicit only a portion of the response (Figure 1-1). Inverse agonists do the opposite by stabilizing inactive

Figure 1-1. Activation states of the receptor. Plotted are functional response graphs illustrating full agonists (●), partial agonists (■), protean agonists (◆), and inverse agonists (◆) at a single type of receptor with varied constitutive activity. A, a receptor at 50% constitutive activity B, the same graph as A in a system with only 12.5% constitutive activity. C, the same graph as A in a system with no observable constitutive activity.


A.
% Response
100
50
0
-50
-100
Full Agonist
Partial Agonist
Constitutive Activity
Protean Agonist
Full Inverse Agonist
B.
% Response
100
50
0
-50
-100
Full Agonist
Partial Agonist
Protean Agonist
Constitutive Activity
Full Inverse Agonist
C.
% Response
100
50
0
-50
-100
Full Agonist
Partial Agonist
Protean Agonist
Full Inverse Agonist
Decreasing Constitutive Activity

conformations and decreasing constitutive activity. Neutral antagonists do not select for inactive or active states of the receptor but instead maintain constitutive activity while occupying the receptor binding pocket. Finally, protean agonist is a term used to describe a subdivision of agonists that stabilize the receptor to an active conformation with less intracellular signaling then the normal constitutive activity of the receptor (Kenakin 2004, Figure 1-1B-C). These are usually identified as inverse or neutral agonists prior to re-characterization in systems with lower constitutive activity. Protean agonists are closely tied to the concept of agonist directed trafficking (sometimes termed functional selectivity) because this form of agonism is related to receptor conformations which are specific for activation of different isoforms of downstream signaling molecules (Lane et al., 2007; Kenakin 2007).

While these terms are useful for describing what response is generated for a ligand-receptor complex within a specific system, they are not as useful and possibly confusing when correlated to the activation states of the receptor. For example, if a receptor has no constitutive activity in heart cell line but high constitutive activity in a lung cell line, a weak partial agonist in the heart cell line may appear to be an inverse agonist in the lung cell line. In this contrived example, this ligand would be termed a protean agonist. However, if only the data from the lung cell line was obtained, the ligand would be labeled an inverse agonist since definitions are based upon the system used and the data available. An example of protean agonism is observed both in vitro and in vivo for the interaction of proxyfan, an H3 ligand, with species dependent isoforms of the H3 receptor (99% sequence homology between rat and human H3 receptors; Gbahou et al., 2003; Arrang et al., 2007). Activation states are harder to qualify with these terms because ligands are thought to activate GPCRs by subtle changes to the conformation of the receptor including breakage of the “ionic lock” between TMs three and six, movement of TMs six and

seven, and coupling to G-proteins. These conformational changes select for certain substates of activation which enhance or limit downstream signaling through G-proteins. In essence, any ligand that breaks the "ionic lock" and enables G-protein coupling is an agonist (protean, partial or full); however, the high constitutive activity of some receptor systems will "mask" the activity of protean agonists. A full inverse agonist would stabilize the ionic lock and force the receptor into a G-protein uncoupled inactive conformation. Although it has been suggested that no antagonist is truly neutral, a neutral antagonist would block the orthosteric receptor pocket without affecting the constitutive state of the receptor in any system that changes the constitutive activity (Kenakin 2004). An allosteric modulator, by comparison, would modify the ability of the receptor to enter certain activation states through a site distinct from the orthosteric site. Although there is some debate about the preferred activation state of sodium sensitive receptors modulated by sodium (see Chapter V), sodium has been experimentally proven to bind an intracellular allosteric site only accessible from the intracellular space (Limbird et al., 1982; Horstman et al., 1990; Ceresa and Limbird, 1994; Neve et al., 2001).

Understanding the molecular structure of GPCRs, and the relation of that structure to intracellular signaling, is important for understanding how cellular communication and physiological processes work. GPCRs are comprised of seven distinct transmembrane segments, three intracellular loops, three extracellular loops, an extracellular amine terminus and an intracellular carboxyl terminus. The high resolution crystal structure of bovine rhodopsin, a Class A GPCR, supports the seven transmembrane model and enables structural comparisons with similar Class A GPCRs (Palczewski et al., 2000). Models of related receptors, i.e. dopamine, were made using the crystal structure of rhodopsin as a template. Further high resolution crystallization studies of β2 adrenergic receptors revealed similar architectures when compared

to the rhodopsin template (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). However, while both bovine rhodopsin and beta adrenergic receptors are similar in both crystalline structure and the positioning of key residues, β2 adrenergic receptors were not observed in an ionically "locked" conformation suggesting that the crystalline structure may not be the inactive state of the receptor. A possible explanation for this result is that carazolol, the ligand stabilizing the crystalline structure of the β2 adrenergic receptor, is a putative protean agonist, and therefore may have the ability to break the ionic lock while maintaining the receptor in one of many possible inactive states (Rasmussen et al., 2007). However, this breakage of the ionic lock by carazolol may represent an activation substate with limited ability to stimulated G_s proteins i.e. an activation substate that couples to G_s proteins at a lower rate then the constitutive substate. This implies that β2 adrenergic receptors may have multiple activation substates with varying ability to couple the G_s protein. An alternate explanation for the breakage of the ionic lock by carazolol is that this activation substate represents an active conformation for an alternative G_s-protein independent pathway (Shukla et al., 2008). These hypotheses not only suggest that secondary messages elicited by a GPCR are tailored to specific activation substates, but also that specific ligands can be utilized to "switch on" certain substates. More recently, the high resolution crystal structure of opsin, the "active" form of rhodopsin formed by the conversion of cis-retinal to all trans retinal and the subsequent dissociation of retinal from the opsin molecule, has been obtained (Park et al., 2008). The opsin structure supports the postulated movement of TMs 5-7 to form the activate state of opsin; however, the structure lacks light activated coupling of rhodopsin to G_t and, therefore, is an incomplete model of an active Class A GPCR.

Heterotrimeric G proteins are divided into several different subtypes each with a different intracellular activity and receptor binding profile. Two classical isoforms of G proteins, G_s and $G_{i/o}$, modulate the production of cyclic adenosine monophosphate (cAMP) by respectively stimulating or inhibiting adenylate cyclase (AC). For example, dopamine receptors, couple in a subtype specific fashion to G_s or $G_{i/o}$ to elicit downstream signaling. A different G protein isoform, G_q, stimulates phospholipase C (PLC) to cleave phosphotidylinositol bisphosphate (PIP_2) into inositol 3-phosphate (IP_3) and diacylglycerol (DAG). These two signaling molecules act in concert: IP3 stimulates an increase in intracellular Ca^{2+} by activation of IP_3 receptors on the endoplasmic reticulum and DAG activates classical protein kinase C isoforms (α, βI, βII, and γ) in response to increased intracellular Ca^{2+} (Steinberg et al., 2008). Other G protein isoforms including transducin (G_t), which is responsible for signaling via rhodopsin, and olfactory (G_{olf}), which couples to olfactory receptors, exist. In addition, GPCRs can undergo G protein independent signaling by beta arrestins (β2 adrenergic, Violin and Lefkowitz, 2007) or by regulating the expression of membrane bound potassium ion channels (D2 dopamine, Einhorn and Oxford 1993; D2 and D3 dopamine, Liu et al., 1996). Although several different types of GPCRs have been shown to be capable of forming homodimers and heterodimers on the plasma membrane, most GPCRs are thought to exist and function as monomers (Javitch 2004). Interestingly, the D3 receptor has recently been shown to heterodimerize with D1 through a combination of co-immunoprecipitation from striatal tissues and bioluminescent energy transfer in co-transfected HEK293 cells (Fiorentini et al., 2008). Interestingly, the only requirement for agonist stimulated β-arrestin mediated internalization of the D1/D3 complex was activation of the D1 receptor. Specific activation of the D3 receptor by (-)-quinpirole did not internalize either the monomer or the heterodimer. In contrast, the D2 receptor homodimerizes by a concerted

array of symmetrical intermolecular interactions TM4 segments of the dimer (Guo et al., 2003; Lee et al., 2003). In COS7 cells expressing truncation mutants that selectively deleted one or more TMs of the D2 receptor, Lee and colleagues found that only mutants that included TM4 were able to homodimerize. This homodimerization remained intact in the presence of reducing agents suggesting that the TM4 homodimer interface is hydrophobic. However, perhaps the most important finding was that disruption of TM4 helix by the addition of a proline kink eliminated homodimerization in the D2-M4.45(155)$P_{TM4\text{-}IC3}$ truncated mutant receptor but not in whole D2-M4.45(155)P mutant receptor. This finding suggests that other TM segments have important molecular interactions that contribute to D2 receptor homodimerization.

Naming wild type and mutant receptors

GPCRs are protein structures composed of a single amino acid chain folded into a three dimensional structure i.e. a tertiary protein. The naturally occurring amino acid sequence for a receptor in a specific species is the *wild type* receptor. Mutant receptors are created by changes to the amino acid sequence of the *wild type* receptor. Amino acid substitutions are traditionally named by counting from the amine terminus to the substitution. The receptor is then written according to the following formula:

[species][receptor]-[Coordinates]

In this system, species is a single letter abbreviation such as h for human or r for rat and coordinates is the numerical position of the amino acid preceded by the *wild type* amino acid and followed by the mutant amino acid. For example, rD2-V91F refers to a rat dopamine receptor subtype 2 with a phenylalanine substitution for the naturally occurring valine 91 positions downstream of the amine terminus. While this simple naming scheme is appropriate for receptor

sequencing, it is difficult to use in cross receptor or cross species comparisons. To aid in these comparisons, Ballesteros and Weinstein (1995) created a system by which the most conserved amino acids of a transmembrane sequence are aligned and given the same coordinates. The system follows a similar structure to the previous system, differing only in how the coordinates are designated. The coordinate system uses the following formula:

[Coordinates] = [A][x].[yy][Z]

A is the amino acid found in the *wild type* at the specified numerical coordinates

Z is the amino acid replacing **A** in a mutated receptor

x is the transmembrane segment

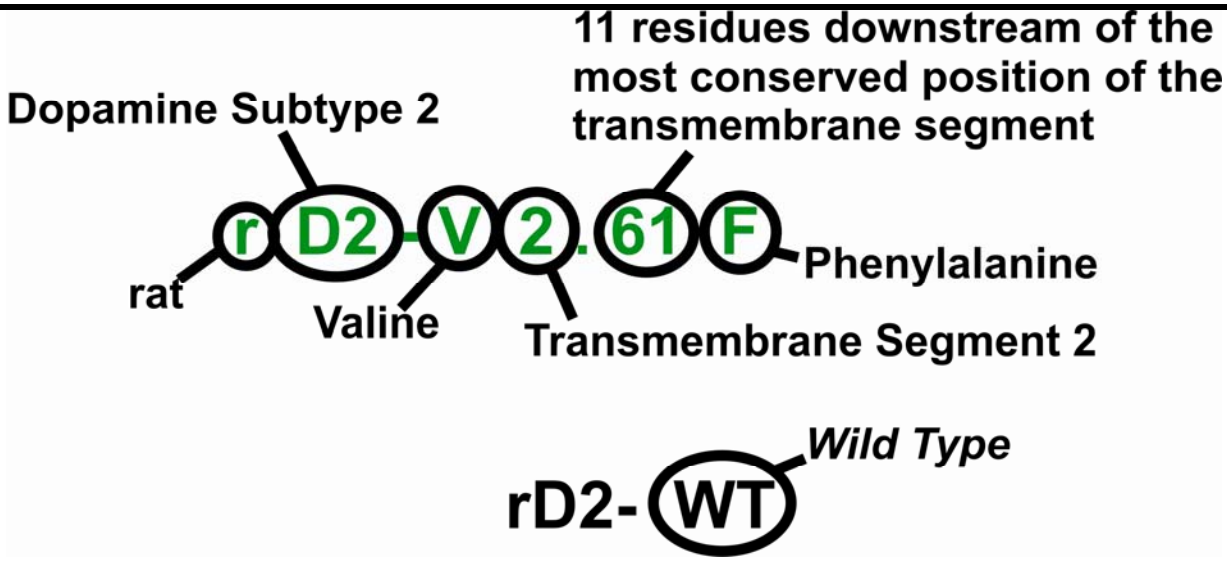
yy is the position of the amino acid relative to the most conserved amino acid of the transmembrane segment. The most conserved amino acid is always given the numerical position 50. Amino acids upstream (towards the carboxyl terminus) of this position are counted by addition while amino acids downstream (towards the amino terminus) are counted by subtraction. Therefore, an amino acid 10 residues upstream of position 50 would be 60 while an amino acid 10 residues downstream would be position 40.

In this new system, the rD2-V91F mutation would be written as rD2-V2.61F. This is illustrated in Figure 1-2. In addition, the actual amino acid position from the carboxy terminus is occasionally written within parentheses as rD2-V2.61(91)F when necessary.

Dopamine receptors

The dopamine receptor is a class A GPCR sharing structural and phylogenetic homology with rhodopsin (Foord et al., 2005; Fredriksson et al., 2003; Bjarnadóttir et al., 2006). Five distinct subtypes of dopamine receptor exist based on preferred G protein-coupling, tissue localization, structure and physiological effect. The D1-like dopamine receptors, D1 and D5, prefer to couple the G_s protein and stimulate cAMP production. Using both tissue mRNA and

Figure 1-2. Examples of receptor nomenclature. The upper diagram shows a rat D2 dopamine receptor with a mutation in transmembrane 2. In the traditional naming system this mutation would be rD2-V91F. The lower diagram illustrates the wild type receptor designation.



[^{3}H]SCH-23390 autoradiographs, D1 receptors in the brains of rats have been localized to the striatum, amygdala, nucleus accumbens, and suprachiasmatic nucleus (Mansour et al., 1991). Expression of D1 receptor mRNA is highest in the caudate putamen, nucleus accumbens, and olfactory tubercles, however, significant populations of D1 receptors also reside in the cerebral cortex, striatum, substantia nigra, amygdala, suprachiasmatic nucleus, limbic system, thalamus,

hypothalamus and hippocampus (Mansour et al., 1991; Fremeau et al., 1991). Independent studies also showed significant populations of D1 receptors in kidneys (Lokhandwala and Amenta, 1991). Similarly, D5 receptors, which bind [^{3}H]SCH-23390 and other D1 selective ligands at equal to or greater affinity, have been localized by mRNA expression to the limbic regions of the brain (Sunahara et al., 1991). Other tested anatomical areas including the kidney, liver and heart were devoid of D5 mRNA expression.

In contrast to the G_s coupled D1 and D5 receptors, D2-like receptors, D2 and D4, prefer to couple $G_{i/o}$ proteins and therefore inhibit the production of intracellular cAMP. D2 receptors are located at synapses within the nigrostriatal, mesocorticalimbic, and tuberoinfundibular pathways (for review see Le Moine and Bloch 1995) while D4 receptors are localized to the retina, prefrontal cortex, hippocampus, amygdala, and hypothalamus (for review see Oak et al., 2000). Significant populations of D2 receptors exist in the substantia nigra, caudate putamen, nucleus accumbens, and olfactory tubercule. D2 receptors, while present in the same tissues as D1 receptors, have been further localized to the Substance P containing neurons in the caudate-putamen, nucleus accumbens, and olfactory tubercle (Le Moine and Bloch 1995). D1 neurons are localized to the enkephalin neurons of these striatal regions. D4 receptor mRNA is highly expressed in the photoreceptors of the retina (Cohen et al., 1992; Ivanova et al., 2008; Pozdeyev et al., 2008; Jackson et al., 2009) as well as the prefrontal cortex, amygdala, hypothalamus and pituitary (Valerio et al., 1994; Asghari et al., 1995).

Subtype selective D4 ligands were synthesized on the basis that these drugs would have antipsychotic properties with minimal side effects after data suggesting that the atypical antipsychotic clozapine has a 5-10 fold increase in D4 receptor affinity relative to the D2 receptor (Van Tol et al., 1991; Asghari et al., 1994; Patel et al., 1997). Spurred by the possibility

of a side-effect free target for atypical antipsychotics, various D4 selective ligands belonging to the 1,4-disubstituted aromatic piperadine/piperazine (1,4-DAP) structural class were developed (Appendix A). Despite the large array of usable subtype selective compounds, D4 selective antagonists have shown little promise as antipsychotics. The D4 selective 1,4-DAP L-745,870 (CPPMA) failed to show noticeable improvements on the hyperactivity of amphetamine challenged rats (Bristow et al., 1997) or in psychological measures of a human phase II clinical trial on 38 acutely psychotic inpatients with previous history of positive response to neuroleptics treatment (Kramer et al., 1997). Sonepiprazole (PNU-101387G), a D4 selective antagonist of the 1,4-DAP structural class, failed to show antipsychotic efficacy in clinical trials and significantly worsened emotional discomfort when given at moderate dosage (Corrigan et al, 2004). A trial testing the clinical efficacy of fanaserin demonstrated that the mixed D4 and serotonin 2A receptor antagonist was unable to treat positive or negative symptoms of schizophrenia (Truffinet et al, 1999). However, PD 168,077, a D4 selective 1,4-DAP showing partial agonism at the D4 receptor, appears to stimulate rat penile erection by increasing dopamine release in the nucleus accumbens and oxytocin release in the ventral tegmental area (VTA, Succu et al., 2007). A BOLD-fMRI (Blood Oxygen Level Dependent-functional Magnetic Resonance Imaging) study has shown that the VTA in humans is a key area for the perception and expectation of reward (D'Ardenne et al., 2008). BOLD-fMRI measures the increase oxygen consumption in neurons that have increased activity due to afferent input i.e. the neurons with high BOLD values are undergoing synaptic transmission. In this study, dehydrated human participants were observed to have increased BTA BOLD values when conditioned to expect water. In a supplementary experiment, increased VTA BOLD values were observed for human participants conditioned to expect monetary reward based on numerical cues. Based on these findings the authors concluded

that the activity of VTA neurons may be responsible for the perception and expectation of rewards. These clues suggest that partial agonists selective for the D4 receptor may aid in the treatment of erectile and libido dysfunctions. Understanding the molecular basis underlying ligand selectivity for the D4 receptor may aid in the rapid development of therapeutics for these, and other, disorders.

Research into the molecular basis for the D4 subtype selectivity of ligands in the 1,4-disubstituted aromatic piperadine/piperazine structural class (1,4-DAP, Kortagere et al., 2004) was tested by examining the non-conserved amino acids in transmembrane segments two and three of the D4 and related D2 receptor. By mutating specific amino acid locations in a N and C terminal tagged human D2 dopamine receptor with the amino acid found at the same location in the D4 receptor, a process termed reciprocal mutation, several non-conserved positions on transmembrane segments two and three were revealed to be important binding determinants for the D4-selective 1,4-DAPs L-745,870, Ro 61-6270, and Ro 10-4548 (Simpson et al., 1999). Reciprocal single point mutations at positions 2.61 and 3.28, but not 3.29 were found to increased ligand affinity at reciprocally mutated D2 receptors to match affinities at *wild type* D4 receptors for L-745,870. Similarly, D2 mutants involving 2.61 and 3.28 were shown to have increased affinity for Ro 61-6270, and Ro 10-4548. At the same time, an extensive study of multiple reciprocal amino acid mutations in the D4 receptor revealed that single or combined reciprocal mutations of the three amino acid positions in the rat D4 receptor decreased the affinity of L-750,667, the iodinated cogener of L-745,870, making the receptor more D2-like in terms of affinity for this 1,4-DAP (Schetz et al., 2000). Together, these findings suggested that the microdomain formed by the amino acids of positions 2.61, 3.28, and 3.29 is an important determinant of 1,4-DAP selectivity for dopamine receptor subtypes. Recently, a much more

comprehensive study of reciprocal mutations at 2.61, 3.28, and 3.29 in the rat D4 receptor illustrated the use of 1,4-DAPs as probes of ligand binding and receptor structure (Kortagere et al., 2004). In this study, nine of the eleven 1,4-DAPs tested showed sensitivity to mutations at these three subtype selective amino acid positions in the D4 receptor background. Later studies of these positions in the D2 receptor background confirmed that these positions are critical to the subtype selective binding affinity of certain 1,4-DAP structures (Floresca et al., 2004; Floresca et al., 2005; Ericksen et al., 2009). Other 1,4-DAP structures including methylspiperone, clozapine, olanzapine, and quetiapine are unaffected by changes at these positions.

While binding data for mutant D2 dopamine receptors bearing 1,4-DAP sensitive mutations in transmembrane segments two and three is available, functional data was not. Therefore I probed the functional properties of a D2 receptor possessing a combined TM2/TM3 reciprocal mutation, D2-V2.61F+FV3.28-3.29LM, and report that it has increased potency for both L-750,667 and RBI-257. I also show that the D2-V2.61F+FV3.28-3.29LM receptor, when compared to the *wild type* D2 receptor, has similar potency and efficacy for the full agonist (-)-quinpirole suggesting that the mutant receptor is fully functional.

My additional research was devoted to experimentally illustrating the specific molecular mechanisms of the TM2 microdomain in the sodium bound D2 receptor using a D2-V2.61F mutant receptor. This mutant receptor exhibited a significant 97-fold increase in affinity for L-745,870 relative to wild type human D2 receptor (Simpson et al., 1999). In a surprising contradiction, later experiments using L-750,667, the iodinated cogener of L-745,870, produced affinities for the rat D2-V2.61F mutant receptor that were the same as the wild type D2 receptor (Floresca et al., 2005). Interestingly, the affinities for these two ligands at the hD2-V2.61F+FV3.28-3.29LM+Y7.35V and rD2-V2.61F+FV3.28-3.29LM mutant receptors were

similar (0.19 nM and 0.14 nM respectively). The D2-Y7.35V mutation alone does not gain significant affinity for L-745,870 in comparison to the wild type D2 receptor (3.5 fold increase; Simpson et al., 1999). Two initial hypotheses were suggested for these discrepancies (Floresca et al., 2005). The first hypothesis suggested that the N and C terminal tags of the human D2 receptor were responsible for the shift in affinity, while the second hypothesis suggested that differences in the two cell lines used for expression of these receptors (HEK293 and COS7 respectively) could explain the observed differences. Careful comparison of the two experimental protocols suggested a third hypothesis: the observed discrepancies in L-745,870 and L-750,667 binding to the D2-V2.61F mutant receptor could be attributed to subtle modifications of the orthosteric binding site by sodium, an allosteric modulator of the D2 dopamine receptor. To test this, I used L-745,870 as a high affinity probe of the D2-V2.61F structure in the presence and absence of 140 mM sodium chloride. Using this methodology I was able to replicate the data observed by Simpson and colleagues confirming that the observed discrepancy was due to the allosteric effects of sodium. The high sodium sensitivity of L-745,870 at the mutant D2-V2.61F receptor suggested that sodium specific molecular changes could be ascertained by using other D4-selective ligands as probes of the orthosteric binding site. The results of these assays were utilized to model ligand-receptor interactions and showed that sodium induces an increase in the movements of the extracellular portions of TM2 and 3 (Ericksen et al., 2009).

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CHAPTER II

THREE AMINO ACIDS IN THE D2 DOPAMINE RECEPTOR REGULATE SELECTIVE LIGAND FUNCTION AND AFFINITY[1]

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KEYWORDS

G-protein coupled receptor, dopamine receptor, erectile dysfunction, attention deficit hyperactivity disorder, schizophrenia, structure-activity relationship

RUNNING TITLE

Regulation of D2 ligand function

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Abbreviations:

CHELPG, CHarges from ELectrostatic Potentials using a Grid-based method;

CLM, Contact Likelihood Matrix;

cyclic AMP, 3'-5'-cyclic adenosine monophosphate;

D2, D2 long receptor;

D4, D4 receptor;

1,4-DAPS, 1,4-disubstituted aromatic piperidines and piperazines;

ENM, elastic network model;

FAUC113, 3 [4 (4 chlorophenyl)piperazin 1 ylmethyl]pyrazolo[1,5 a]pyridine;

FAUC213, 2 [4 (4 chlorophenyl)piperazin 1 ylmethyl]pyrazolo[1,5 a]pyridine;

GROMACS, GROningen Machine for Chemical Simulations;

IC50, half maximal inhibitory concentration;

L-745,870, 3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine and is also known as CPPMA, which stands for chlorophenylpiperazinyl methylazaindole;

L-750,667, 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine;

L-BFGS, low-memory Broyden-Fletcher-Goldfarb-Shanno (quasi-Newtonian algorithm for energy minimization);

Methylspiperone,8-[4-(4-Fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride;

NGD 94-1, 2-phenyl-4(5)-[4-(2-pyrimidinyl)-piperazin-1-yl)-methyl]-imidazole;

NMA, normal mode analysis;

NOMAD-Ref, Normal Mode Analysis Deformation and Refinement);

OPLS-AA, Optimized Potentials for Liquid Simulations – All-Atom (Molecular Mechanics Forcefield);

PD168,077, N [[4 (2 Cyanophenyl) 1 piperazinyl]methyl] 3 methylbenzamide;

PME, particle-mesh Ewald;

(-)-Quinpirole, (4aR-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline;

RBI-257, 1 [4 iodobenzyl] 4 [N (3 isopropoxy 2 pyridinyl) N methyl] aminopiperidine;

Ro61-6270, 2-Amino-benzoic acid 1-benzyl-piperidin-4-yl ester;

TMS, transmembrane segment

ABSTRACT

The D2 dopamine receptor is an important therapeutic target for the treatment of psychotic, agitated and abnormal behavioral states. To better understand the specific interactions of subtype-selective ligands with dopamine receptor subtypes, seven ligands with high selectivity (>120 fold) for the D4 subtype of dopamine receptor were tested on wild type and mutant D2 receptors. Five of the selective ligands were observed to have 21-fold to 293-fold increases in D2 receptor affinity when three non-conserved amino acids in TM2 and TM3 were mutated to the corresponding D4 amino acids. The two ligands with the greatest improvement in affinity for the D2 mutant receptor (i.e., L-750,667 and RBI-257) were investigated in functional assays. Consistent with their higher affinity for the mutant than for the wild type receptor, concentrations of L-750,667 or RBI-257 that produced large reductions in the potency of quinpirole's functional response in the mutant did not significantly reduce quinpirole's functional response in the wild type D2 receptor. In contrast to RBI-257, which was an antagonist at all receptors, L-750,667 was a partial agonist at the wild type D2 but an antagonist at both the mutant D2 and wild type D4 receptors. Our study demonstrates, for the first time, that the TM2/3 microdomain of the D2 dopamine receptor not only regulates the selective affinity of ligands, but can, in select cases, also regulate their function. Utilizing a new docking technique that incorporates receptor backbone flexibility, the three non-conserved amino acids that encompass the TM2/3 microdomain were found to account in large part for the differences in intermolecular steric contacts between the ligands and receptors. Consistent with the experimental data, this model illustrates the interactions between a variety of subtype-selective ligands and the wild type D2, mutant D2, or wild type D4 receptors.

INTRODUCTION

Modulation of dopaminergic pathways is a principal aim of numerous pharmacological interventions because dopamine receptors play an integral role in movement, emotional cognition, memory, and attention. Parkinson's disease and schizophrenia are but two examples of severely debilitating disorders mitigated by dopaminergic treatments (Ustun TB et al., 1999) that prolong life and improve its quality. Still there is much to be learned concerning the molecular basis of selective ligand actions on dopamine receptors and analogous heterotrimeric G-protein coupled receptor systems.

Dopamine receptors are biogenic amine heterotrimeric G protein-coupled receptors (GPCRs) sharing structural homology with rhodopsin. Five genes coding for five subtypes of the dopamine receptor (D1-D5) are known to exist in mammals. With the exception of the D2 receptor partial agonist aripiprazole, the most commonly prescribed neuroleptics are thought to attenuate psychosis by antagonizing the D2 receptor subtype (Lawler et al., 1999; Shapiro et al., 2003; Schetz and Sibley, 2007). Although the D4 receptor was previously considered a drug target for the treatment of psychosis (Sanyal and Van Tol, 1997), the failure of three clinical trials suggests that this is not the case (Kramer et al., 1997; Truffinet et al., 1999; Corrigan et al., 2004). However, the D4 receptor has remained a potentially important clinical target due to its roles in regulating hyperactivity and CNS mediated penile tumescence (Schetz and Sibley, 2007; Schetz, 2009).

Previous work on the D4 receptor revealed that the non-conserved amino acids at positions 2.60, 2.61, 3.28 and 3.29 provide key structural determinants for drug selectivity between D2 and D4 receptor subtypes (Schetz et al., 2000). This conclusion was reached by analyzing reciprocal mutations, i.e., mutations where one or more amino acids in the D4

sequence were substituted with the corresponding amino acid of the D2 sequence. A more comprehensive study focusing on a large number of D4-selective ligands from the 1,4-disubstituted aromatic piperazine/piperidine (1,4-DAP) structural class revealed that a majority of 1,4-DAPs have a pattern of molecular recognition (mode-1) dominated by amino acids occupying positions 2.61, 3.28 and 3.29 (Kortagere et al., 2004). Related studies in a D2 receptor background have corroborated the importance of amino acids at position 2.61, 3.28, and 3.29 in the D2 receptor as binding determinants for several D4-selective ligands (Simpson et al., 1999); however, the functional properties of D2 mutant receptors with improved affinity for specific D4-selective drugs have never been investigated.

We report here for the first time the functional properties of a combined TM2/TM3 reciprocal mutant D2 receptor (D2-V2.61F+FV3.28-3.29LM) and demonstrate that it not only has increased affinity and improved functional sensitivity for D4-selective ligands, but that such ligands (L-750,667 and RBI-257) exert the same functional effects on the D2 mutant as they do on the wild type D4 receptor. These findings suggest that a D2 receptor microdomain which controls a ligand's selectivity can also control a ligand's function.

METHODS

Reagents. Cell culture media was purchased from Hyclone (Logan, UT, USA). [3H]Methylspiperone ([3H]MSP) (NET-856, 70-80 Ci/mmol) was purchased from Perkin Elmer (St. Louis, MO, USA). Other drugs were purchased from Sigma (St. Louis, MO, USA) or Tocris (Ellisville, MO, USA).

Site directed mutagenesis and Transfections. Mutagenesis was accomplished using a QwikChange^{TM} kit (Stratagene, CA, USA) and confirmed by full length sequencing. Mutant receptors were named by slight modification of the receptor nomenclature system as described previously (Ballesteros and Weinstein, 1995; Ericksen et al., 2009): each amino acid in a TMS is described by coordinates based on its position relative to the most conserved amino acid of that TMS (position 50). For example, D2-V2.61F denotes a valine to phenylalanine mutation at position 61 of TMS 2 in the D2 receptor. Plasmid DNA was transfected into cells by calcium phosphate precipitation as described previously (Ericksen et al., 2009). Stable receptor expression was maintained under 100 μg/mL G418 drug selection pressure.

Membrane Preparation and Radioligand Binding Assays. Cell membranes were prepared as described previously (Ericksen et al., 2009) by suspension in lysis buffer (5 mM Tris, 5 mM $MgCl_2$, pH 7.4, 4°C) for 5-10 minutes followed by homogenization with eight strokes of a dounce homogenizer and centrifugation at 28,000 x *g* for 45 min. The resulting pellet was resuspended in cold binding buffer and recentrifuged. The final membrane pellet was resuspended in cold binding buffer, homogenized with four strokes and stored on ice until use. Radioligand binding buffer consisted of 50 mM Tris adjusted to pH 7.4 at 25°C with 1N KOH or 1N HCl (Ericksen et al., 2009). For saturation isotherms, cell membranes were equilibrated (90 min) with increasing concentrations of [^{3}H]MSP in the presence or absence of 5 μM (+)-butaclamol, a drug used to define non-specific binding. For competition binding experiments, a fixed concentration of 0.5 nM [^{3}H]MSP was equilibrated (90 minutes) with increasing concentrations of non-radiolabeled competing ligand. Equilibrated samples were rapidly filtered through Whatman GF/C filters (Brandel, Gaithersburg, MD, USA)pretreated with 0.3%

poly(ethyleneimine) (10 min) and washed three times with 3 mL of ice cold binding buffer (50 mM Tris pH 7.4, 0°C). Radioactivity was quantified in a scintillation counter. Membrane protein concentration was determined by bicinchoninic acid assay (Pierce, IL, USA).

Cyclic Adenosine Monophosphate Functional Assays. Intracellular cyclic AMP concentrations were determined using a cyclic AMP Alphascreen™ detection kit (Perkin Elmer, Waltham, MA, USA) as described previously (Ericksen et al., 2009). Briefly, HEK293 cells were seeded at 50,000 cells/well in a poly-L-lysine coated 96-well microtiter plate. Stimulation buffer (DMEM, 20 mM HEPES, 100 µM sodium metabisulfite, 30 µM Ro 20-1724), cell lysis buffer (0.3% Tween 20, 20 mM HEPES, 1 µg/µl BSA), and bead buffer (20 mM HEPES, 30 µM Ro 20-1724, 1 µg/µl BSA, 1X Hank's Balanced Salt Solution) were prepared the next morning and adjusted to pH 7.4 with 1N cell culture tested NaOH (Sigma-Aldrich, St. Louis, MO, USA). Drug dilutions prepared in stimulation buffer containing 6 µM forskolin were equilibrated (37 °C, 5% CO_2) for 30 minutes. Cells were challenged with the equilibrated dilutions for 20 minutes (37 °C, 5% CO_2) followed by 5 minute centrifugation at 1500 x *g*. Cells were lysed by 90 minutes of shaking in 100 µL of cell lysis buffer. Prior to quantification, half units of acceptor and donor beads preincubated with biotinylated cyclic AMP (3.76 nM) were equilibrated (1 h) with 30 µL of each sample in an opaque 96-well Costar plate (Corning, NY, USA) protected from light. Samples were read on a Perkin-Elmer Alpha Fusion™ plate reader in alpha mode using a 2 second window (680 nm excitation at 0.6 sec/sample and 520-620 emission 1.4 sec/sample).

Calculations and Data Analysis. All data points for each experiment were sampled in triplicate and each experiment was repeated three times, unless noted otherwise. All data were analyzed using Prizm version 4.0 (Graphpad Software Inc., CA). Geometric means and standard deviations are reported in the tables and standard errors of the mean (S.E.M.) are plotted in the graphs. For saturation isotherm analysis, specifically bound [^{3}H]MSP was calculated by subtracting non-specifically bound [^{3}H]MSP (defined as binding in the presence of 5 μM (+)-butaclamol) from the total bound [^{3}H]MSP at each concentration of [^{3}H]MSP. The equilibrium dissociation constant (K_D) for [^{3}H]MSP and the receptor density (B_{max}) were determined by best-fitting the specific binding data versus the concentration for free radioligand to a single-site square hyperbola curve. Inhibition constants (K_i) for unlabeled ligands were calculated using the Cheng-Prusoff equation: $K_i = IC_{50}/(1+[\text{radioligand}]/K_D)$. For cases where drug solubility was limited, IC_{50} values were generated by extrapolating concentration-response curves to zero. All cAMP values were converted to the amount of cyclic AMP generated per milligram of sample protein (cyclic AMP/mg) then normalized relative to the amount of cyclic AMP generated by 6 μM forskolin in the absence of agonist. Efficacy was determined by subtracting the best-fit values for the lowest horizontal asymptote from the highest horizontal asymptote of the fitted sigmoidal semi-log concentration response curves. Statistical analyses of the curve fitting procedures included the run test, F-test, and Pearson's correlation coefficient. Values were analyzed by one-way ANOVA and Dunnett's post-hoc analysis with significance established at the 95% confidence level ($p \leq 0.05$).

Construction of Receptor Homology Models. D2 and D4 receptor structures were constructed with Modeller 9v1 (Šali and Blundell, 1993) using both the bovine rhodopsin (Li et al., 2004)

(*1GZM*) and β_2 adrenergic (Cherezov et al., 2007) (*2RH1*) structures as templates. For each dopamine receptor model, 1000 structures were generated and ranked using Modeller's objective function. The models were then aligned and clustered using the GROningen Machine for Chemical Simulations (GROMACS) (v3.3; Uppsala, Sweden) (Van der Spoel et al., 2005) *gcluster* utility (cutoff = 0.20 Å). The most representative (central) structure from the first (wild type D2 receptor) and second (wild type D4 receptor) most-populated clusters were used for minimization, normal mode analysis (NMA), and docking. These structures were minimized *in vacuo* using the L-BGFS method in two stages, each applied to convergence (F_{max} < 10 kJ mol^{-1} nm^{-1}) using GROMACS v3.3 (Van der Spoel et al., 2005) with the molecular systems parameterized according to the Optimized Potentials for Liquid Simulations (OPLS) all-atom forcefield (Jorgensen et al., 1996). Electrostatic interactions were treated by the particle-mesh Ewald (PME) method. In the first stage all backbone protein atoms were restrained with half-harmonic force restraints (k = 1000 kJ mol^{-1} nm^{-1}). In the second minimization stage, only C_α atoms were restrained.

Building Ensembles of Receptor Conformers. To obtain receptor conformers based on perturbations along low-frequency normal modes, each minimized receptor model was reduced to a C_α-only elastic network model (ENM) and submitted to NOMAD-Ref web server for NMA and automated decoy generation using default parameters with the exception of the distance weighting parameter. For the distance weighting parameter a non-default value of 3.0 Å was applied as recommended for C_α-only models and the approximate average C_α root mean square deviation (Å) of the decoy value was set to 1 Å to focus on smaller backbone motions near the initial conformation. (http://lorentz.immstr.pasteur.fr/decoys/submission.php) (Lindahl et al.,

2006). Based on the motion of the first five, non-trivial (non-zero-eigenvalue), normal modes, 1000 decoy structures were generated from random linear combinations of movements along the five mode eigenvectors. These five modes embody TMS movements that appeared to influence binding crevice geometry. Vibrational modes beyond the lowest five frequencies were excluded because they involved motion in the non-binding cleft regions or relatively low amplitude motions in the TM core region. For the resulting 1000 decoys generated for each receptor we rebuilt all-atom structures on the C_α frames. Backbone and side chain atoms were built onto the fixed C_α template with Modeller 9v1, followed by minimization, a short 15 ps MD run, and minimization with C_α atoms fixed for each procedure. The structure was then minimized to convergence with positional restraints on the C_α carbons in GROMACS, as described above. For use in docking, the 1000 rebuilt and minimized decoys for each receptor were clustered, as described above, into a more manageable subset of 24-25 representative conformers.

Docking Ligands into Receptor Conformers. The ligands were constructed in Discovery Studio (Accelrys, Inc., San Diego, CA, USA) and geometry optimized in Gaussian03 with ab initio quantum mechanical calculations using the Hartree-Fock 6-31G** basis set (Frisch et al., 2004). The partial charges were set according to the automated Gasteiger partial charge assignment of AutoDockTools 1.4.5 (The Scripps Research Institute, La Jolla, CA, USA). Each ligand was docked 25 times to each receptor conformation (24-25 conformers) using the default Lamarckian genetic algorithm search routine in AutoDock 4.0 (Morris et al., 1998) with non-default parameters for increased genetic algorithm population size (increased to 500) and increased maximum number of energy evaluations (ramped up to 2.5×10^6). Selective side chain flexibility was allowed within the docking routine: the rotation of dihedrals in the side chains of

2.61, 3.28, 3.29, 3.32, and 6.51 were explored because of their apparently critical positions in the ligand binding site and influences on cleft shape. After docking with each receptor conformation, poses were clustered using an Root Mean Square Deviation (RMSD) tolerance of 3.0 Å and then ranked by mean energy. Poses with reinforced ionic interaction between the ligand's protonatable amine and the conserved aspartate at position 3.32 (D3.32) were visually screened for mode-1 (Kortagere et al., 2004) orientations in the binding cleft (see *Results* for a description). Complexes with poses meeting these criteria were included in the subset used to obtain the contact-likelihood matrices (CLMs) from the intermolecular contacts extracted with Ligplot v4.4.2 (Wallace et al., 1995). PyMOL v0.99 (DeLano Scientific LLC, San Carlos, CA, USA) was used for rendering figures.

RESULTS

To characterize the subtype selective binding domain in a D2 receptor background, we tested an extensive panel of D4-selective 1,4-DAPs that were previously examined in the D4

Figure 2-1. D4-selective ligands L-750,667 and RBI-257 have significantly enhanced affinity for the mutant D2-FV3.28-3.29LM and D2-V2.61F+FV3.28-3.29LM receptors relative to the wild type D2 receptor. (-)-Quinpirole did not display significantly enhanced affinity at these mutants. Graphs are the averaged values of three radiolabeled competition binding experiments between [^{3}H]methylspiperone and A) L-750,667, B) RBI-257, or C) (-)-quinpirole; at the wild type D2 (●), mutant D2-FV3.28-3.29LM (■), or mutant D2-V2.61F+FV3.28-3.29LM (▼) receptors. Data is graphed as the geometric mean ± S.E.M. Corresponding affinity values and fold changes relative to the wild type D2 receptor are listed in Table 2-1.

Figure 1

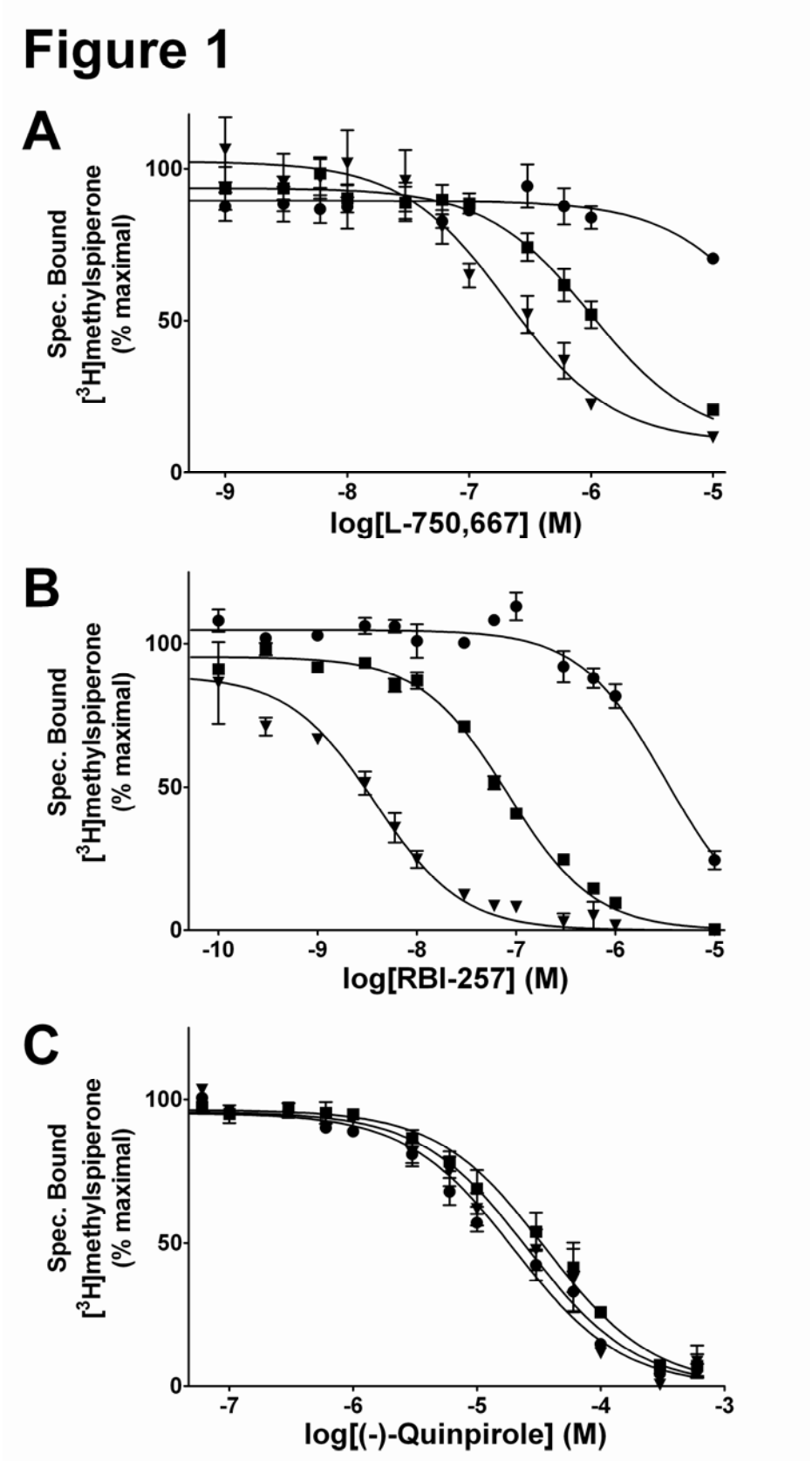
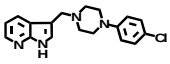
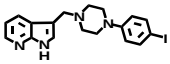
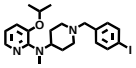
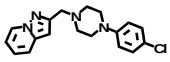
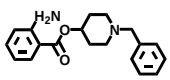
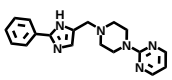
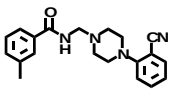
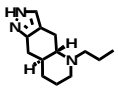

A
Spec. Bound
[3H]methylspiperone
(% maximal)
100
50
0
-9
-8
-7
-6
-5
log[L-750,667] (M)
B
Spec. Bound
[3H]methylspiperone
(% maximal)
100
50
0
-10
-9
-8
-7
-6
-5
log[RBI-257] (M)
C
Spec. Bound
[3H]methylspiperone
(% maximal)
100
50
0
-7
-6
-5
-4
-3
log[(-)-Quinpirole] (M)

TABLE 2-1 Affinities of D4-selective ligands for wild type D2, mutant D2-FV3.28-3.29LM or mutant D2-V2.61F+LM3.28-3.29FV receptors expressed in COS-7 cells. Affinity values (K_i or K_D) are the geometric mean ± S.D. of three experiments. Affinity values for the wild type D4 receptor are listed for comparison. Fold change relative to the wild type D2 receptor is shown within parentheses below the corresponding affinity values. Decreases in K_i values (corresponding to increased affinity) are denoted with ↓ arrows and increases in K_i values (corresponding to decreased affinity) are denoted with ↑ arrows. Significant changes in mutant receptor affinity relative to the wild type D2 receptor were evaluated by Dunnett's Multiple Comparison Test (*p<0.05). N.D. means not determined.

Ligand	Structure	D2-WT	D2-FV3.28-3.29LM	D2-V2.61F+FV3.28-3.29LM	D4-WT
L-745,870 (CPPMA)		**656 ± 227 (1)**	**117 ± 95* (↓5.6)**	**11 ± 4.3* (↓60)**	**0.32 ± 0.14 (↓2050)**
L-750,667[c]		**1400 ± 950 (1)**	**150 ± 87 (↓9.3)**	**14 ± 4.2* (↓100)**	**0.11 ± 0.02**[a] **(↓13400)**
RBI-257		**85 ± 12 (1)**	**3.8 ± 0.73* (↓22)**	**0.29 ± 0.07* (↓293)**	**0.27 ± 0.10**[b] **(↓315)**
FAUC213[c]		**1300 ± 640 (1)**	**190 ± 100 (↓6.8)**	**32 ± 16* (↓41)**	**1.1 ± 0.22**[b] **(↓1030)**
Ro61-6270		**655 ± 274 (1)**	**136 ± 43* (↓4.8)**	**31 ± 2.5* (↓21)**	**0.89 ± 0.12**[b] **(↓736)**
NGD 94-1		**817 ± 284 (1)**	**64 ± 34* (↓13)**	**127 ± 17* (↓6.4)**	**0.3 ± 0.04**[b] **(↓2720)**
PD168,077		**1380 ± 64 (1)**	**41 ± 4.4* (↓34)**	**287 ± 30 (↓4.8)**	**1.5 ± 0.41**[b] **(↓540)**
(-)-Quinpirole		**812 ± 617 (1)**	**2461 ± 2230 (↑3.0)**	**3075 ± 3009 (↑3.8)**	**N.D.**

[a] Schetz et al., 2000; [b] Kortagere et al., 2004; [c] Floresca et al., 2005

receptor background (Kortagere et al., 2004). When mutant D2-FV3.28-3.29LM and D2-V2.61F+FV3.28-3.29LM receptors were transiently expressed in COS-7 cells, the levels of expression and the affinities for the moderately D2-selective 1,4,-DAP [^{3}H]MSP were comparable to the wild type D2 receptor (1.1-fold to 1.3-fold and 1.7-fold to 2.3-fold, respectively, Floresca et al., 2005). The affinities of the mutant D2 receptors for the agonist (-)-quinpirole, which lacks a 1,4-DAP structural motif, were similar to those of the wild type D2 receptor (1.2-fold to 3.8-fold, Figure 2-1 and Table 2-1). The small to moderately improved affinities (4.8-fold to 22-fold) observed for the mutant D2-FV3.28-3.29LM receptor for five of the 1,4-DAPs (L-745,870, L-750,667, FAUC213, Ro61-6270, RBI-257) improved further (21-fold to 292-fold) for the mutant D2-V2.61F+FV3.28-3.29LM receptor (Table 2-1). The two remaining 1,4-DAPs (NGD 94-1 and PD168,077) had higher affinities for the mutant D2-FV3.28-3.29LM receptor (13-fold and 34-fold, respectively), than for the mutant D2-V2.61F+FV3.28-3.29LM receptor (4.8-fold to 6.4-fold, respectively) whose affinities were closer to the wild type D2 receptor (Table 2-1). RBI-257 and L-750,667, the compounds with the largest improvements in affinity for the D2-V2.61F+FV3.28-3.29LM receptor relative to the wild type D2, were selected for further characterization in functional assays.

Although identification of binding site residues that could impart 1,4-DAP recognition and D2/D4 preference was important, we were more interested in characterizing changes in subtype specific function related to the subtype selective binding domain of the D2 receptor. COS-7 cells, while appropriate for binding experiments, were unable to inhibit cyclic AMP formation through activation of D2-like dopamine receptors (data not shown). Therefore the HEK293 line, which lacks endogenous dopamine receptors but contains G proteins capable of mediating the cyclic AMP responses of transfected D2 or D4 dopamine receptors (Gazi et al.

1998, Chemel et al., 2006), was selected for functional assessments. Wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors were stably expressed in HEK293 cells and characterized by receptor binding studies. The cell surface density of dopamine receptors expressed in several stable HEK293 clones was determined by [^{3}H]MSP saturation isotherm analysis. The corresponding [^{3}H]MSP affinity values (K_D) were 0.78 ± 0.39 nM for the wild type D2 receptor and 1.6 ± 0.76 nM for the D2-V2.61F+FV3.28-3.29LM receptor. Note that while absolute affinity values for [^{3}H]MSP were quite difference for wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors expressed in COS-7 and HEK293 cells, the relative difference between wild type and mutant D2 receptors within a cell type were essentially the same (2.3-fold vs. 2.1-fold, respectively). To limit any ambiguity caused by unequal receptor expression, clones expressing wild type or mutant receptors were matched by receptor density. HEK293 cell lines expressing 4.6 ± 1.6 pmol/mg protein of wild type D2-WT receptor or 4.6 ± 2.5 pmol/mg protein of mutant D2-V2.61F+FV3.28-3.29LM receptor were analyzed for their ability to inhibit cyclic AMP formation.

Because RBI-257 had strikingly different affinity for the wild type and mutant D2 receptors, preliminary tests to determine the most appropriate concentration range for investigating the antagonism of (-)-quinpirole function at the wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors were performed (data not shown). Based on these results, the following concentrations of RBI-257 were selected: 0.2 μM and 2 μM for the D2-V2.61F+FV3.28-3.29LM receptor and 2 μM and 20 μM for the D2-WT receptor. Similar tests performed for L-750,667 resulted in the selection of 0.5 μM L-750,667 as an appropriate single concentration for comparing functional antagonism of (-)-quinpirole at the wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors. Concentration-response curves demonstrated that

TABLE 2-2 Potency and percent efficacy values for (-)-quinpirole alone and with competing ligand (L-750,667 or RBI-257) at the wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors. Potency (EC_{50}) and percent efficacy are expressed as the geometric mean ± S.D. of three experiments. Fold changes relative to (-)-quinpirole response in the absence of competing ligand are in parentheses with decreases denoted by ↓ arrows (higher relative potency or lower relative efficacy) and increases denoted with ↑ arrows (lower relative potency or higher relative efficacy). Significance relative to the wild type D2 receptor was evaluated by one-way ANOVA with Dunnett's post hoc analysis *p<0.05. N.D. means not determined.

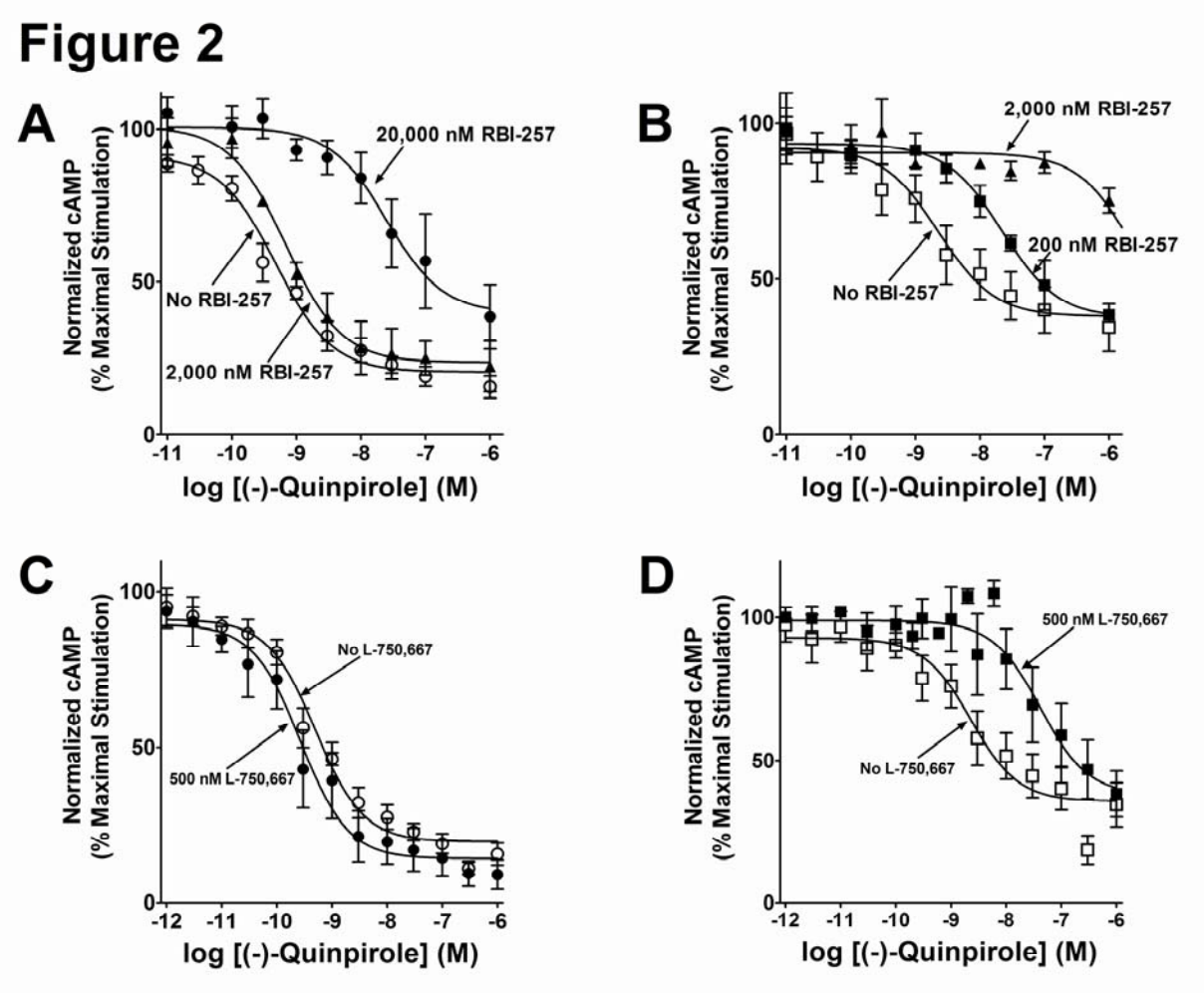
	D2-WT		**D2-V2.61F+FV3.2-3.29LM**	
Ligands	**EC_{50} (nM)**	**% Efficacy**	**EC_{50} (nM)**	**% Efficacy**
(-)-Quinpirole	**0.46 ± 0.06 (1)**	**73 ± 2.0 (1)**	**2.0 ± 0.94 (1)**	**60 ± 6.5 (1)**
(-)-Quinpirole + 0.2 µM RBI-257	**N.D.**	**N.D.**	**21 ± 5.4 (↑11)**	**60 ± 8.0 (1)**
(-)-Quinpirole + 2.0 µM RBI-257	**0.70 ± 0.20 (↑1.5)**	**78 ± 5.2 (↑1.1)**	**>10,000* (↑ >5000)**	**N.D.**
(-)-Quinpirole + 20 µM RBI-257	**55 ± 26* (↑120)**	**64 ± 9.9 (↓1.2)**	**N.D.**	**N.D.**
(-)-Quinpirole + 0.5 µM L-750,667	**0.37 ± 0.14 (↓1.2)**	**64 ± 8.7 (↓1.2)**	**98 ± 50* (↑49)**	**68 ± 7.8 (↑1.1)**

the mutant receptor had 4.3-fold lower potency and 18% less efficacy for the agonist (-)-quinpirole than did the wild type D2 receptor (Table 2-2). Further, the right shift in (-)-quinpirole potency induced by RBI-257 occurs at a much lower RBI-257 concentration in the mutant D2-V2.61F+FV3.28-3.29LM than in the wild type D2 receptor: a >5000-fold potency shift is observed in the presence of 2 µM RBI-257 for the mutant D2-V2.61F+FV3.28-3.29LM receptor

with no significant shift for the wild type D2 receptor (Figure 2-2A-B and Table 2-2). However, a 10-fold higher concentration of RBI-257 was able to significantly shift the potency (120-fold) of (-)-quinpirole for the wild type receptor (Table 2-2 and Figure 2-2A). A similar pattern of shifted (-)-quinpirole potency was observed for L-750,667 (Table 2-2 and Figure 2-2D). Neither RBI-257 nor L-750,667 had statistically significant effects on the efficacy of (-)-quinpirole (≤ 20% effect, Table 2-2 and Figure 2-2). No attempt was made to quantify the functional antagonism of these ligands as pK_b values (Schild analysis) because D4 selective ligands and (-)-quinpirole do not compete for the same binding site (Table 2-1) which is an assumption that must be met to yield meaningful values (Kenakin et al. 1997).

Assays of cyclic AMP accumulation designed to differentiate the functional properties of D4-selective 1,4-DAPs revealed that RBI-257 was an antagonist at the wild type D2, wild type

Figure 2-2. Much lower concentrations of RBI-257 are needed to antagonize the (-)-quinpirole induced functional activation of the mutant D2-V2.61F+FV3.28-3.29LM in comparison to the wild type D2 receptor. In the presence of 2 μM RBI-257 a greater than 5000-fold decrease in (-)-quinpirole potency was observed for the mutant but not the wild type receptor. A) Functional activation of the wild type D2 receptor by (-)-quinpirole alone (○) or co-incubated with 2 μM (▲) or 20 μM (●) of RBI-257. B) Functional activation of the mutant D2-V2.61F+FV3.28-3.29LM receptor by (-)-quinpirole alone (□) or co-incubated with 0.2 μM (■) or 2 μM (▲) of RBI-257. C) (-)-Quinpirole in the absence (○) or presence (●) of 500 nM L-750,667 at the wild type D2 receptor. D) (-)-Quinpirole in the absence (□) or presence (■) of 500 nM L-750,667 at the mutant D2-V2.61F+FV3.28-3.29LM receptor. Graphs represent the geometric mean ± S.E.M of three experiments. The corresponding potency and efficacy values are listed in Table 2-2.

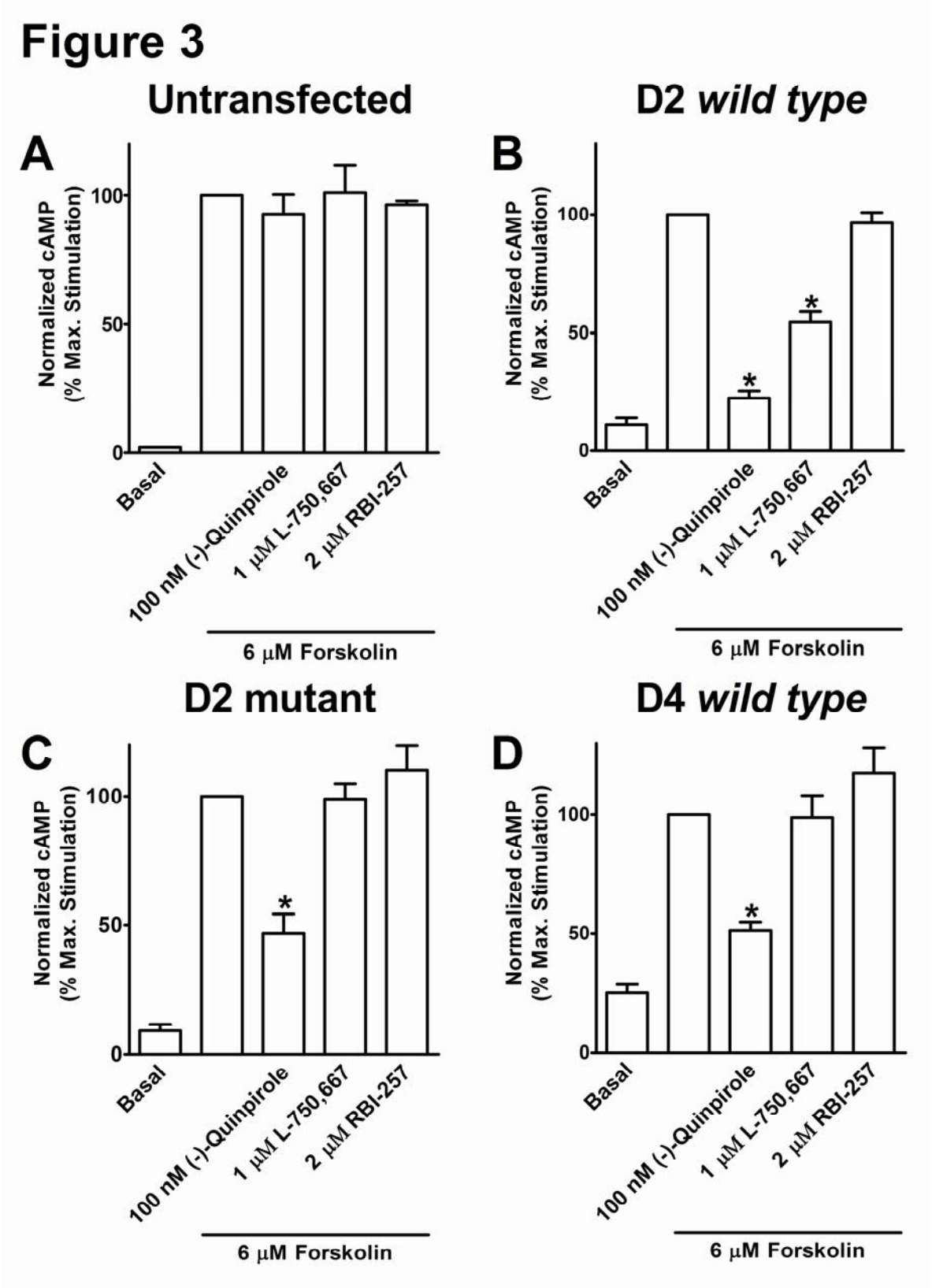


D4, and the mutant D2-V2.61F+FV3.28-3.29LM receptors (Figure 2-3B, D). In contrast, L-750,667, which acted as an antagonist at the wild type D4 and mutant D2-V2.61F+FV3.28-3.29LM receptors, exhibited partial agonism at the wild type D2 receptor (Figure 2-3B-D). Neither (-)-quinpirole nor L-750,667 were able to reduce forskolin-stimulated cyclic AMP accumulation in untransfected HEK293 cells (Figure 2-3A), i.e., those lacking dopamine receptors. Further confirmation of the partial agonist properties of L-750,667 at the wild type D2, but not the mutant D2-V2.61F+FV3.28-3.29LM receptor, was established by evaluating the effect of increasing concentrations of L-750,667 at a fixed 60 nanomolar concentration of (-)-

quinpirole (Figure 2-4). At higher concentrations in the mutant D2-V2.61F+FV3.28-3.29LM receptor, L-750,667 completely reversed the (-)-quinpirole-stimulated functional response confirming functional antagonism of the mutant. In contrast, the highest concentrations of L-750,667 were only able to partly decrease (-)-quinpirole-stimulated wild type D2 receptor inhibition of cyclic AMP formation. This reduced maximal functional response confirmed that L-750,667 was a partial agonist of the wild type D2 receptor. The EC_{50} for antagonism of the (-)-quinpirole functional response by L-750,667 was 3.2-fold higher for the mutant than the wild type receptor (0.57 ± 0.27μM and 1.9 ± 2.3μM respectively, Figure 2-4).

Figure 2-3. L-750,667 exhibits partial agonism at only the wild type D2 receptor. While L-750,667 was identified as a partial agonist of the wild type D2 receptor, this ligand behaved as an antagonist at both the mutant D2-V2.61F+FV3.28-3.29LM and the wild type D4 receptors. Overall, the functional profile observed for the mutant D2 receptor was similar to the wild type D4 receptor but not the wild type D2 receptor. No significant response was observed for A) untransfected HEK293 cells. HEK 293 cells stably expressing either B) wild type D2 receptors, C) mutant D2-V2.61F+FV3.28-3.29LM receptors, or D) wild type D4 receptors, when stimulated by (-)-quinpirole, had respective 65 ± 1.4%, 53 ± 7.4%, and 49 ± 5.1% decreases in the concentration of forskolin stimulated cyclic AMP. Only at the wild type D2 receptor, did L-750,667 significantly decrease the concentration of forskolin-induced cyclic AMP (42 ± 9.2%, *$p<0.05$). Values are the geometric mean ± S.E.M. of three experiments. Statistically significant decreases in cyclic AMP relative to the forskolin control were determined at 95% confidence by one-way ANOVA with Dunnett's post-hoc analysis and indicated by asterisks (*).

Figure 3

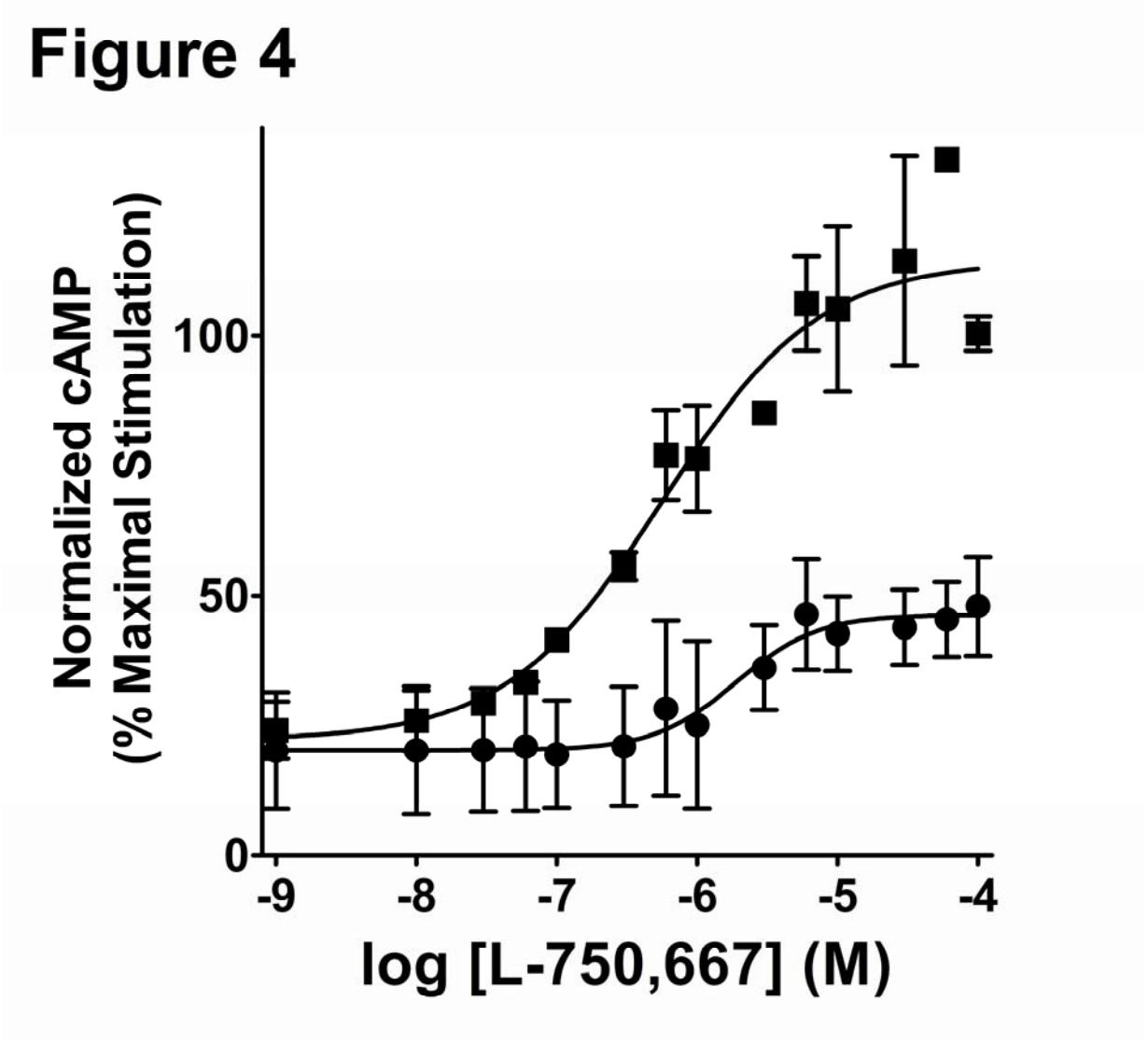

Untransfected
A
Normalized cAMP (% Max. Stimulation)
100
50
0
Basal
100 nM (-)-Quinpirole
1 μM L-750,667
2 μM RBI-257
6 μM Forskolin
D2 wild type
B
Normalized cAMP (% Max. Stimulation)
100
50
0
*
*
Basal
100 nM (-)-Quinpirole
1 μM L-750,667
2 μM RBI-257
6 μM Forskolin
D2 mutant
C
Normalized cAMP (% Max. Stimulation)
100
50
0
*
Basal
100 nM (-)-Quinpirole
1 μM L-750,667
2 μM RBI-257
6 μM Forskolin
D4 wild type
D
Normalized cAMP (% Max. Stimulation)
100
50
0
*
Basal
100 nM (-)-Quinpirole
1 μM L-750,667
2 μM RBI-257
6 μM Forskolin

To examine the role that residues conferring D4/D2 selectivity have on the positioning of 1,4-DAPs in the binding cleft, several 1,4-DAPs (L-750,667, RBI-257, NGD 94-1, and PD168,077) were docked into *w*ild type D2, mutant D2-V2.61F+FV3.28-3.29LM, and wild type D4 homology models. The resulting complexes (600-625) were screened using two geometric criteria: a) an H-bond reinforced ionic interface formed by the protonatable amine group of the ligand interacting with the conserved aspartate at position 3.32 of biogenic amine receptors (for review see Floresca and Schetz 2004) and b) pose orientations where the short arm of the 1,4-DAP (Arm-B, Kortagere et al., 2004) was directed, in accordance with prior experimental evidence from our lab, at the region of TMS 2 and 3 rather than TMS 5 and 6 (Kortagere et al., 2004). These criteria focused our investigations to the ligand poses that were most suitable for residue contact analysis.

When ligand poses that met our criteria were examined, consistent patterns of intermolecular contacts emerged that reconciled some of the observed selectivity features attributed to positions 2.61, 3.28 and 3.29. The poses observed for L-750,667 in both the mutant D2-V2.61F+FV3.28-3.29LM and wild type D4 receptors direct the *p*-iodophenyl ring of Arm-B

Figure 2-4. The concentration dependent partial reversal of (-)-quinpirole agonism by L-750,667 confirms this ligand as a partial agonist of the wild type D2 receptor. The full agonist effect of 60 nM (-)-quinpirole, was concentration-dependently reversed by L-750,667 in wild type D2 (●) and mutant D2-V2.61F+FV3.28-3.29LM (■) receptors. In contrast, to the complete reversal (EC_{50} = 0.57 μM) of (-)-quinpirole agonism at the D2-V2.61F+FV3.28-3.29LM receptor, the partial reversal (EC_{50} = 1.9 μM) of (-)-quinpirole agonism at the wild type D2 receptor confirmed L-750,667 as an unequivocal wild type D2 receptor partial agonist. Data is graphed as the geometric mean ± S.E.M.

Figure 4

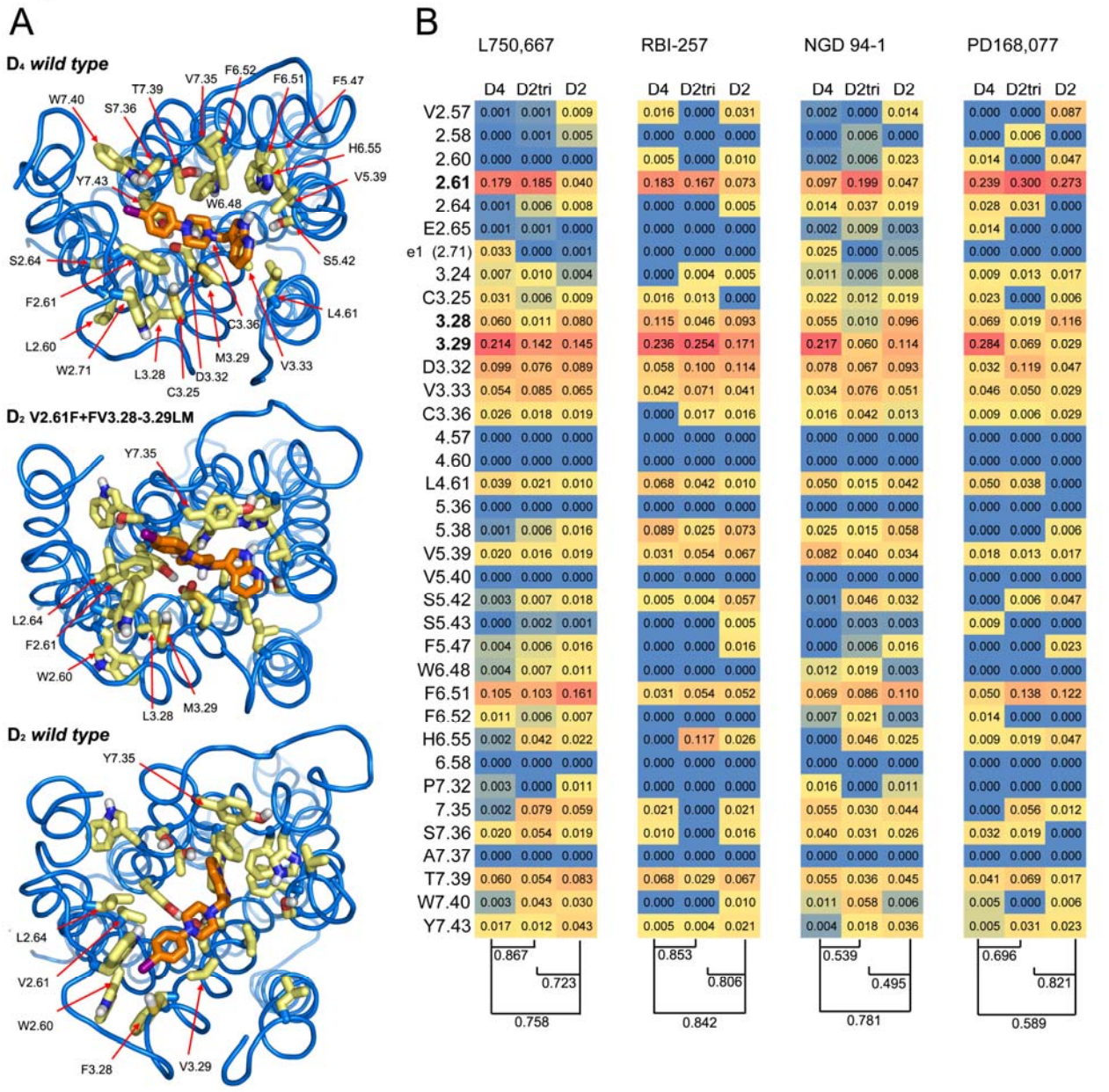


toward F2.61, a resident of the cleft formed by TMS 2, 3, and 7 (Figure 2-5A top and middle panels). A similar orientation was observed for L-745,870, the *p*-chloro analogue of L-750,667, in the D2-V2.61F receptor (Ericksen et al., 2009). However, despite similarities between the ligand orientation and contact residues in wild type D4 and mutant D2-V2.61F+FV3.28-3.29LM receptors, in the wild type D2 receptor L-750,667 was rotated counterclockwise in the binding pocket which allowed the *p*-iodophenyl ring to make significant contact with the phenylalanine of 3.28 within the TM2/3 cleft (Figure 2-5A). These changes in the preferred binding

Figure 2-5. The TM2/3 microdomain accounts for a large portion of the intermolecular contacts between 1,4-DAPs and the D2 or D4 receptor backgrounds. A) Representative poses of L-750,667 bound to: Top panel) wild type D4, Middle panel) mutant D2-V2.61F+FV3.28-3.29LM, or Bottom panel) wild type D2 receptors. Blue ribbons represent the receptor backbones, yellow sticks represent the side chains of primary contact residues for each pose, and orange sticks represent L-750,667. In achieving a similar set of intermolecular contacts, the orientation of L-750,667 bound to the mutant D2 receptor was very similar to the ligand orientation in the wild type D4 receptor. In contrast to the orientation in the wild type D4 receptor, the counterclockwise rotated orientation of L-750,667 (from an extracellular perspective) in the wild type D2 receptor was achieved by the promotion of contact between the para-halogenated Arm-B aryl moiety and the TM2/3 interface. Thus, the binding orientation of L-750,667 was found to be sensitive to the identity of positions 2.61, 3.28 and 3.29. B) Normalized Ligand-Receptor Contact Likelihood Matrices (CLMs). The contact likelihood index, a measure of the probability the ligand will interact with specific binding cleft residues, is taken from the summation of intermolecular contacts (< 3.9 Å) counted for the subset of docking poses that meet the experimentally-suggested interaction criteria as defined in results section. Matrix elements are colored according to the normalized likelihood of contact index value and scaled from blue (low) to red (high). No residue type is specified for positions that vary between the wild type D2 and D4 backgrounds. Correlations for contact index value distributions between receptor constructs and each ligand are given at the bottom of the columns. The two para-halogenated (L-750,667 and RBI257) and two orthoelectronegative (NGD 94-1 and PD168,077) 1,4-DAPs used to construct this matrix show that Arm-B positions 2.61, 3.28, and 3.29 appear to provide key determinants for the D2/D4-selectivity of L-750,667 and RBI257, based on their

influence on the ligand-receptor contact distributions. The role for these positions is less clear for NGD 94-1 and PD168,077 based on this contact model, which falls in line with experimental observations of these orthoelectronegative 1,4-DAP.

Figure 5



configuration for L-750,667 and RBI-257 (5B, right panel; wire model not shown) suggest that positions 2.61, 3.28, and 3.29 provide recognition features integral to positioning 1,4-DAPs with para-halogenated Arm-B moieties in the binding pocket.

To quantitatively compare the observed intermolecular contact distributions for 1,4-DAPs docked to the receptor models, tendencies within a ligand-receptor pairing for ligand-residue interactions were plotted as Contact Likelihood Matrices (CLMs, Figure 2-5B). Each CLM was constructed from the sum of specific ligand–residue contacts (< 3.9 Å) normalized against the total amount of residue contacts (< 3.9 Å) achieved within the entire subset of ligand-receptor poses that passed the screening criteria (see details above). For L-750,667 and RBI-257, the distribution of CLM index values was better correlated between the wild type D4 and mutant D2-V2.61F+FV3.28-3.29LM receptors (correlation = 0.867), than the wild type D2 and mutant D2-V2.61F+FV3.28-3.29LM receptors (correlation = 0.723). This showed quantitatively that the distribution of contacts achieved for poses of L-750,667 in the mutant D2-V2.61F+FV3.28-3.29LM receptor were more like the poses observed at wild type D4 receptor and less like the poses observed at the wild type D2 receptor. A similar trend (0.863 versus 0.806) in the CLM was observed for RBI-257. While this trend was consistent for para-halogenated 1,4-DAPs (L-750,667 and RBI-257) the results for ortho-electronegative 1,4-DAPs (NGD 94-1 and PD168,077) exhibited complex patterning of the CLM in which mutant D2-V2.61F+FV3.28-3.29LM receptor poses were dissimilar to poses in both wild type D2 and wild type D4 receptors. These findings for NGD 94-1 and PD168,077 suggested that 1,4-DAPs with an orthoelectronegative Arm-B group did not adopt D4-like binding modes in the mutant D2-V2.61F+FV3.28-3.29LM receptor and that mutations of the TM2/3 microdomain were not sufficient for conferring orthoelectronegative 1,4-DAP selectivity to the mutant receptor. Our

observation was supported by the revertant affinities of NGD 94-1 and PD168,077 for the mutant D2-V2.61F+FV3.28-3.29LM receptor meaning that this mutant's affinity profile looked more like the wild type D2 receptor than did the mutant D2-FV3.28-3.29LM (Table 2-1).

Despite specific differences in ligand interaction between receptor subtypes, Figure 2-5B revealed a general qualitative similarity within the CLMs. For 1,4-DAPs, the contact residues with the strongest ligand-residue interactions were typically the following positions (in order of general prominence in contact index value): V/F2.61, V/M3.29, F6.51, D3.32, F/L3.28, V3.33, T7.39, V5.39, L4.61, Y/V7.35, H6.55, F/Y5.38, S7.36, S5.42, Y7.43, C3.36, W7.40, V2.57, C3.25, and L/S2.64. Interestingly, 2.61, 3.28, and 3.29 represented three of the top five contact sites in terms of CLM index values and comprised three of the six contact positions that varied in residue identity between the wild type D2 and wild type D4 receptors.

DISCUSSION

The three amino acid TM2/3 microdomain that forms the extracellular portion of the binding site crevice has been suggested, on the basis of radioligand binding studies, to be a site of interaction for ligands with high selectivity for the D4 subtype of dopamine receptor (Kortagere et al. 2004; Schetz et al., 2000; Simpson et al., 1999). Recognition that the receptor background (i.e., D2 versus D4), ligand structures, and binding reaction conditions (Floresca et al., 2005; Ericksen et al, 2009) greatly influenced the experimental outcome of previous TM2/3 microdomain studies (Kortagere et al. 2004; Schetz et al., 2000; Simpson et al., 1999) lead us to ask whether the difference in the observed binding affinities for D4 selective ligands were the result of localized changes in the TM2/3 microdomain or global changes in the receptor complex. It was reasoned that rigorous assessment of the overall state of the mutant receptor

should include a measure of its functional properties relative to the wild type receptor, as has only been done here. Assessment of the functional properties of a combined TM2/3 reciprocal D2 mutant receptor (D2-V2.61F+FV3.28-3.29LM) revealed improved sensitivity of the mutant receptor for the D4-selective ligands L-750,667 and RBI-257. Further, these ligands produced the same functional outcome at both the D2 mutant and wild type D4 receptors. In contrast, non-selective ligands, such as methylspiperone and (-)-quinpirole, were insensitive to these TM2/3 microdomain alterations. These findings demonstrated that the three amino acids exert localized effects while regulating the affinity and function of D4-selective ligands. A molecular docking technique that permits flexibility in the receptor backbone was used to enhance the accommodation of 1,4-DAPs into representations of the homology-modeled crystalline receptor structure. Consistent with the empirical results, the docking studies suggested that the three amino acid TM2/3 microdomain accounts, not only for a large portion of the differences in receptor phenotypes, but also for a large portion of the differences in steric contacts between D4-selective ligands, L-750,667 and RBI-257, and wild type or mutant dopamine receptors.

Similar to D2 and D4 dopamine receptors, positions 2.61, 3.28 and 3.29 are implicated in various biogenic amine receptor ligand interaction domains. For example, in muscarinic receptors positions 2.61, 3.28 and 3.29 are significant mediators of muscarinic ligand selectivity and function (Drubbisch et al, 1992; Matsui et al., 1995; Lu et al., 1999). However, the pattern of recognition between ligands with selectivity for D1 versus D2 receptor subtypes appears to be more complex. In the macaque D1 dopamine receptor, reciprocal mutations at position 3.28 with residues of the rat D2L receptor revealed moderate to large increases in affinity for the D2-like selective ligands spiperone, domperidone and YM-09151-02 (224-fold, 45-fold and 24-fold, respectively), but less than 4-fold changes in affinity for D2-like selective ligands haloperidol,

piquindone, raclopride, sulpiride and tropapride, as well as the D1-like selective ligand SCH23390 (Lan et al., 2006). However, the reciprocal mutant D2 receptor, D2-F3.28W, did not replicate the substantial changes in affinity observed for the mutant D1 receptor, i.e., less then a 2-fold change for any of the listed ligands.

Positions 2.61, 3.28 and 3.29 play an important role in rhodopsin folding and conformational stability. For instance, a T2.61I mutation causes congenital night blindness (al-Jandal et al., 1999), while a G3.29D mutation results in retinitis pigmentosa (Millan et al., 1995). The increased reactivity of both the T2.61I and E3.28A mutants to hydroxylamine in the dark state combined with decreased meta II state decays suggests that these mutant receptors exhibit pathological instability of the rhodopsin resting-state (Sakmar et al., 1991; Han et al., 1996; Ramon et al., 2003). The poorly expressed mutants E3.28V, E3.28I, E3.28M, E3.28W and T2.61K did not reconstitute to form pigments in the presence of 11-*cis*-retinal (Han et al., 1996; Ramon et al., 2003). These findings demonstrated that positions 2.61, 3.28 and 3.29 are located in conformationally-sensitive regions of rhodopsin. In the case of 2.61 and 3.28, the proximity of the retinal Schiff base allows for potential proton exchange with an appropriate amino acid residue. This releases 11-cis-retinal from the binding pocket in a manner analogous to the dissociation of a ligand from a biogenic amine receptor. As a whole, these experiments demonstrate the importance of these three positions for understanding ligand interactions with certain biogenic amine receptors.

While a great deal had been revealed in previous studies concerning the discriminant structural features of 1,4-DAP ligands and the receptor residues governing the strength of ligand interactions with specific D2 or D4 receptor microdomains, virtually nothing was known in this context about receptor activation states (Floresca and Schetz, 2004). Thus, we measured the

functional properties of a combined TM2/3 reciprocal mutation D2 receptor and report here for the first time that the D2-V2.61F+FV3.28-3.29LM mutant not only has increased affinity for the D4-selective ligands L-750,667 and RBI-257, but also requires a much lower concentration of these antagonists to produce a rightward shift of the agonist functional response. We observed that both of these ligands exert the same functional effects on the D2-V2.61F+FV3.28-3.29LM mutant as they do on the wild type D4 receptor. In the case of L-750,667, this amounts to conversion of the D2-V2.61F+FV3.28-3.29LM mutant from a D2 receptor background that was activated by this ligand to one, like the wild type D4 receptor, that was not. The switch in the functional effect of L-750,667 for the D2-V2.61F+FV3.28-3.29LM mutant was not due to a concentration effect, because L-750,667 had higher affinity and potency for this mutant than it did for the wild type D2 receptor. Further, the switch in functional effect for the mutant persisted at concentration ranges well beyond that needed to produce an observable reversal of (-)-quinpirole's functional effect for the mutant or the wild type D2 receptor. In contrast to L-750,667, the functional profile of RBI-257 was not changed in the mutant receptor; however, since RBI-257 is an antagonist at both the D2 and D4 receptor subtypes, no change in functional properties was expected for the mutant receptor.

To gain a molecular perspective on the interactions of 1,4-DAPs with the dopamine receptor subtypes, we docked several 1,4-DAPs into our initial wild type D4, wild type D2, and mutant D2-V2.61F+FV3.28-3.29LM receptor homology models. However, we initially failed to observe any 1,4-DAP poses satisfying our experimental interaction criteria. This failure was attributed to the limitations of our initial docking strategy. In general, ligand docking strategies have been plagued by two major limitations that hinder their applicability and accuracy (Perola et al., 2004, Sousa et al., 2006). One problem is the poor performance of scoring functions which

rank the likelihood of ligand-receptor complex geometries by attempting to estimate the relative pose energies. The other major problem (one we appeared to encounter here) is the static receptor approximation as currently implemented in grid-based docking approaches. Lately, some progress has been made in addressing this latter issue by expanding the ligand docking search over multiple receptor conformations based on normal mode analysis (NMA) (Cavasotto et al., 2005, Lindahl and Delarue, 2005). In a strategy similar to that used by Cavasotto and colleagues we explored relatively small backbone conformational changes by docking not into merely one receptor conformer, but by docking into an ensemble of receptor conformers generated by movement along a combination of the lowest-frequency normal mode vectors. By incorporating receptor backbone flexibility into our docking study, we were able to identify 1,4-DAP binding poses that produced the expected ionic interaction with D3.32, and furthermore, were able to utilized this well-established interaction as a selection criterion (Floresca and Schetz, 2004) rather than only relying on the scoring function.

The poses that resulted from the enhanced strategy, in agreement with the current data and related studies of D2 and D4 dopamine receptors (Simpson et al., 1999; Schetz et al., 2000; Kortagere et al., 2004), suggested that positions 2.61, 3.28 and 3.29 are primary determinants of 1,4-DAP recognition in the D2 dopamine receptor. These three positions accounted for a large proportion of the intermolecular contacts made by docked 1,4-DAPs. For L-750,667 and RBI-257, positions 2.61, 3.28 and 3.29 appeared to be responsible for mimicry of wild type D4 binding in the D2-V2.61F+FV3.28-3.29LM receptor. For Ro61-6270, L-745,870, and FAUC213, a similar pattern holds: the increase of affinity with the addition of a phenylalanine mutation at 2.61 suggested a similar process of interaction for all five of these ligands at the mutant D2-V2.61F+FV3.29-3.29LM receptor (Table 2-1). However, this trend did not hold true

for NGD 94-1 and PD168,077. These ligands lost affinity when the valine at 2.61 was mutated in the mutant D2-FV3.28-3.29LM receptor to create the mutant D2-V2.61F+FV3.29-3.29LM receptor. Careful examination of ligand molecular structure revealed that ligands possessing para-halogenated or un-halogenated Arm-B aryl rings exhibited a trend of increased affinity when more subtype selective amino acids were mutated. This trend did not hold true for ligands with ortho-electronegative groups (ring nitrogens or nitrile groups).

From close inspection of the CLMs (Figure 2-5B), it is interesting to note that the differences in ligand positioning (Figure 2-5A) could potentially explain functional differences for certain 1,4-DAPs. For example, contact between the D2 receptor and the pyrrolopyridine ring of L-750,667 yielded the index values observed for residues F5.47, W6.48, F6.51, and F6.52 (comprising 19.6% of total intermolecular contacts). These contact positions are thought to influence the rotamer state of W6.48 and thus play a role in triggering early steps in the cascade of events leading to receptor activation (Lin and Sakmar, 1996; Ebersole and Sealfon, 2001). The CLM index values for these four residues decreased for the D2-V2.61F+FV3.28-3.29LM mutant (12.2%) and *w*ild type D4 (12.4%) in agreement with the finding that L-750,667 lost partial agonism in the mutant D2 receptor (Figure 2-3C). While it is speculative to suggest a role between docking contacts and receptor efficacy, it remains quite likely that ligand positioning plays a key role in receptor activation. More informed conclusions may be drawn from assessing the dynamic nature of ligand-receptor interactions to show how the “trigger” positions are specifically affected by a given ligand’s occupancy. In summary, substitution of amino acids in the TM2/3 microdomain of the D2 receptor for the corresponding amino acids in the D4 receptor yielded a mutant D2-V2.61F+FV3.28-3.29LM receptor with pharmacology and function that reflected that of the wild type D4 receptor.

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CHAPTER III

1,4-DISUBSTITUTED AROMATIC PIPERADINE/PIPERAZINES ARE USEFUL PROBES OF D2 RECEPTOR DYNAMICS

The major challenge of my research was to identify the specific contributions made by transmembrane segments two and three to the affinity and function of the D2 dopamine receptor. To examine this, two compounds possessing high affinity for the D2-V2.61F+FV3.28-3.29LM mutant receptor, RBI257 and L-750,667, were selected for functional assessment. However, the initial functional assessment of RBI257 revealed cell line dependent cAMP elevation for concentrations of ligand greater then 10 μM (Appendix B-3). To overcome this obstacle, concentrations of RBI257 were kept below the minimum threshold (approximately 10 μM) for endogenous cAMP activation (Appendix B-3). In contrast, the initial testing of L-750,667 revealed partial agonism at only the D2-WT receptor (Figure 2-4). This discovery was interesting because L-750,667 is an antagonist at the D4-WT receptor (Appendix B-6). Coincidently, further experimentation showing that L-750,667 is antagonistic to D2-V2.61F and D2-V2.61F+FV3.28-3.29LM mutant receptors supports the concept that these three amino acid positions, alone or in combination, can cause a swap in the D2 and D4 receptor 1,4- DAP affinity and function (Appendix B-7; Figure 2-5). However, further work in the D4-F2.61V+LM3.28-3.29FV mutant receptor showed that the converse is not true: L-750,667 does not gain agonist function at the D4 receptor by mutation of these three amino acids (Appendix B-6).

As mentioned in Chapter I, sodium modulation was hypothesized to modify the orthosteric site of the D2-V2.61F mutant receptor to accommodate 1,4-DAP ligands. If true, the data would not only explain the literature discrepancy (Simpson et al., 1999; Floresca et al., 2005) but also help to subtly deduce how transmembrane segments two and three move in response to allosteric sodium modulation. The sodium binding site in the D2 receptor background is an intracellular binding pocket formed by residues in transmembrane segments two, three and seven (D2.50, S3.39, N7.45, and S7.46; Neve et al., 2001). Based on evidence that a charge neutralizing mutation (D2.50N or D2.50A) eliminates D2 receptor sodium sensitivity while a charge sparing mutation (D2.50E) preserves the sensitivity, sodium putatively requires direct intracellular access to D2.50 to modulate the receptor (Neve, 1991; Schetz and Sibley 2001). Using the basic principles of the previous work (Chapter II), I used 1,4-DAPs to probe the molecular structure of the D2-V2.61F receptor in the presence or absence of 140 mM sodium chloride. This concentration was selected to be consistent with the previous data generated by the Javitch lab and the physiological levels of extracellular sodium in normotensive individuals (Simpson et al., 1999). While it is not reasonable to assume that pathological concentrations of intracellular sodium would be equivalent to normotensive levels of extracellular sodium, intracellular concentrations up to 50 mM have been associated with salt sensitive hypertension. Additionally, vasopressin has been shown to substantially elevate intracellular sodium in hypertensive rats (35 mM) beyond the vasopressin challenged levels observed for normotensive rats (20 mM) (Okada et al., 1993). Similarly, in oubain or monensin challenged renal epithelia, increasing cytoplasmic sodium to 13 mM resulted in significant up-regulation of the D1 dopamine receptor with concomitant down-regulation of angiotensin receptor I when compared to 9 mM cytoplasmic sodium controls (Efendiev et al., 2003). While these experiments were

done in whole cell preparations, they serve to illustrate the potential effect of small changes in intracellular sodium concentration. To experimentally control the effects of 140 mM sodium on the dopamine receptor, sodium free and 140 mM N-methyl-d-glucamine (NMDG) buffers were used as controls. Sodium-free buffer was utilized because earlier data suggested that extremely low concentrations of sodium (2 mM) decreased the affinity of [^{3}H]-methylspiperone (personal communication, John A. Schetz, PhD). To control for the change in ionic gradient, 140 mM NMDG was used as a sodium charge substitute (Lin et al., 2006). Using these parameters as guidelines, I probed the D2-V2.61F mutant for changes in transmembrane segment positioning induced by sodium bound to the intracellular allosteric binding pocket.

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CHAPTER IV

LIGAND SELECTIVITY OF D_2 DOPAMINE RECEPTORS IS MODULATED BY CHANGES IN LOCAL DYNAMICS PRODUCED BY SODIUM BINDING[2]

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RUNNING TITLE PAGE

Running Title: Sodium-induced dynamics modulate D_2 receptor function

[2] This project is taken in part from a thesis submitted to the University of North Texas Health Science Center Graduate School of Biomedical Sciences in partial fulfillment of the requirements for the degree Master of Science. This work has been published in the Journal of Pharmacology and Experimental Therapeutics. Ericksen SS, Cummings DF, Weinstein H and Schetz JA (2009) Ligand selectivity of D2 dopamine receptors is modulated by changes in local dynamics produced by sodium binding. J Pharmacol Exp Ther 328:40-54.

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Abbreviations:

cAMP, Cyclic adenosine monophosphate;

CHELPG, CHarges from ELectrostatic Potentials using a Grid-based method;

1,4-DAPs, 1,4-disubstituted aromatic piperidines and piperazines;

D_2, D_2 long wild type dopamine receptor;

D_4, D_4 wild type dopamine receptor;

Dopamine, 3,4-Dihydroxyphenethylamine;

ENM, elastic network model;

Epinephrine, 3,4-Dihydroxy-α-(methylaminomethyl)benzyl alcohol;

FAUC113, 3-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine;

FAUC213, 2-[4-(4-chlorophenyl)piperazin-1-ylmethyl]pyrazolo[1,5-a]pyridine;

Gpp(NH)p, 5′-Guanylyl-imidodiphosphate;

GROMACS, Groningen Machine for Chemical Simulations;

IC_{50}, half maximal inhibitory concentration;

p-Iodoclonidine, 2-[(2,6-Dichloro-4-iodophenyl)imino]imidazoline;

L-745,870, 3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine and is also known as CPPMA, which stands for chlorophenylpiperazinyl methylazaindole;

L-750,667, 3-{[4-(4-iodophenyl) piperazin-1-yl]methyl}-1H-pyrrolo[2,3-b]pyridine;

L-BFGS, low-memory Broyden-Fletcher-Goldfarb-Shanno (quasi-Newtonian algorithm for energy minimization);

Methylspiperone, 8-[4-(4-Fluorophenyl)-4-oxobutyl]-(3-methyl-1-phenyl)-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride;

NGD 94-1, 2-phenyl-4(5)-[4-92-pyrimidinyl)-piperazin-1-yl)-methyl]-imidazole;

NMA, normal mode analysis;

NOMAD-Ref, Normal Mode Analysis Deformation and Refinement;

OPLS-AA, Optimized Potentials for Liquid Simulations – All-Atom (Molecular Mechanics Forcefield);

PD168,077, N-[[4-(2-Cyanophenyl)-1-piperazinyl]methyl]-3-methylbenzamide;

PME, particle-mesh Ewald;

PTX, pertussis toxin;

(-)-Quinpirole, (4aR-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline;

(-)-Raclopride, 3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide;

RBI-257, 1-[4-iodobenzyl]-4-[N-(3-isopropoxy-2-pyridinyl)-N-methyl]-aminopiperidine;

Ro61-6270, 2-Amino-benzoic acid 1-benzyl-piperidin-4-yl ester;

UK14304, 5-Bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine

ABSTRACT

We have uncovered a significant allosteric response of the D2 dopamine receptor to physiologically relevant concentrations of sodium (140 mM), characterized by a sodium-enhanced binding affinity for a D4-selective class of agonists and antagonists. This enhancement is significantly more pronounced in a D2-V2.61(91)F mutant and cannot be mimicked by an equivalent concentration of the sodium replacement cation N-methyl-D-glucamine. This phenomenon was explored computationally at the molecular level by analyzing the effect of sodium binding on the dynamic properties of D2 receptor model constructs. Normal mode analysis (NMA) identified one mode (M19), which is involved in the open/closed motions of the binding cleft, to be particularly sensitive to the sodium effect. To examine the consequences for D2 receptor ligand recognition, one of the ligands, L-745,870, was docked into conformers along the M19 trajectory. Structurally and pharmacologically well established ligand-receptor interactions, including the ionic interaction with D3.32(114) and interactions between the ligand aryl moieties and V2.61(91)F, were achieved only in "open" phase conformers. The docking of (-)-raclopride suggests that the same binding cleft changes in response to sodium-binding perturbation account as well for the enhancements in binding affinity for substituted benzamides in the wild type D2 receptor. Our findings demonstrate how key interactions can be modulated by occupancy at an allosteric site and are consistent with a mechanism in which sodium binding enhances the affinity of selected ligands through dynamic changes that increase accessibility of substituted benzamides and 1,4-DAP ligands to the orthosteric site and accessibility of 1,4-DAPs to V2.61(91)F.

INTRODUCTION

Sodium ions have been shown to modulate dopamine receptors, and allosteric modulation by sodium ions has been shown to drive the conformational equilibrium of heterotrimetric G protein-coupled receptors (GPCR) toward an agonist low-affinity state (for a review see Schetz, 2005). In dopamine receptors, like in other heterotrimeric GPCRs, the highly conserved and negatively-charged aspartic acid at position 2.50 (the generic numbering system is defined in Ballesteros and Weinstein, 1995) has been identified as a sodium interaction site. For example, charge-neutralizing mutations in the D_2 or the D_4 receptor (e.g., D2.50(80)N or D2.50(80)A) make them sodium–insensitive, while a charge sparing mutation (e.g., D2.50(80)E) retains much of the sodium-sensitivity (Neve et al., 1991; Schetz and Sibley 2001). D_2 receptor mutations at other positions (e.g., S3.39(121)A and S7.46(391)A) also diminish sensitivity, presumably by reducing the H-bonding capacity at the sodium binding site (Neve et al., 2001). The latter studies, in the structural context of the high resolution crystalline structure of bovine rhodopsin (Palczewski et al., 2000), led to a revised model of the sodium binding site (Neve et al., 2001), in which sodium is at the center of a square-pyramidal hydrogen-bonding network whose vertices are formed by D2.50(80), S3.39(121), N7.45(390), and S7.46(391); sodium binding is thought to neutralize the negative charge centered at D2.50(80). Allosteric modulation of dopamine receptors by sodium has been shown previously to reduce the affinity of endogenous agonists and zinc, increase the affinity or binding capacity (B_{max}) of substituted benzamide antagonists, and alter the rate of chemical modification (Neve, 1991; Schetz et al., 1999; Schetz et al., 2001; Vivo et al., 2006). However, despite the prevalence of studies across multiple GPCR families indicating allosteric modulation by sodium, an exhaustive search of the literature failed to identify a mechanism for the sodium-induced effects.

In the process of determining the source of large discrepancies in binding affinity reported for 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAP) for the D_2-V2.61(91)F mutant (Simpson et al., 1999; Floresca et al., 2005), we discovered a change, elicited by sodium binding, in the dynamic properties of the receptor that correlate with a dramatic increase in sodium-sensitivity for both agonists and antagonists belonging to a similar structural class. This finding offers an opportunity to understand the allosteric mechanism of the effect produced by sodium binding. To this end, we carried out a normal mode analysis (NMA) of the dynamic properties of various D_2 receptor constructs using 3D molecular models of the receptor. Normal modes are calculated from the molecular structure model and provide information about the component harmonic motions or vibrations of the molecule that characterize its dynamic fluctuation as it occupies a stable conformational state (e.g., inactive state, etc.). The modes constitute a set of orthogonal vectors ranked by energy (or the corresponding frequency), which indicates the direction in which each particle (the component atoms, or residues, or C_α) is moving at that particular level of energy (frequency). Thus, the superposition of all the normal mode vectors describes the entire intrinsic motion of the molecule based on its shape and molecular connectivity, but often one or a few low frequency (low energy) modes contribute most significantly to this thermal "breathing" motion of the molecule. It was demonstrated for many proteins that the directions of the lowest frequency modes also tend to indicate the path of molecular movements associated with functionally relevant conformational changes (Cui and Bahar, 2006). Here, the comparison of the normal modes between sodium-bound and sodium-free structures of the receptor models allowed us to identify a specific sodium-responsive normal mode motion that indicates distinct dynamic changes in the environment of position 2.61(91) that accounts for the hypersensitivity in the mutant D_2-V2.61(91)F. Thus, when backbone movements

associated with this mode are explored as an additional degree of freedom in the ligand docking process, the "open" conformations produced within the trajectory of this normal mode are found to promote ligand binding poses that are consistent with experimentally verified interactions. These findings connect the dynamic properties characterized by the NMA of the wild type and highly sensitive mutant receptor with experimentally observed sodium dependent allosteric effects, and suggest a mechanism by which the presence of sodium alters ligand affinity.

METHODS

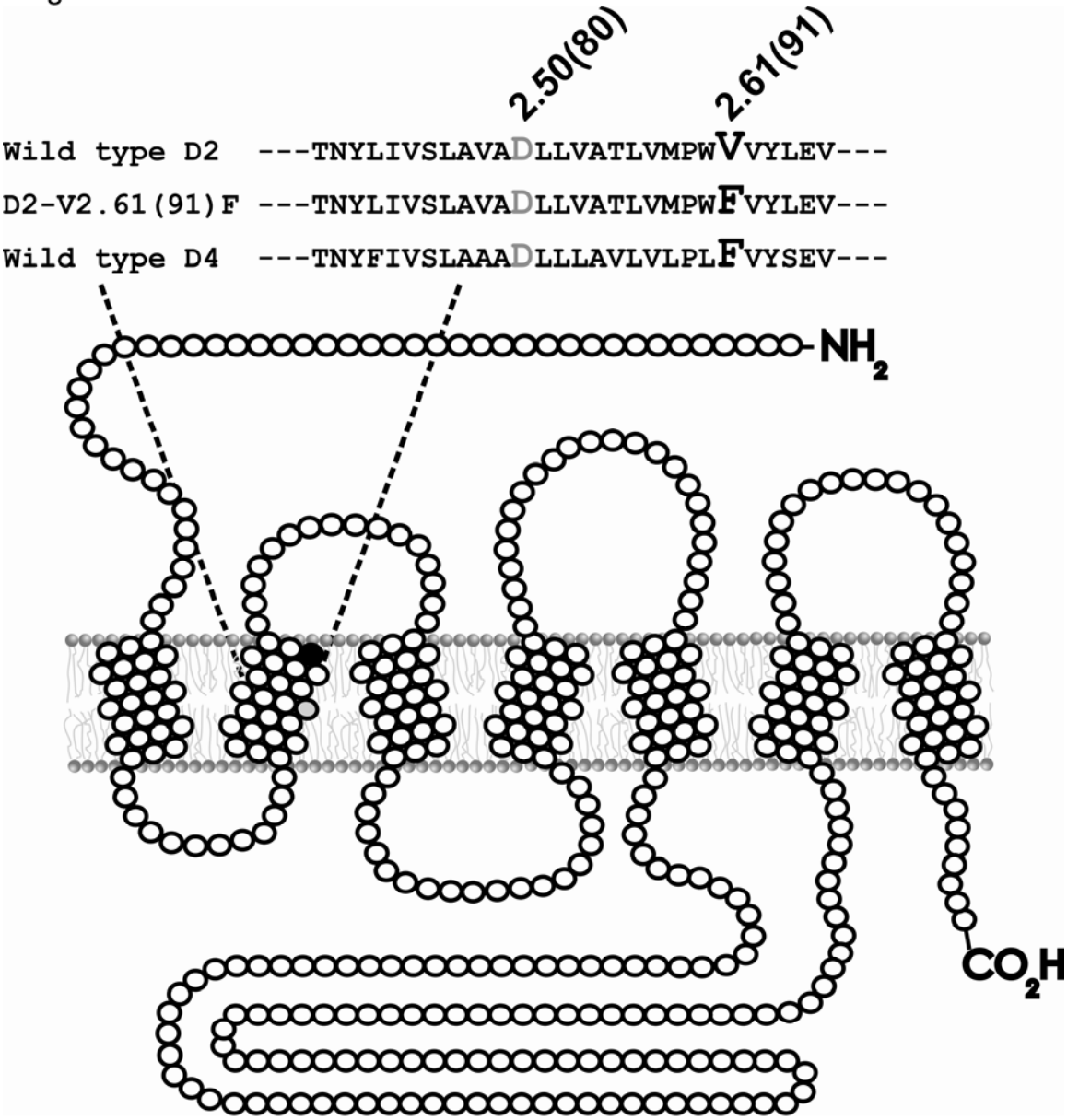
Reagents. Cell culture medium was purchased from Hyclone Laboratories (Logan, UT). For radioligand studies [^{3}H]methylspiperone (NET-856, 70-80 Ci/mmol) was purchased from Perkin Elmer Life Sciences (St. Louis, MO) and wash buffer reagents were purchased from US Biological (Swampscott, MA). The source of (-)-quinpirole and forskolin was from Sigma Chemical Company (St. Louis, MO). Other drugs were purchased from Tocris Cookson, Inc. (Ellisville, MO).

Site-Directed Mutagenesis. Mutagenesis was accomplished using a QuikChange^{TM} kit (Stratagene, CA). The integrity of mutations and the lack of unwanted mutations were confirmed by full length sequencing at the University of Maine DNA sequencing facility (Orono, ME). Mutant receptors are named employing the system created by Ballesteros and Weinstein (1995) and other nomenclature conventions. Briefly, for each residue in a transmembrane segment, the first digit denotes the transmembrane segment (TMS), followed by a period and a relative position index within the transmembrane segment. The most conserved amino acid in a TMS is assigned the position index 50 and the other amino acids within this TMS are numbered relative

to the conserved amino acid. The number in parentheses is the residue number in the sequence of the rat D_2 dopamine receptor short isoform. Our naming system for the mutants begins with a letter to designate the species (e.g. "r" for rat or "h" for human) followed by the receptor subtype abbreviation, e.g., rD_2 for rat D_2 dopamine receptor. Next the single letter abbreviation for the amino acid is listed followed by its position and then amino acid substitution. For example, rD_2-V2.61(91)F denotes a rat D_2 receptor with valine at position 2.61(91) being substituted for phenylalanine (Figure 4-1).

Figure 4-1. Depiction of the D_2-V2.61(91)F mutant dopamine receptor as a monomer in a section of lipid bilayer. This figure represents the unfolded D_{2L} receptor showing the amino terminus (**–NH_2**) on the extracellular side and the carboxyl terminus (**–CO_2H**) on the intracellular side. Open circles (○) are used to indicate position wild type amino acids while closed circles (● and ●) are used represent specific amino acids in the D_2-V2.61(91)F receptor. As shown in the sequence, a valine to phenylalanine mutation at amino acid residue 91 (●) results in the D_2-V2.61(91)F mutant dopamine receptor. The purpose of the V2.61(91)F mutation is to modify the binding pocket of the D_2 receptor with the corresponding residue of the D_4 receptor and make it more accommodating to D_4-selective 1,4-DAPs (Simpson et al., 1999; Schetz et al., 2000; Kortagere et al., 2004; Floresca et al., 2005). While most ligands bind an orthosteric binding site accessible from the extracellular face of the receptor, (Floresca and Schetz, 2004), the sodium ion binds the receptor through an intracellular allosteric binding site formed by the interactions of transmembrane segments two, three and seven (Neve et al., 2001). Also shown in the diagram and the sequence is the relative position of the conserved negatively charged D2.50(80) (●) that is critical for the interaction of sodium ions with the dopamine receptor (Neve et al., 1991; Neve et al., 2001).

Figure 1



Transfections. DNA constructs subcloned into the pcDNA3.1 vector were transfected into HEK293 cells by $CaPO_4$ precipitation. Briefly, 20 μg of DNA was mixed with 60 μL of 2M $CaCl_2$ and the mixture was added to an appropriate volume of sterile water to make 500 μL of solution. This DNA-$CaCl_2$ solution was then added dropwise to 500 μL of 2x Hepes buffered

saline (HBS) while bubbling the HBS with a 1 mL serological pipet. The resultant final transfection mixture was allowed to sit for 30 minutes before dropwise addition to 150 cm^2 culture dishes seeded with an appropriate number of cells. For stable transfections, HEK293 cells were seeded at a density of 200,000 cells/150 cm^2 culture dish. For transient transfections, COS7 cells were seeded at a density of 1.5 million cells/150 cm^2 culture dish. Both cell lines were allowed to grow overnight in 20 mL of sterile growth medium containing DMEM supplemented with 10% BOVINE CALF SERUM, 100 μM sodium pyruvate, and 1% penicillin/streptomycin (5000 units). This resulted in about 30% confluent COS-7 cells and less than 5% confluent HEK293 cells. The transfected cells were incubated overnight with the final transfection mixture, after which, the medium was replaced. Two to four hours prior to transfection, the medium was replaced with 20 mL of new sterile growth medium. Again, the following day, the medium was replaced. For the HEK293 cells the medium was supplemented with 2 mg/mL G418 to allow for clonal selection. Stable clones of HEK293 cells containing the mutant receptor were generated after several weeks of G418 selective pressure and expanded for further use in radioligand binding and functional assays.

Membrane Preparation. Cell membranes were prepared by first detaching healthy cells with lifting buffer (Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+}; 5 mM EDTA), and then pelleting the detached cells by centrifugation in a sterile conical tube for 10 minutes at 700 x *g*. After centrifugation, the supernatant was decanted from the pellet. The pellet is then resuspended in 10 mL of lysis buffer (5 mM Tris, 5 mM $MgCl_2$, pH 7.4 at 4°C), and allowed to lyse on ice for 5-10 minutes before transfer to an ice cold Dounce homogenizer. Eight full strokes of the dounce homogenizer are used to disrupt the whole cells by glass on glass

homogenization. The resulting homogenate was poured into a centrifugation tube, balanced by the addition of cold binding buffer (50 mM Tris pH 7.4 at 4°C), and then centrifuged at 28,000 x *g* for 45 minutes. The supernatant is decanted from the resulting membrane pellet. This membrane pellet was then resuspended in cold binding buffer and recentrifuged. The final membrane pellet obtained was then resuspended in an appropriate amount of cold binding buffer for the experiment, rehomogenized by four strokes in an ice cold dounce homogenizer, and then stored on ice for same day use.

Radioligand Binding Studies. Both receptor saturation experiments and radiolabeled competition assays were used to characterize the receptors in this study. Briefly, the binding and wash buffers consist of 50 mM Tris pH 7.4 at 25°C with 1 N KOH used for the fine pH adjustment. For sodium shift assays, the binding and wash buffers were supplemented with 140 mM NaCl. The membrane density of the receptors and their affinity for the radioligand [^{3}H]methylspiperone ([^{3}H]MSP) was assessed by saturation isotherm analysis. For this type of assay, the cell membranes were allowed to equilibrate with increasing nanomolar concentrations of [^{3}H]MSP in the presence or absence of 5 μM (+)-butaclamol, a dopamine receptor antagonist used to define the nonspecific interactions of [^{3}H]MSP. After 90 minutes of equilibration at room temperature, the samples were rapidly filtrated and washed with ice cold binding buffer (50 mM Tris pH 7.4 at 0°C) through GF/C filters pretreated for 10 minutes with 0.3% PEI. The filters were allowed to dry before cutting them into vials. Vials were then filled with 3.5 mL of scintillation fluid and mixed prior to quantifying the amount of radioactivity in a scintillation counter. Radiolabeled competition assays were performed in a similar fashion to saturation assays, except that a fixed concentration of 0.5 nM [^{3}H]MSP was utilized in conjunction with

increasing concentrations of non-radiolabeled competitive ligand. Membrane protein concentration was determined by bicinchoninic acid assay (Pierce, IL) according to the manufacturer's instructions.

Cyclic Adenosine Monophosphate Functional Assays. Intracellular cyclic adenosine monophophate (cAMP) concentrations were determined using a Perkin Elmer Fusion^{TM} plate analyzer and a cAMP Alphascreen^{TM} detection kit (Perkin Elmer, MA). The assay was performed essentially according to the manufacturer's specifications, except for adaptations we devised to measure cAMP levels in attached cells. Briefly, HEK293 cells stably expressing mutant receptor were resuspended in sterile growth media (DMEM with 10% BOVINE CALF SERUM, 1% penicillin/streptomycin, 100 μM sodium pyruvate) and then plated at a density of 50,000 cells/well in 96 well microtiter plates coated with poly-L-lysine (Sigma P4832; St. Louis, MO). The following morning, stimulation buffer (DMEM, 20 mM HEPES, 100 μM sodium metabisulfite, 30 μM Ro 20-1724; pH 7.4 at 25 ^{o}C), cell lysis buffer (0.3% Tween 20, 20 mM HEPES, 1 μg/μl BSA; pH 7.4 at 25 ^{o}C), and bead buffer (20 mM HEPES, 30 μM Ro 20-1724, 1 μg/μl BSA, 1X Hank's Basic Salt Solution; pH 7.4 at 25 ^{o}C) were freshly prepared and pH adjusted to 7.4 with 1 N cell culture tested sodium hydroxide (Sigma-Aldrich, St. Louis, MO). To examine the G_i protein-mediated inhibition of adenylyl cyclase, the levels of cAMP were first raised with 6 μM forskolin, a direct stimulator of adenylyl cyclase. Drug dilutions were prepared in stimulation buffer and 200 μL of dilution was added per well in an empty 96 well microtiter plate, and allowed to equilibrate in the 37 ^{o}C incubator for 30 minutes. Culture medium was removed from the cells and the temperature- and carbon dioxide-equilibrated drug dilutions were rapidly added to the cells using a multichannel pipet. Cells were then incubated the presence of

the drug dilutions at 37 ^{o}C for 20 minutes and then centrifuged at 1500 x *g* for 5 minutes. Drug dilutions were carefully removed by pipetting and 100 μL of cell lysis buffer was added to each well. The cells were lysed by shaking at 600 rpm on a microtiter plate shaker for 1.5 hr. A portion of the resulting lysate (30 μL) was transferred to an opaque 96 well Costar plate (cat. # 07-200-309; Corning, NY) and challenged with 0.5 units of acceptor and donor beads (9.35 μg/mL and 12.5 μg/mL respectively) containing 5 units of biotinylated cAMP (3.76 nM). Before reading, this reaction was allowed to equilibrate for one hour with shaking at 600 rpm protected from light with aluminum foil.

Calculations and Data Analysis. Data points for each experiment were sampled in triplicate and each experiment was repeated three times, except where noted. The geometric mean and standard deviation are reported for each experiment; however, the errors in the graphs are standard errors of the mean (S.E.M.). The equilibrium dissociation constant (K_D) of [^{3}H]MSP was determined from saturation isotherm analysis. The inhibition constants (K_i) for all radioligand competition assays were calculated with the Cheng-Prusoff equation: $K_i = IC_{50}/(1+[\text{radioligand}]/K_D)$, where K_D is the equilibrium dissociation constant of the radioligand. A $K_{0.5}$ value is reported in cases where the Hill slope is significantly different from unity. All data were analyzed using Prizm version 4.0 (Graphpad Software Inc., CA). For the inhibition assays, data from three or more assays were combined and then interpreted by extrapolating all concentration response curves to zero to generate IC_{50} values. These were subsequently converted to K_i values before analysis by one-way ANOVA with a Dunnett's post hoc analysis. For saturation isotherm binding assays, specific binding curves were obtained by subtracting non-specific binding (defined as binding in the presence of 5 μM (+)-butaclamol) from the total

binding at each concentration of radioligand. Values for K_D and B_{max} were determined from the specific binding curve. Sodium shift binding assays were analyzed by comparing the receptor data with and without sodium in a paired two tail t-test. Cyclic AMP functional assays were assessed by first quantifying the amount of cAMP generated per mg of protein in each sample, and then normalizing this value as a percentage of the cAMP generated by unopposed 6 μM forskolin. Efficacy was determined by subtracting the best-fit values for the bottom of the curve (lowest horizontal asymptote) from the top of the curve (highest horizontal asymptote). Functional assays are graphed as sigmoidal semi-log concentration response curves. Statistical analyses of the curve fitting procedure included the run test, F-test, and Pearson's correlation coefficient. Potency and efficacy values generated from three or more replicate curves were analyzed by one-way ANOVA with a Dunnett's post hoc analysis. Significance was established at the 95% confidence level ($p \leq 0.05$).

Construction of Receptor Homology Models. A wild type D_2 receptor model was constructed using Modeller 9v1 (Šali and Blundell, 1993) simultaneously using as templates the (*1GZM)* structure of bovine rhodopsin (Li et al., 2004) and the recently determined structure of the β_2 adrenergic receptor (Cherezov et al., 2007) (*2RH1*). Initially, 1000 D_2 receptor structures were generated and ranked by Modeller's objective function. The models were then structurally aligned and clustered using the GROMACS v3.3 package (van der Spoel et al., 2005) *gcluster* utility (cutoff = 0.20 Å). The most representative (central) structure from the best scoring cluster was selected for further analysis and mutated to V2.61F(91) prior to energy minimization runs.

Building Sodium-Bound D_2 Receptor Models. Sodium-bound models were constructed with the sodium cation placed at the putative sodium binding pocket near D2.50(80) proposed by Neve et al. (2001). One negative control was constructed by positioning the sodium far from the TM region, at the intracellular carboxylate terminus (C415) where it would not affect the dynamic properties through direct interaction with the TM region. A second negative control used in this study is the sodium-free "null" system. All the structures were subjected to energy minimization runs *in vacuo* with the L-BGFS method, in three stages, each carried out to convergence (F_{max} < 10 kJ mol^{-1} nm^{-1}) using the GROMACS v3.3 (van der Spoel et al., 2005) with the molecular systems parameterized according to the OPLS all-atom forcefield (Jorgensen et al., 1996). Electrostatic interactions were treated by the particle-mesh Ewald (PME) method (Essman et al., 1995). In the first stage all heavy protein atoms were restrained with half-harmonic force restraints (k = 1000 kJ mol^{-1} nm^{-1}) with only the sodium ion unrestrained. In the second minimization stage, sodium and all sidechains of residues within 6 Å of the sodium were unrestrained. Finally, sodium and all atoms of residues within 6 Å of the sodium position were unrestrained during minimization with residues outside of this region restrained.

Normal Mode Analysis. To examine the effect of sodium binding on the dynamic properties of the receptor molecule, we used normal mode analysis (NMA) which determines a spectrum of independent harmonic (vibrational) motions available to a particular stable molecular conformation within a harmonic approximation (see further description in the Introduction). Our analysis focused on the lower frequency modes which, comprise more facile motions (thus higher amplitude) along directions which coincide with the more shallow curvatures along the potential well (Tama and Sanejouand, 2001) and have been shown to indicate function-related

dynamics of proteins (Cui and Bahar, 2006). Each minimized structure was submitted to NOMAD-Ref webserver for NMA (Lindahl et al., 2006). Elastic network models (ENMs) (Tirion, 1996) were built from C_α positions with the sodium ion represented as an additional C_α at its optimized binding position. The first 106 normal modes ($M_{1\text{-}106}$) were calculated for each ENM using default parameters with the exception of the distance weighting parameter in which a non-default value of 3.0 Å was applied as recommended for C_α-only models.

To examine divergence in dynamical behavior between sodium-bound and control (null) structures, dot products were computed for each sodium-bound normal mode vector against all computed modes from the null system. A window of the null spectrum was then chosen by centering it at the most analogous mode M_j^{Null} (highest dot product with M_i^{Na+}). The dot product squares were then summed over the selected null spectrum window to provide a P_{ij} value ($P_{ij} = \sum_{i,j(window)} |M_i^{Na+} \bullet M_j^{Null}|^2$). A range of vectors from the control structures must be included because what might appear as a unique sodium-bound mode can be recapitulated by a set of such vectors, when combined. This overcomes a potential pitfall in comparative NMA resulting from a comparison of only pairs of corresponding modes between structures (Ming and Wall, 2005). P_{ij} was then plotted as a function of M_i^{Na+} using various window sizes to identify the difference in normal mode(s) between sodium-bound structures and control.

Ligand Docking into D_2 Receptor Conformers from the M_{19}^{Na+} trajectory. The trajectory of the sodium-sensitive M_{19}^{Na+} was selected to explore effects of sodium-related motions on ligand docking, because (i) its P_{ij} value demonstrates significant divergence from the conformational space of the other modes; (ii) it was the lowest-frequency mode among those showing divergence and thus represents a softer, and thus higher amplitude, collective domain motion;

and (iii) visual inspection revealed that as part of its characteristic motion, an "open" phase appears to widen the binding pocket, increasing accessibility (from the extracellular milieu) and its volume in the V2.61(91)F mutant. We therefore constructed a series of D_2 receptor models representing points in the trajectory of this particular mode, by rebuilding all-atom structures on the C□ frames from the M_{19} trajectory (output from NOMAD-Ref). Backbone and sidechain atoms were built onto the fixed C□ template with Modeller 9v1, followed by minimization, a short 15 ps MD run, and minimization with C_α atoms fixed for each procedure. The structure was then minimized to convergence with positional restraints on the C□carbons in GROMACS, as described in Methods.

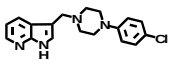
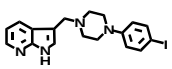
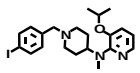
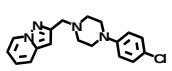
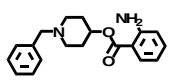
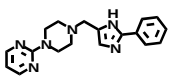
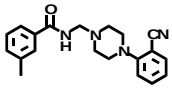
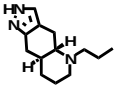
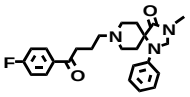
Ligands were constructed in Discovery Studio (Accelrys, Inc.) and their geometries optimized with *ab initio* quantum mechanical calculations using the HF6-31G** basis set in Gaussian03 (Frisch et al. 2004). The partial charges were set according to the AutoDockTools 1.4.5 automated Gasteiger partial charge assignment. Ligands were then docked 50 times into each M_{19}-based D_2 receptor frame using the Lamarckian genetic algorithm search routine in AutoDock 4.0 (Morris et al., 1998) with default parameters, and a maximum number of energy evaluations of 4.0×10^6. Selective sidechain flexibility was allowed within the docking routine: we explored the rotation of dihedrals in the sidechains of F2.61(91), F3.28(110), V3.29(111), D3.32(114), W6.48(358), F6.51(361), and H6.55(375), chosen because of their apparently critical position in the ligand binding site. After docking into each receptor frame, poses were clustered using an RMSD tolerance of 3.0 Å and clusters ranked by mean energies.

RESULTS

When transiently expressed in COS-7 cells, the D2-V2.61(91)F mutant receptor had a level of expression and an affinity for the moderately D2-selective 1,4-DAP [^{3}H]MSP that was similar to the wild-type D2 receptor (1.1- and 4.1-fold, respectively) (Table 4-1; Figure 4-2). It also had an affinity comparable with that of the wild-type receptor for the agonist (-)-quinpirole, which lacks a 1,4-DAP structural motif (1.2-fold, Table 4-1). The 1,4-DAP structural motif consists of two aromatic rings linked to positions 1 and 4 of a central six-membered piperidine or piperazine ring. L-745,870, for example, has two distinct aryl substituents that extend from positions 1 and 4 of the central piperazine ring directly and via a methylene spacer (Table 4-1). One of these ring nitrogens is likely protonated at physiological pH and interacts with the acidic pocket residue D3.32(114). For all ligands tested within this structure class, the D2-V2.61(91)F mutant had affinities very similar to those measured for the wild-type D2 receptor, including L-745,870 and six other D4-selective 1,4-DAPs (1.1–3.3-fold changes, Table 4-1). All of these 1,4-DAPs have been tested in previous binding studies designed to investigate molecular determinants of ligand selectivity for D4 receptors versus D2 receptor subtypes (Schetz et al., 2000; Kortagere et al., 2004), but only one of them (L-745,870 or CPPMA) had been tested on the D2-V2.61(91)F mutant, and it was reported to have a large improvement in binding affinity (Simpson et al., 1999).

In an effort to determine the source of the discrepancy between the reported 97-fold increase in affinity for L-745,870 for an N- and C-terminally epitope-tagged human D2-V2.61(91)F mutant (Simpson et al., 1999) and the lack of change for the identical mutation in the rat receptor for the same ligand (Table 4-1) and several other ligands belonging to the same structural class (Table 4-1; Floresca et al., 2005), we systematically eliminated differences

Table 4-1. Affinity of D_4-selective ligands for the *wild type* D_2 and D_2-V2.61(91)F mutant receptors expressed in COS-7 cells. Affinities for the *wild type* D_4 receptor are shown for comparison. Affinity values (K_i or K_D, nM) are expressed as geometric averages of the mean of three experiments ± S.D. Significant differences between values for *wild type* D_2 and D_2-V2.61(91)F mutant receptors were determined at 95% confidence by Dunnetts multiple comparison test and are marked by an astericks. N.D. means not determined. The fold changes in affinity values relative to the *wild type* D_2 (D_2-WT) are shown in parentheses with the direction of the change indicated by arrows: the ↑ means an increase in K_i value corresponding to a decreased affinity, while the ↓ means a decrease in K_i value corresponding to an increased affinity.

Ligand	Structure	D_2-WT	D_2-V2.61(91)F	D_4-WT
L-745,870 (CPPMA)		**656 ± 227 (1)**	**482 ± 251 (↓1.4)**	**0.32 ± 0.14 (↓2050)**
L-750,667[c]		**1400 ± 950 (1)**	**1100 ± 510 (↓1.3)**	**0.11 ± 0.02**[a] **(↓13400)**
RBI-257		**85 ± 12 (1)**	**78 ± 3.3 (↑1.1)**	**0.27 ± 0.10**[b] **(↓315)**
FAUC213[c]		**1300 ± 640 (1)**	**1200 ± 730 (↓1.1)**	**1.1 ± 0.22**[b] **(↓1030)**
Ro 61-6270		**655 ± 274 (1)**	**1121 ± 70* (↑1.7)**	**0.89 ± 0.12**[b] **(↓736)**
NGD 94-1		**817 ± 284 (1)**	**3358 ± 266* (↑3.3)**	**0.3 ± 0.04**[b] **(↓2720)**
PD 168,077		**1380 ± 64 (1)**	**3601 ± 474* (↑2.6)**	**1.5 ± 0.41**[b] **(↓540)**
(-)-Quinpirole		**812 ± 617 (1)**	**673 ± 466 (↓1.2)**	**N.D.**
[^{3}H]methylspiperone		**0.016 ± 0.0032 (1)**	**0.066 ± 0.030* (↑4.1)**	**0.29 ± 0.030**[a] **(↑18)**

[a] Schetz et al., 2000; [b] Kortagere et al., 2004; [c] Floresca et al., 2005

between the two experimental systems. Initially, we expressed our rat D2-V2.61(91)F mutant in the same HEK293 cell line used in the previous report. Changing the cell background had only a moderate effect on the relative affinity measured for L-745,870 at the rD2-V2.61(91)F mutant versus the wild-type rD2 receptor that was difficult to accurately quantify because of low affinity and limited drug solubility under the conditions tested (~5.5-fold increase based on estimates from extrapolated values, Figure 4-2, A and B; Table 4-2).

The stable expression of these receptors in the HEK293 cell line also allowed us to study D2-like dopamine receptor cAMP functional responses. Although useful for the initial binding studies, transient expression in a COS-7 cell line lacked a suitable functional response in our assay system (data not shown). The HEK293 cell line lacks endogenous receptors for dopamine but can mediate a cAMP functional response for transfected dopamine receptors (data not shown). In preparation for functional assays, the cell surface density of dopamine receptors expressed in several stable HEK293 clones was determined with [^{3}H]MSP saturation isotherm analysis, after which selected clones were matched by receptor density to avoid discrepancies because of spare receptors. HEK293 cell lines expressing 8.6 ± 3.4 pmol/mg protein of the wild-type D2 receptor and 11.6 ± 0.23 pmol/mg protein of the D2-V2.61(91)F receptor were selected for use in all subsequent experiments. The corresponding [^{3}H]MSP affinity values (KD) were 74 ± 9.7 pM for the wild-type D2 receptor and 95 ± 18 pM for the D2-V2.61(91)F receptor. The absolute affinities of [^{3}H]MSP for the D2-V2.61(91)F mutant receptor expressed in COS-7 and HEK293 cells were similar (1.4-fold different), although small differences were found for the wild-type receptor (4.6-fold different). For this same reason, the relative differences between wild-type and mutant D2 receptors within the same cell line are more pronounced in COS-7 than in HEK293 cells (4.1- versus 1.3-fold, respectively).

Figure 4-2. L-745,870, L-750,667 and NGD 94-1 display sodium sensitive binding to the D_2-V2.61(91)F mutation. Shown are parallel [^{3}H]methylspiperone competition binding experiments with L-745,870, L-750,667 or NGD 94-1 competing for wild type D_2 or D_2-V2.61(91)F receptors stably expressed in HEK293 cells. The following graphs are composites of three or more parallel runs statistically examined by one way ANOVA with Dunnett's post hoc analysis (*$p < 0.05$). (A) L-745,870 at the wild type D_2 receptor in the presence (●) and absence (○) of sodium. Sodium does not significantly increase the affinity for L-745,870 at the wild type D_2 receptor. (B) L-745,870 at the D_2-V2.61(91)F receptor in the presence (■) and absence (□) of sodium. Sodium significantly increases ($p \leq 0.05$, 37-fold increase) the affinity of L-745,870 for the D_2-V2.61(91)F mutant receptor. (C) L-750,667 at the wild type D_2 receptor in the presence (●) and absence (○) of sodium. In the absence of sodium binding to the wild type D_2 receptor is negligible. In the presence of sodium very weak binding is observed. (D) L-750,667 at the D_2-V2.61(91)F receptor in the presence (■) and absence (□) of sodium. Only in the presence of sodium does L-750,667 have significant binding ($p < 0.05$, 35-fold increase). (E) NGD 94-1 at the wild type D_2 receptor in the presence (●) and absence (○) of sodium. In the absence of sodium the wild type D_2 receptor has negligible binding. In the presence of sodium, weak binding is observed. (F) NGD 94-1 at the D_2-V2.61(91)F receptor in the presence (■) and absence (□) of sodium. Only in the presence of sodium does NGD 94-1 have detectable binding ($p < 0.05$, greater than a 16-fold increase). All affinity values are given in table 2.

Figure 2

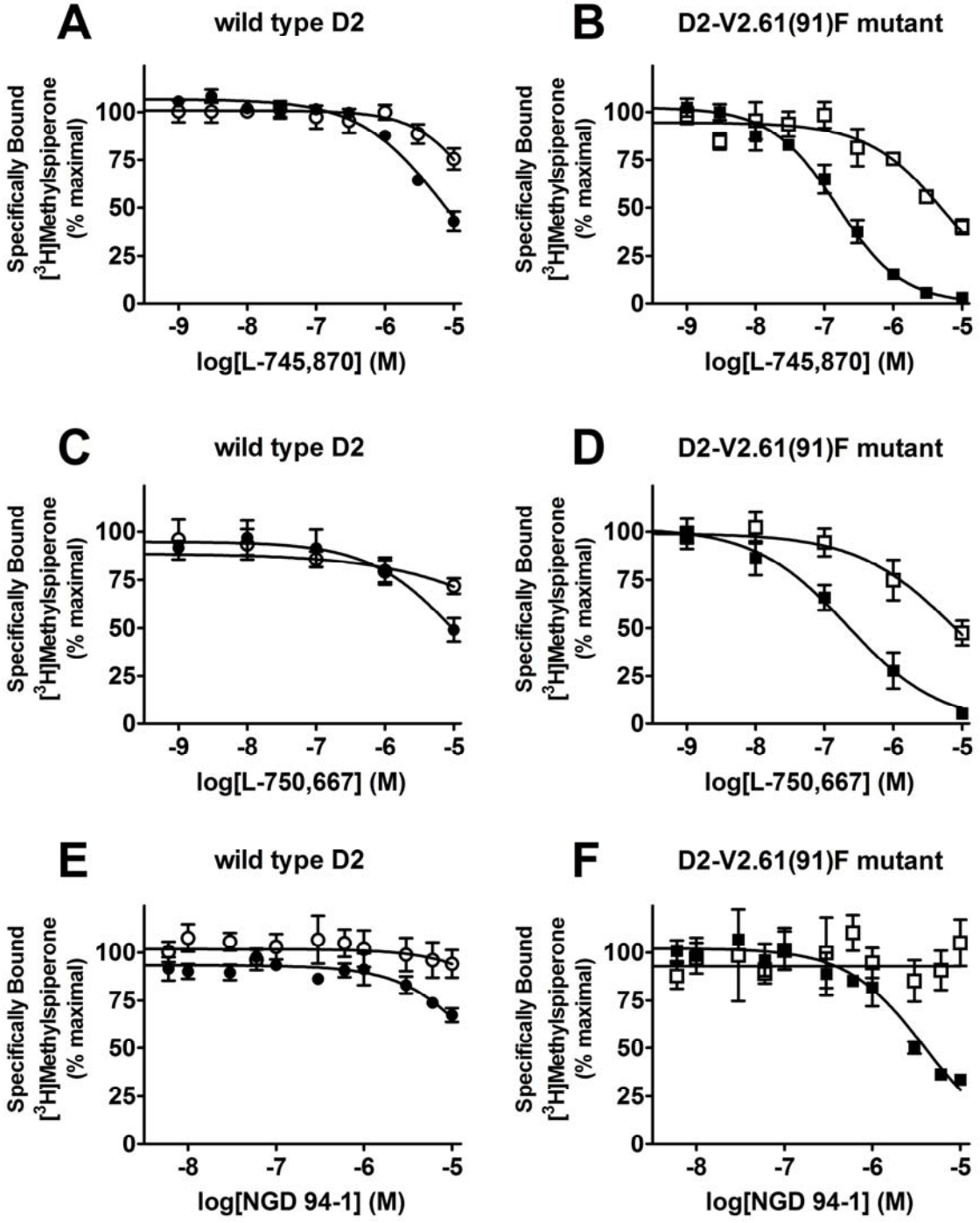

A
wild type D2
B
D2-V2.61(91)F mutant
C
wild type D2
D
D2-V2.61(91)F mutant
E
wild type D2
F
D2-V2.61(91)F mutant
Specifically Bound [3H]Methylspiperone (% maximal)
log[L-745,870] (M)
log[L-750,667] (M)
log[NGD 94-1] (M)

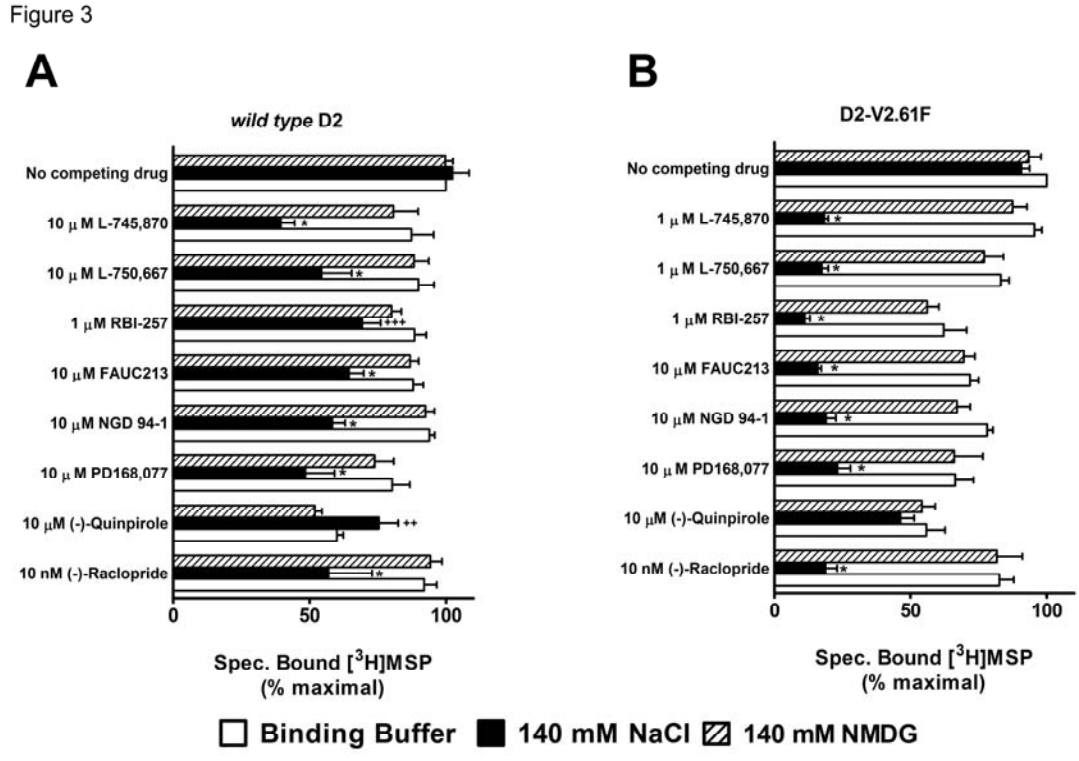
Table 4-2. The affinities of L-745,870, L-750,667 and NGD 94-1 for the D_2-V2.61(91)F mutant receptor are significantly enhanced in the presence of 140 mM NaCl. Receptors were stably expressed in the HEK293 cell line. Binding affinities (K_i) were calculated from the Cheng-Prusoff equation ($K_i = IC_{50}/(1+[radioligand]/K_D)$ and expressed as geometric mean values (nM) ± S.E.M. (n = 3). The D_2-V2.61(91)F was run in three paired experiments while *wild type* D_2 was run only in two paired experiments. *These are approximate values based upon extrapolation. The fold changes in affinity values relative to the *wild type* receptor, i.e., *wild type* D_2 (D_2-WT) or D_2-V2.61(91)F, are shown in parentheses with the direction of the change indicated by arrows: the ↓ means a decrease in K_i value corresponding to an increased affinity.

	D_2-WT*		**D_2-V2.61(91)F**	
	No NaCl	**140 mM NaCl**	**No NaCl**	**140 mM NaCl**
L-745,870	**5232 ± 8419**	**778 ± 89**	**943 ± 328***	**27.0 ± 4.34**
	(1)	**(↓7)**	**(1)**	**(↓35)**
L-750,667	**>10,000**	**1331 ± 927**	**1271 ± 924***	**34 ± 24**
	(1)	**(> ↓7)**	**(1)**	**(↓37)**
NGD 94-1	**>10,000**	**3827 ± 3299**	**>10,000***	**616 ± 128***
	(1)	**(> ↓3)**	**(1)**	**(> ↓16)**

Our next step was to assess whether differences in the binding buffer could account for the large difference observed between our initial COS-7 binding data (Table 4-1) and published HEK293 binding data from Simpson et al. (1999). The striking finding was that the addition of 140 mM sodium chloride to the binding (and wash) buffer, to mimic the buffer conditions in the previous report (Simpson et al., 1999), resulted in a large (35-fold) increase in L-745,870 affinity for the rD2-V2.61(91)F mutant receptor (Figure 4-2B; Table 4-2), although the wild-type D2 receptor displayed only a very limited sodium sensitivity for this ligand (~7-fold, Figure 4-2A; Table 4-

2). These affinity values in the presence of sodium are consistent with those for hD2 wild-type and hD2-V2.61(91)F published in an earlier report (920 ± 200 and 9.5 ± 4.0 nM, respectively, Simpson et al., 1999). A similarly strong pattern of sodium-sensitive binding is evident for L-750,667, the iodinated derivative of L-745,870 (Figure 4-2, C and D; Table 4-2); the affinity of L-750,667 for the rD2-V2.61(91)F mutant was increased 37-fold in the presence of 140 mM

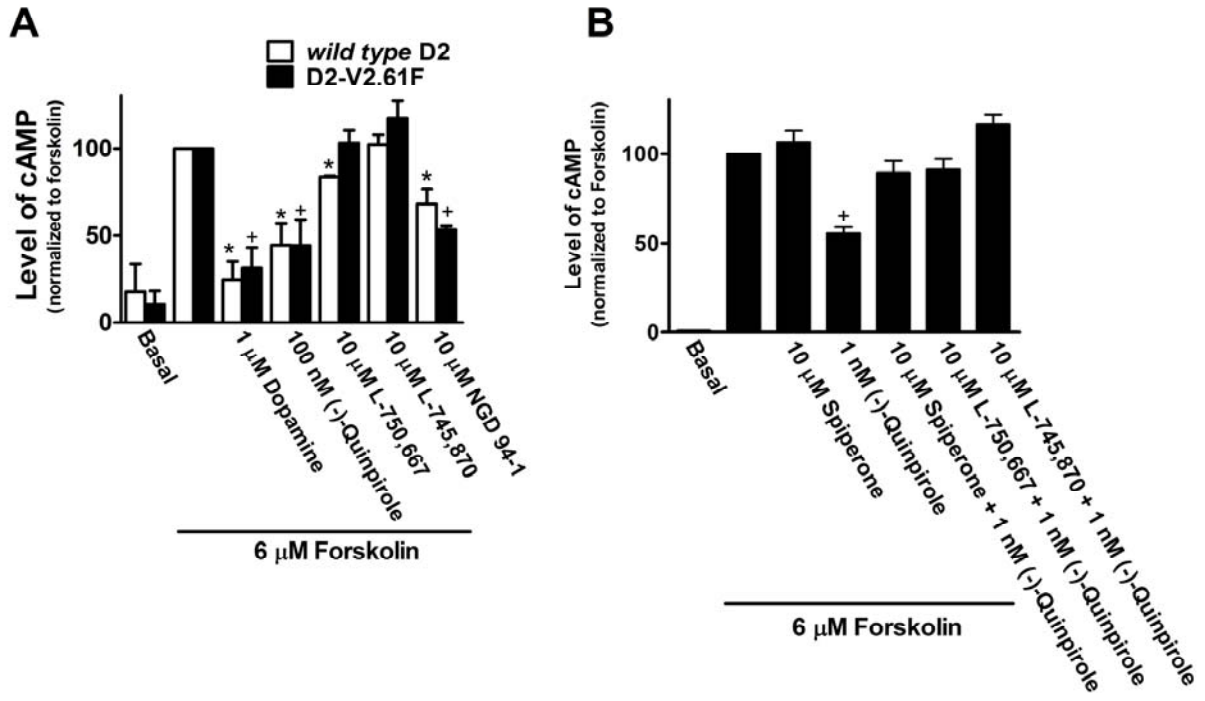
Figure 4-3. Single-point competition measurements for displacement of [^{3}H]-methylspiperone by various 1,4-DAPs. [^{3}H]-Methylspiperone-bound D_2 receptors were incubated with various 1,4-DAPs in the presence of different buffer conditions. (A) The presence of 140 mM sodium enhances the affinity of D_2 receptor for 1,4-DAP ligands . (B) The effect of sodium is greatly enhanced in the D_2 V2.61(91)F mutant. As a control for ionic strength and non-specific charge effects, NMDG was tested and displayed no significant changes from binding buffer alone.



sodium, even though there is only a moderate sodium sensitivity for this ligand at the wild-type receptor (>~7-fold). Another D4-selective 1,4-DAP, NGD 94-1, chosen because its structure is less similar to L-745,870 and L-750,667 (Table 4-1), still displayed comparably enhanced affinity for the D2-V2.61(91)F mutant in the presence of sodium (Figure 4-2, E and F; Table 4-2) but only a very small sodium-dependent increase in affinity for the wild-type receptor (Figure 4-2E; Table 4-1). Similar patterns of sodium sensitivity were also observed for several other D4-selective 1,4-DAPs and the sodium-sensitive substituted benzamide antagonist, (-)-raclopride (Figure 4-3), and these patterns were not mimicked by the same concentration of N-methyl-D-glucamine, a sodium replacement ion (Figure 4-3). Note that differences in (in the absence of

Figure 4-4. L-750,667 has agonist activity at only the wild type D_2 receptor. (A) The agonists dopamine, (-)-quinpirole and NGD 94-1 inhibit forskolin-stimulated cAMP response for wild type D_2 and D_2-V2.61(91)F mutant receptors stably expressed in HEK293 cells, indicating that both receptors are functional. L-745,870 is an antagonist at both receptors, but L-750,667 displays (partial) agonist activity at only the D_2 wild type receptor. (B) Blockade of a low concentration (-)-quinpirole functional response by L-750,667 indicates that, like L-745,870 and spiperone, it is an antagonist at the D_2-V2.61(91)F receptor. * means significantly different than forskolin alone for the wild type D_2 receptor, and + means significantly different than forskolin alone for the D_2-V2.61(91)F mutant receptor at $p \leq 0.05$. For each experiment the values are an average of triplicate determinations. The data are expressed as the geometric means from four separate experiments (n = 4) for all groups in 4A, except for dopamine, L-750,667 and NGD 94-1 where n = 3, and n = 3 for all groups in 4B. In Figure 4B, all groups are statistically different from (-)-quinpirole alone at $p \leq 0.05$.

Figure 4



sodium) affinities appear in Tables 4-1 and 4-2, but these are not comparable. The affinities for expressed wild-type and mutant D2 receptors in Table 4-1 are from membranes isolated from COS7 cells, whereas those in Table 4-2 are from membranes isolated from HEK293 cells.

In whole-cell attached functional assays, the full agonists dopamine and (-)-quinpirole both strongly reversed forskolin-stimulated increases in cAMP for both wild-type and D2-V2.61(91)F mutant receptors (Figure 4-4A). Spiperone was able to fully reverse the (-)-quinpirole-stimulated inhibition of cAMP (Figure 4-4B), but neither (-)-quinpirole nor L-750,667 were able to reduce forskolin-stimulated cAMP accumulation in untransfected HEK293 cells (data not shown).

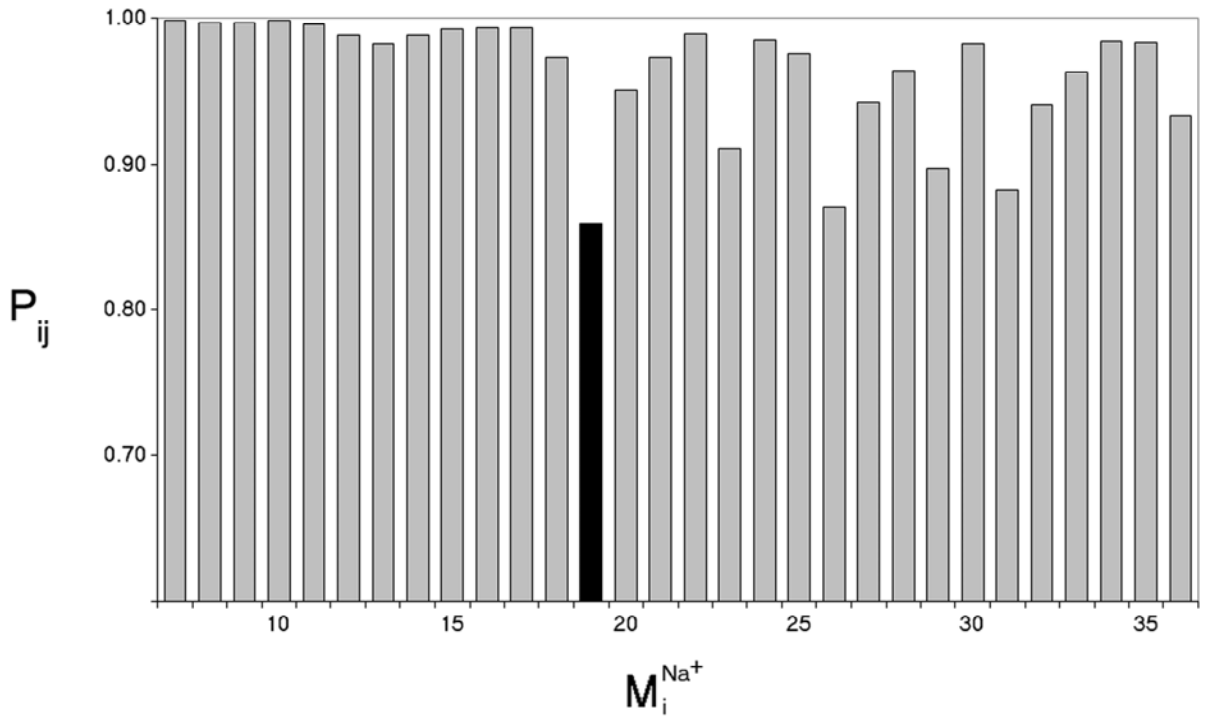
It is notable that we found that L-750,667 has partial agonist properties at the wild-type D2 receptor (Figure 4-4A) but acts as an antagonist at the D2-V2.61(91)F mutant receptor (Figure 4-4B). However, the chlorinated derivative, L-745,870, has no agonist properties at either receptor, whereas NGD 94-1 has agonist properties at both receptors (Figure 4-4A). Concentrations of L-745,870 five times higher than that shown in Figure 4-4 did not change its functional profile (data not shown). Despite these drugs having enhanced affinity in the presence of a high concentration of sodium (Figure 4-2), no attempt was made to measure such sodium effects in the functional assays.

Dynamic Properties of the Receptor Constructs

NMA performed on molecular model constructs of the D2 receptor was used to examine the dynamic response to sodium ion binding. In an effort to characterize changes in the receptor's harmonic motions imparted by the perturbation of sodium ion, we compared the spectrum of normal modes calculated for: 1) the sodium-bound structure [near D2.50(80)], 2) a negative control with sodium ion bound outside the protein core (at the carboxylate terminus), and 3) a system without any sodium, hereafter referred to as the null system. To ascertain which intrinsic low-frequency (and relatively high-amplitude) motions are sensitive to the perturbation from sodium binding within the receptor, we calculated the sum of dot product squares, $P_{ij} = \sum_{i,j(window)} |M_i^{Na+} \bullet M_j^{Null}|^2$ (see Figure 4-5) for each sodium-bound normal mode (M_i^{Na+}) over ranges (windows) of modes in the null spectrum M_j^{Null} (for details, see Materials and Methods). A plot of Pij as a function of sodium-bound normal mode vectors (M_i^{Na+}) indicates that sodium ion binding influences the characteristic movements of only a few low-frequency modes. Pij values < 0.9 are indicative of motions different from those described by the normal modes of the null

Figure 4-5. Divergence in sodium-bound normal modes M_i^{Na+} from the null spectrum, M_j^{Null}, calculated from the normal mode analysis of the control (no sodium in binding site) system. Dot product squares were computed for each sodium-bound normal mode vector against modes within a window of the null spectrum centered at the most analogous mode M_j^{Null} (highest dot product with M_i^{Na+}). Each dot product square is then summed over the entire null spectrum window to provide a P_{ij} value ($P_{ij} = \sum_{i,j(window)} |M_i^{Na+} \bullet M_j^{Null}|^2$). Plotted here is P_{ij} for each normal mode M_i^{Na+}, calculated over a window size of seven null modes. Among the first non-trivial normal modes (7-18), only M_{19}^{Na+} exhibits significant divergence, as it is not reproduced by a set of normal modes from the null system. A similar trend in P_{ij} values is obtained regardless of the window size used in the P_{ij} calculation.

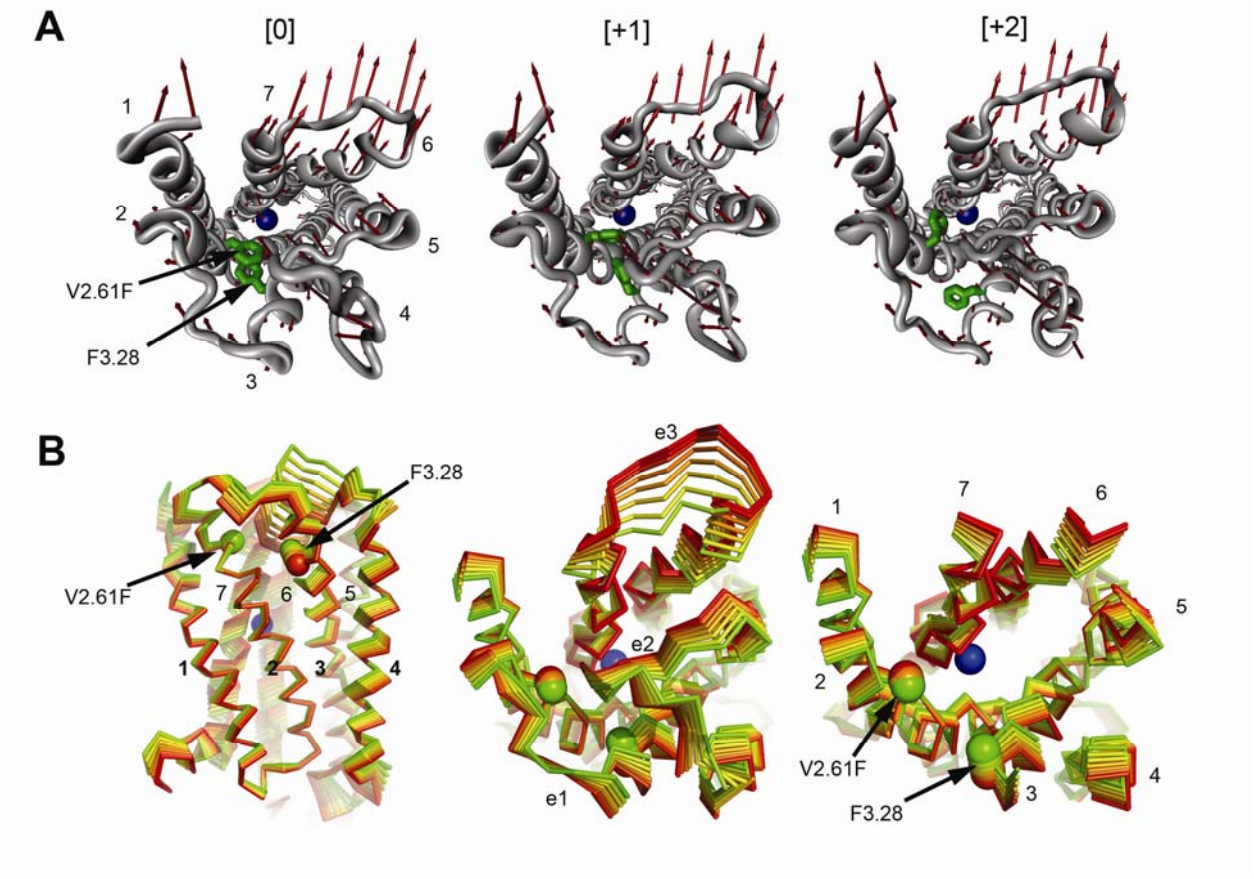
Figure 5



system. Such values were observed for modes 19, 26, 29, and 31 in the sodium-bound structure, hereafter referred to as M_{19}^{Na+}, M_{26}^{Na+}, and so forth. We explored different window sizes (window = 7, 15, 21, and 106) in the null spectrum to see whether divergent M_i^{Na+} could be recapitulated ($P_{ij} \sim 1.0$) by comparison against larger windows of the null spectrum. M_{19}^{Na+}, however, consistently provided P_{ij} values significantly lower than the other M_i^{Na+} modes. P_{ij} calculations for the negative control spectrum ($M_i^{Control}$) against the null spectrum (not shown) do not yield uniquely low Pij values comparable with M_{19}, which also suggests that the M_{19}

Figure 4-6. The sodium-sensitive normal mode vector M_{19}^{Na+}. (A) Motion along vector M_{19}^{Na+} disrupts the "hydrophobic brace": red arrows indicate the directionality of the "open" motion vectors for C_α atoms (grey ribbon) in M_{19}^{Na+}. The sodium ion in the binding site is represented by a blue sphere. Green sticks depict the side chains of V2.61(91)F and F3.28(110) at the TM2-TM3 interhelical junction (helices identified by numbers). A small movement along the M_{19}^{Na+} path from [0] (*left*) to [+1] (*middle*) to [+2] (*right*) leads to disruption of interactions at this TM2-TM3 junction. (B) The range of motions and flexibilities. *Left and middle*: opening interval of M_{19}^{Na+} from the initial (green = 0) to fully open conformation (red = +7). In this motion, the kinking in TM2 becomes more pronounced as the intracellular segment of TM2 moves with TM3 while the extracellular segment moves with TM1. A significant vertical movement (perpendicular to membrane plane) of TMs 6 and 7 upon opening pushes the extracellular loop 3 (e3) up and out, away from the cleft; lateral motions (in the plane of the bilayer) of TMs 3-5 lead to a clamping down of the e2 loop on the binding site (Shi and Javitch, 2004). Note that the sodium ion is fixed to reduce visual clutter. *Right*: extracellular vantage with loop regions removed. The shearing motion at the extracellular region of TMs 2 and 3 is apparent in the increased spacing between C_α atoms of V2.61(91)F and F3.28(110) (spheres).

Figure 6



divergence is site specific in regards to sodium perturbation. M_{19}^{Na+} had no clearly equivalent mode in the entire spectrum calculated for the null system. The modes M_{19}^{Null} and M_{16}^{Null} were most analogous, with $P^{Na+/Null}_{ij}$ values of 0.25 and 0.36, respectively. Together, these findings suggest that the different motion described by M_{19}^{Na+} is due to the effect of sodium binding on the dynamic properties of the receptor molecule.

It is interesting that visual inspection of the M_{19}^{Na+} trajectory shows that it deforms the binding pocket and as a result facilitates the potential contact of V2.61(91)F with the ligand (Figure 4-6, A and B). The trajectory describes a concerted TM2 kinking motion at the proline kink (P2.59) along with the lateral TM3/TM4 motion away from the cleft in the plane of the

bilayer, which could potentially disrupt the TM2-TM3 interhelical packing to expose V2.61(91)F for interaction with the ligand. Therefore, sodium induction of this particular mode must be considered relevant to the observed enhancement of L-745,870 affinity in the D2-V2.61(91)F mutant receptor.

The dynamic response of the receptor to sodium binding, as measured by the divergence between normal mode spectra from sodium-bound and null structures, agrees with dynamic perturbation studies in other systems showing that significant changes in conformational equilibrium can be triggered by what would appear to be only small perturbations at "dynamical control points" (Ming and Wall, 2005, 2006). In the D2 receptor, the allosteric site is likely coupled to instabilities near the proline kink in TM2. Despite sequence divergence for the templates rhodopsin and β2-adrenergic receptor in this region, the TM2s are virtually superimposable. In the β2-adrenergic receptor, an H-bond between W3.28 and the backbone carbonyl oxygen of V2.57 stabilizes the Pro kink. In our homology model of the D2 receptor, the corresponding interactions are missing because of differences in sequence, but the dynamics of the TM2 region could still be coupled to the sodium binding site near D2.50(80) via intrahelical H-bonding and/or local conformational arrangements involving interhelical (TM2–3) side chain packing, interhelical H-bonding, or structural waters near the sodium binding pocket. To examine how dynamic flexibility expressed in the various receptor conformations visited by the M_{19}^{Na+} trajectory may influence L-745,870 binding in the D2-V2.61(91)F mutant receptor, we developed a docking protocol to explore a series of conformations determined by this trajectory.

Ligand Docking Guided by Conformations from M_{19}^{Na+}

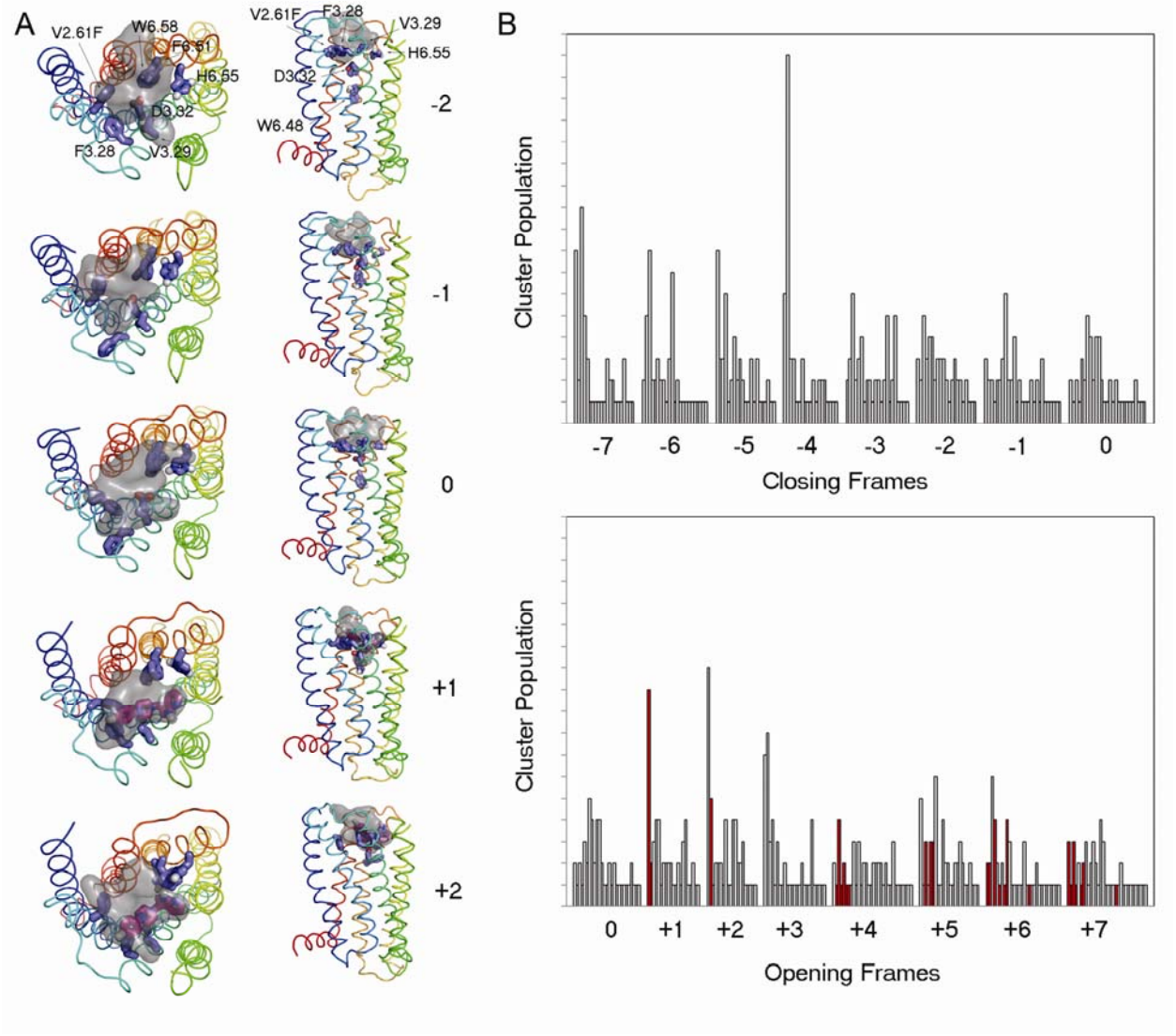
Based on preliminary results of L-745,870 docking into our initial model of the D2 receptor (frame 0), the docking poses with the highest ranking lacked the expected interactions: π-stacking between the p-chlorophenyl moiety of L-745,870 and V2.61(91)F (Figure 4-6, A and B) and the H-bond reinforcement of the ionic interaction between the piperazine ammonium proton and D3.32(114). This is because in the "closed" conformation of the D2 receptor model, V2.61(91)F is adjacent to D2-F3.28(110), with which it can maintain stable interactions (π - stacking or T-type interactions) that reduce the ability of the ligand to interact with it. In addition, the cleft in which the protonatable amine of the piperazine ring must fit to achieve this salt bridge is effectively occluded by residues F3.28(110), V3.29(111), and F6.51(361). These observations agree with our experimental data showing low affinity for L-745,870 in wild-type D2 and D2-V2.61(91)F in the absence of high concentrations of sodium.

To explore the changes in ligand binding attributable to the dynamic effects produced by sodium, we used the M_{19}^{Na+} trajectory to construct as described under Materials and Methods, 15 receptor conformers (frames -7 to +7) representing the structure of the receptor along the motion described by M_{19}^{Na+} (Figure 4-7A). Each of these conformations was used to dock L-745,870. The protocol involves 50 separate dockings into each of these conformations, and the resulting ligand poses generated within each frame were binned into clusters based on similarity of binding position and orientation (Figure 4-7B). Receptor frames -1 and -2 in Figure 4-7A represent the closed interval (-7 being maximally closed) (see Figure 4-6, A and B), and +1 through +7 represent the "opened" frames (+7 being maximally opened) (Figure 4-7B). Because the amplitude of the M_{19}^{Na+} trajectory was set arbitrarily in the NMA calculation, we were most interested in the smallest backbone movements away from the initial

Figure 4-7. Docking of L-745,870 into D_2 V2.61(91)F receptor conformers visited by the M_{19}^{Na+} trajectory. (A) Frames shown from top to bottom correspond to closed (negative) and open (positive) steps in the M_{19}^{Na+} trajectory, respectively. The D_2 receptor structure is rendered as a ribbon and the TM segments are colored according to the rainbow spectrum—TM1=blue, to TM7=red. The transparent grey blob indicates the cumulative van der Waals space occupied by the top 5 most favorable binding poses. Initial (0) and closed frames -1 and -2 prevent ligand access for direct interactions with V2.61(91)F/F3.28 and D3.32(114), and restrict occupancy to regions extracellular of the presumed binding site. In contrast, in the frames corresponding to the "open" phase of the harmonic motion of M_{19}^{Na+} (frames +1, +2), L-745,870 is accommodated in orientations that support experimentally suggested interactions with V2.61(91)F, F3.28(110), and D3.32(114). Maroon stick represents L-745,870 bound in experimentally validated poses with the *p*-chlorophenyl ring toward F2.61(91) and F3.28(110). (B) Histograms of docking pose clusters. L-745,870 was docked into various conformers of the D_2 receptor based on the M_{19}^{Na+} trajectory. Frame 0 represents the initial homology model structure. Frames -1 to -7 (upper histograms) represent various receptor conformers designated as "closed," and frames +1 to +7 (lower histograms) represent "open" frames of the receptor. The histogram collects results from 50 poses obtained from each independent L-745,870 docking performed on each receptor conformation taken from the trajectory and ranked with the AutoDock4 energy-based scoring function. The poses are binned into clusters by similarity (3.0 Å RMSD), with the vertical bar height indicating the number of cluster members (population). Clusters in each docking run are also ranked most favorable to least favorable (left-to-right) based on the lowest energy representative within each cluster. Red bars highlight clusters that represent suitable binding conformations that achieve the expected binding geometries defined for the 1,4-DAP class of

ligands docked to the D_4 receptor (Kortagere et al., 2004). Note that proper binding geometries are only obtained in "opened" frames, indicating the need for a conformation change in the D_2 receptor to obtain the high-affinity state.

Figure 7



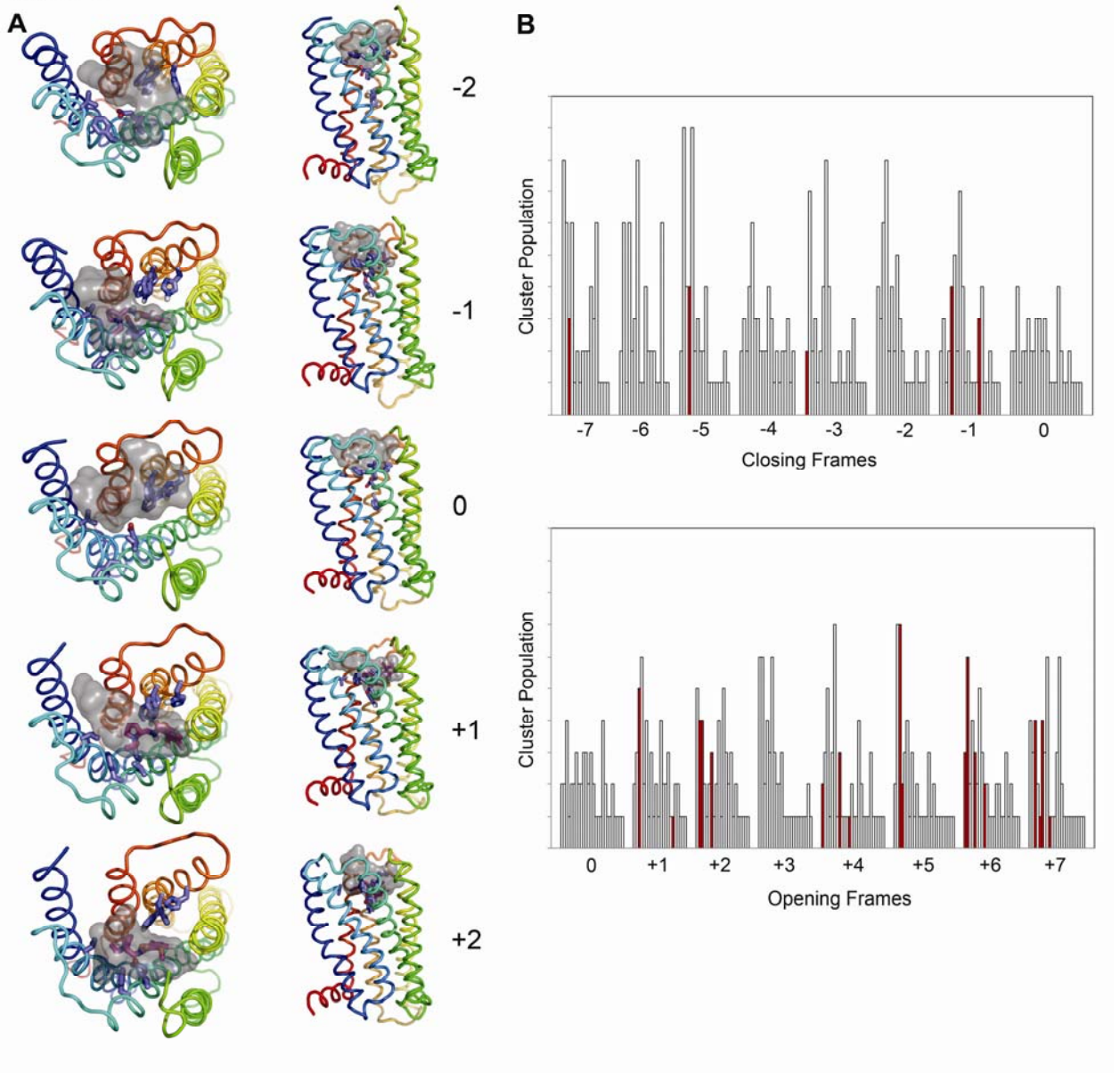
structure (±1, 2, 3) rather than the extremes (±7). Docking of L-745,870 into the various frames of the receptor oscillating along the M_{19}^{Na+} harmonic motion shows that even a minimal excursion into the open phase of M_{19} better accommodates the expected ligand binding poses

(see Kortagere et al., 2004) and, hence, facilitates a direct interaction between either of the ligand's aryl moieties and the phenyl ring in the V2.61(91)F mutant. Within these receptor conformations visited by the M_{19}^{Na+} trajectory, L-745,870 can achieve reasonable binding geometries that are not available in the static D2 receptor model. It is notable that proper ligand accommodation in the open frames is because of the increase in the TM2 proline kink bend angle and TM3 lateral translation and increased accessible depth of the binding cleft that is noticeable in Figure 4-7A (note the position of the gray regions that depict regions favorable to ligand occupancy). As the receptor opens (frames +1 and +2), this ligand occupancy region moves intracellularly into the vicinity of D3.32(114) and W6.48(358). Thus, the movement provides increased space for the ligand to access the entire binding cleft spanning from TM2,3,7 to TM3,5,6. Therefore, while maintaining the previously identified contacts in the D2 receptor, L-745,870 can associate with a series of TM cleft-lining residues including F2.61(91), L2.64(94), C3.25(107), F3.28(110), V3.29(111), D3.32(114), V3.33(115), C3.36(118), F5.38(189), V5.39(190), S5.42(193), F5.47(198), W6.48(358), F6.51(361), T7.39(386), G7.42(389), and Y7.43(390) and potentially with e2 loop residues L143(171), E153(181), C154(182), I155(183), and I156(184) (when the loop is replaced after docking). This enhancement of the binding pocket by the effect of sodium binding on the dynamic properties of the receptor makes some previously inaccessible binding sites available to the ligand and results in the higher affinity observed in the presence of sodium.

Figure 4-8. (-)-Raclopride docked into wild type D_2 receptor conformers visited by the M_{19}^{Na+} trajectory. (A) Initial (0) and “closed” frames (-1, -2) restrict (-)-raclopride occupancy in the primary binding pocket (situated between TMs 4, 5, and 6) that is expected to accommodate the substituted benzamide ring moiety of (-)-raclopride (Lan et al., 2006). Although a few docking

poses were observed in "closed" conformers (-7 to -1) with the signature H-bond reinforced ionic interaction between the ligand's pyridyl ammonium group and the carboxylate sidechain of D3.32(114), the benzamide ring remained extracellular of the presumed primary binding site– without deeper penetration into the cleft primary cleft. In contrast, the frames corresponding to the "opened" phase of the harmonic motion of M_{19}^{Na+} (frames +1, +2) accommodate (-)-raclopride in orientations that satisfy the experimentally suggested interactions, such as the H-bond reinforced ionic interaction, an H-bond interaction between the ligand's hydroxyl group and the sidechain of Y7.43(388) (not shown), and the deeper access of the benzamide ring into the primary pocket (Lan et al., 2006). (B) Histograms of docking poses for (-)-raclopride in wild type D_2 receptor. (-)-Raclopride was docked into various conformers of the D_2 receptor based on the M_{19}^{Na+} trajectory. Frame 0 represents the initial homology model structure. Frames -1 to -7 (upper histograms) represent various receptor conformers designated as "closed," and frames +1 to +7 (lower histograms) represent "opened" frames of the receptor. 50 (-)-raclopride docking poses were obtained on each receptor conformation taken from the trajectory and ranked with the AutoDock4 energy-based scoring function. The poses are binned into clusters by similarity (3.0 Å RMSD), with the vertical bar height indicating the number of cluster members (population). Clusters in each docking run are also ranked most favorable to least favorable (left-to-right) based on the lowest energy representative within each cluster. Red bars highlight clusters that represent suitable binding conformations that achieve the expected binding geometries defined for the substituted benzamide class of ligands docked to the D_2 receptor. Note that validated binding geometries are more frequently obtained in opened frames (78% versus 22% in the closed frames), indicating a suitable collective motion vector for conformation change in the D_2 receptor to obtain the high-affinity states for (-)-raclopride.

Figure 8



(-)-Raclopride was also docked into the wild-type D2 receptor conformations visited by the M_{19}^{Na+} trajectory (Figure 4-8). We find that receptor conformations generated along the opening path of this motion also increase the likelihood of proper binding interactions with (-)-raclopride; an H-bond-reinforced ionic interaction is achieved with D3.32, and the ligand's phenyl substituent penetrates more deeply into the primary binding pocket situated among TM 3,

5, and 6. These findings support our observation that (-)-raclopride affinity for wild-type D2 receptor is enhanced in the presence of sodium (Figure 4-3).

DISCUSSION

Our finding that the affinity of L-745,870 for the rD2-V2.61(91)F mutant is drastically increased in the presence of high sodium concentration (140 mM) is significant for two main reasons. First, it explains the discrepancy (~100-fold differences) in the reported affinities of L-745,870 and several other structurally similar D4-selective 1,4-DAPs for the D2-V2.61(91)F mutant and thus resolves an apparent contradiction in the literature (Simpson et al., 1999; Floresca et al., 2005). Second, it demonstrates how key molecular interactions between a ligand and a specific GPCR microdomain are influenced by occupancy of an allosteric site. In our case, the interactions that become accessible through the allosteric effect of sodium binding involve a π-stack or T-type interaction between the ligand's aryl moiety and F2.61(91) of the receptor as we proposed previously (Kortagere et al., 2004). However, we found that in the D2-V2.61(91)F receptor, this favorable interaction can occur only if an interhelical π-stack between the F2.61(91) and the adjacent F3.28(110), which forms a "hydrophobic brace," is disrupted. We show that this disruption, achieved by sodium occupancy near D2.50(80), induces new dynamics that appear to widen the junction between TM2 and TM3 in the extracellular region of the receptor and thus disrupt the F2.61/F3.28 interaction. It is important that each of the 1,4-DAP compounds tested here (L-745,870, L-750,667, NGD 94-1, RBI-257, PD168,077, FAUC213, and Ro61-6270) is predicted from molecular models to engage in similar interactions when phenylalanine occupies position 2.61(91); in agreement, we found experimentally that all

compounds tested display drastic sodium-dependent increases in affinity for the D2-V2.61(91)F mutant.

Although previous studies have revealed the affinity relationship between the discriminant structural features of 1,4-DAPs and the 1,4-DAP D4/D2 selectivity-conferring positions 2.61(91), 3.28(110), and 3.29(111), the effects of mutations in this microdomain on the activity of 1,4-DAP ligands had not been described previously (Simpson et al., 1999; Schetz et al., 2000; Kortagere et al., 2004; Floresca et al., 2005). Therefore, because the D2-V2.61(91)F receptor exhibits sodium-sensitive affinity changes to 1,4-DAPs, we examined here the functional properties of L-745,870, L-750,667, and NGD 94-1 at the wild-type and D2-V2.61(91)F receptor. In contrast to the reports showing reduction in agonist affinities as a result of allosteric modulation by sodium, we report here sodium-dependent enhancement of affinities for the 1,4-DAP agonists (and antagonists) similar to that observed for the substituted benzamide antagonists (Neve, 1991). Moreover, we report here that although the D2-V2.61(91)F mutant can be activated by dopamine and (-)-quinpirole, it cannot be activated by L-750,667, which exhibits weak partial agonist properties on the wild-type D2 receptor. Rather, L-750,667 acts as an antagonist of the D2-V2.61(91)F mutant. The relatively small influence of the D2-V2.61(91)F mutation on the binding affinity of (-)-quinpirole and methylspiperone demonstrated here suggests that neither ligand is likely to directly contact 2.61(91). Furthermore, this mutation had little effect on the activation of the receptor by (-)-quinpirole and its reversal by methylspiperone. This suggests that the 2.61(91)-3.28(110) hydrophobic brace can prevent the receptor from being activated by some agonists if their binding brings them near position 2.61(91). Because the affinities of other 1,4-DAPs, which retained their agonist properties at the D2-V2.61(91)F mutant receptor, were also enhanced in the presence of sodium, we reviewed the

literature concerning the connection between agonist high- and low-affinity states, G protein coupling, and sensitivity to sodium and GTP.

In broken membrane preparations, high concentrations of either sodium ions (120 mM) or GTP and its related analogs (100 μM) decrease the affinity of agonists for catecholaminergic GPCRs (Paris et al., 1989; Neve, 1991; Neve et al., 2001; Schetz and Sibley, 2001). However, the similarities between the agonist affinity shift for sodium and GTP are coincidental because sodium binds an allosteric site on dopamine receptors accessible from the intracellular side (Neve, 1991; Neve et al., 2001; Schetz and Sibley, 2001), whereas GTP binds G proteins that then complex with the receptor at a different intracellular allosteric site. Under conditions (e.g., no GTP) in which a ternary complex is formed (receptor + G protein), kinetic studies of detergent-solubilized or broken membrane α adrenergic receptors demonstrate that both sodium ions and Gpp(NH)p accelerate the rate of agonist dissociation, and a synergistic increase in the rate is observed in the presence of both of these modulators (Limbird et al., 1982). In broken membrane equilibrium studies of α adrenergic receptors, agonist affinity decreases in the presence of either sodium or Gpp(NH)p, and a further decrease in affinity is observed in the presence of both of these modulators. These results were cited as evidence that sodium ions do not compete with GTP for its binding to G proteins (Limbird et al., 1982). Later studies by the same group demonstrated that substitution of the negative charge of the conserved aspartic acid in TM2 [D2.50(79)] to a neutral asparagine in the α2 adrenergic receptor resulted in a total loss of epinephrine's sensitivity to sodium (Horstman et al., 1990). When tested for its ability to stimulate GTPase activity, this same mutant receptor had a 7.5-fold decrease in potency for the agonist UK14304 with no change in efficacy (Ceresa and Limbird, 1994) and no change in affinity relative to the wild-type receptor. Increasing concentrations of Gpp(NH)p decrease the

agonist p-[^{125}I]iodoclonidine's binding to the wild-type but not the mutant receptor (Ceresa and Limbird, 1994). These data suggest that the sodium-insensitive D2.50(79)N mutant α adrenergic receptor can still couple to G proteins but that the state of coupling (coupled or uncoupled) no longer influences agonist high- and low-affinity states of the receptor. Thus, it would appear that, at least in the case of α2 adrenergic receptors, sodium ions control the agonist high- and low-affinity states of the receptor but do not uncouple G proteins from their receptor.

Broken membrane equilibrium studies demonstrate that dopamine's affinity for the D2 receptor is decreased when pertussis toxin or GTP uncouple G proteins from the receptor and that the addition of sodium ions further decreases dopamine's affinity (Neve et al., 1989). This was cited as evidence that sodium ions interact directly with dopamine receptors. Later studies by the same group demonstrated that substitution of the conserved aspartate in TM2 [D2.50(80)] for alanine results in a mutant receptor whose affinities for agonists and substituted benzamide antagonists are sodium insensitive (Neve et al., 1991); however, this same mutant receptor is unable to efficiently couple to G proteins, making it impossible to carry out the same types of studies as described above for α2 adrenergic receptors. Finally, the rate of chemical modification of D2 dopamine receptors by the thiolreactive agent N-ethylmaleimide is altered in the presence of sodium (Neve, 1991), and this is consistent with our finding [utilizing the D2-V2.61(91)F mutant receptor as a molecular probe of the 1,4-DAP binding pocket] that the binding of sodium ions to the allosteric site [D2-D2.50(80)] causes significant conformation changes in the receptor in the region of the orthosteric binding pocket.

The changes in dynamic properties of the receptor that can explain the observed pharmacological consequences of sodium binding are illustrated by our findings from NMA. Thus, the presence of sodium was found from this analysis to give rise to a unique, high-

amplitude (low-frequency) normal mode that is not observed for the wild-type structure. Following the motions represented by this unique, sodium-related mode shows that it produces a wider cleft opening in the region responsible for ligand binding. These "opening dynamics" allow larger ligands to be accommodated in the pocket for ligand recognition and make room for better interaction of the ligand with the sites in the receptors at which it forms H-bonds, aromatic interactions, and hydrophobic matches. This leads to the measured improvement in the ligand's affinity for the receptor. Specifically, the effects of sodium binding near D2.50(80) on the dynamics in the microdomain surrounding the 2.61(91) position were shown by NMA to involve an increase in the scissors-like helical movements of the extracellular portions of TMs 2 and 3. This motion accounts for the enlarged opening and, consequently, for the large affinity changes observed at the D2-V2.61(91)F mutant, where these movements disrupt the hydrophobic brace formed by the interaction between V2.61(91)F and F3.28(110). The result of these local rearrangements is a receptor conformation more suitable for accommodating a ligand like L-745,870, which can now make stabilizing interactions as found in the optimal docking poses. Similar sodium-enhanced motions are predicted to occur in the wild-type receptor, which lacks a hydrophobic brace, but cleft opening would lead to better ligand access to the conserved aspartic acid at D3.32(114) for a reinforced ionic bond interaction with the protonatable piperizinyl amine portion of the docked 1,4-DAP. This prediction is supported experimentally by our findings for the wild-type D2 receptor whose affinities for L-745,870, L-750,667, and NGD 94-1 are somewhat increased (~3–7-fold) in the presence of sodium. Moreover, the docking of the substituted benzamide (-)-raclopride, which belongs to a distinct structural class of D2 antagonists, into conformations along the sodium-induced normal mode produced very similar results. Again, suitable binding poses were more readily achieved in wild-type D2 receptor

conformations visited along the open phase of the sodium-induced mode (Figure 8), thus accounting for the enhanced affinity for (-)-raclopride (Figure 3) and other substituted benzamides at the D2 receptor when modulated by sodium (Neve, 1991).

How these dynamic changes affect ligand binding is clearly demonstrated by exploring the binding poses of L-745,870 in a series of receptor conformers built along the characteristic vector of M_{19}^{Na+}. It is notable that involving NMA in a docking protocol to provide a basis for exploring collective backbone movements in ligand docking has also been used successfully to reproduce known conformations from ligand-bound crystal structures (Lindahl and Delarue, 2005). Here, we found that docking into conformations generated from the open interval of M_{19}^{Na+} accommodates L-745,870 in the expected binding modes for 1,4-DAPs in D4 dopamine receptors (Kortagere et al., 2004) because the opening motion disrupts contact between F2.61(91) and F3.28(110), which in turn creates access to these residues that appeared inaccessible to the p-chlorophenyl ("mode 1") or pyrrolopyridinyl group ("mode 2") of L-745,870 in the initial D2 receptor homology model. The access is established by the dynamic changes and enables favorable aromatic-aromatic interactions between the ligand moieties and the receptor sites. This result suggests, therefore, that the specific perturbation propagated from the sodium binding site influences ligand affinity by dynamically changing the interactions in the orthosteric site. The backbone movement observed in our model might capture the phenomenon of sodium allostery by achieving the high-affinity receptor conformational state along the M_{19}^{Na+} vector path and/or by disrupting the brace that stabilizes the low-affinity state without a significant, time-averaged, conformational change, i.e., through the sodium-induced amplitude increase along this vector (Cooper and Dryden, 1984; Popovych et al., 2006). In any case, the presence and effect of endogenous allosteric modulators (e.g., sodium, G protein, GPCR dimer

partner, etc.) should be considered when selecting a receptor representation for in silico library screening. It is also important to point out that the use of target receptor conformations based on normal mode trajectories could inform the design of drugs working through allosteric mechanisms.

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FOOTNOTES

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CHAPTER V

THE ROLE OF TRANSMEMBRANE SEGMENTS TWO AND THREE IN D2 RECEPTOR BINDING AND FUNCTION

Molecular Determinants of Subtype Selective D2 Receptor Function are located in Transmembrane Segments Two and Three

My work in the D2 receptor microdomain formed by transmembrane segments (TM) two and three yielded several findings that provide significant contributions to the literature for both dopamine receptors and G protein coupled receptors (GPCRs). First, I found that this microdomain is important for both the binding and function of specific ligands of the 1,4-disubstituted aromatic piperadine/piperazine (1,4-DAP) structural class to the D2 dopamine receptor. Reciprocally mutating the positions 2.61, 3.28, and 3.29 in the D2 receptor background yielded the expected correlation between increasing number of amino acids and increased subtype selective 1,4-DAP affinity for many 1,4-DAPs with the exception of 1,4-DAPs with orthoelectronegative Arm B groups and methylspiperone (Table 2-1 and Table 4-1). An affinity shift was not expected for methylspiperone based on the results of previous studies which suggested that this compound is not significantly affected by mutation of these residues (Kortagere et al., 2004). These results support previous data which pioneered the idea that 1,4-DAPs were highly sensitive to certain amino acid positions in the D2 and D4 receptor backgrounds (Simpson et al., 1999, Schetz et al., 2000, Kortagere et al., 2004, Floresca et al., 2005). However, these previous studies lacked functional data examining whether changes in

binding affinity are correlated to positive (D2 to D4-like function) or negative (no change in D2 function) changes in D2 receptor function. The results for L-750,667 suggest that, in the D2 receptor background, changes in binding affinity can be positively correlated to changes in D2 receptor affinity and function yielding a receptor that is not only more D4-like in binding but also more D4-like in function (Figures 2-1, 2-4, 2-5; Table 2-1, 2-2). However, the results for D2-V2.61F+FV3.29-3.29LM appear to be specific to only the D2 receptor background since L-750,667 does not gain agonist properties at the D4-F2.61V+FV3.28-3.29LM receptor (Appendix B-6). This suggests that other residues may be involved in the orientation of this ligand in the D4 receptor binding pocket and that these ligands prevent L-750,667 from activating the D4-F2.61V+LM3.28-3.29FV receptor. Additionally, the affinity data obtained for CP226,269 and NGD94-1 suggest that 1,4-DAPs with orthoelectronegative groups on Arm A (Appendix A) have less favorable interactions with the D2 receptor binding pocket in the D2-V2.61F+FV3.28-3.29LM mutant receptor when compared to the D2-FV3.28-3.29LM mutant receptor (Table 2-1). The substitution of the phenylalanine at position 2.61, as suggested by the affinity data in Table 2-1, creates an unexpected negative steric interaction between these two ligands and other residues in the binding pocket perhaps by subtly changing the binding pocket orientation of these ligands. This premise is supported by previous data in the D4 receptor background (Kortagere et al., 2004) and the contact likelihood matrix for NGD94-1 in the D2-V2.61F+FV3.28-3.29LM receptor (Figure 2-6). Taken together, these results show that reciprocal modification of specific amino acids within the TM2/3 microdomain of the D2 receptor enables the mutant receptor to both bind and function similar to the D4 receptor for D4-selective 1,4-DAPs that do not possess orthoelectronegative groups.

Antagonism or weak partial agonism of the D2 receptor is the primary functional modality of neuroleptic drugs used for the treatment of schizophrenia. Typical antipsychotics, such as the butyrophenone haloperidol, block mesocorticalimbic D2 receptors to affect a cessation of symptomatology (Creese et al., 1976; Seeman et al., 1976; Seeman 1987). Atypical antipsychotics such as clozapine and aripiprazole, act in a similar fashion but possess a favorable side effect profile which minimizes the risk of tardive dyskinesia. Aripiprazole, a D2 selective 1,4-DAP, was once thought to be free of extrapyramidal side effects, however, recent clinical examples in female patients with prior antipsychotic use (risperidone or substituted benzamides) have refuted this claim (Abbasian and Power, 2008; Wang et al., 2009). Interestingly, Wang and colleagues (2009) proposed the hypothesis that chronic blockade of D2 receptors with long term use of substituted benzamides may result in D2 receptor hypersensitivity. This hypersensitivity is then exacerbated by the partial agonism of aripiprazole. However, this hypothesis conflicts with a case report from Caykoylu and colleagues (2009) where aripiprazole was used to treat an incidence of tardive dyskinesia in a female patient with a history of risperidone therapy. Incidentally, while substituted benzamides have been shown to increase their affinity for membrane preparations expressing D2 receptors in the presence of sodium or in mutant D2 receptors possessing modification of the intracellular allosteric binding site, antagonists and inverse agonists rarely exhibit significant sodium-sensitive changes in wild type D2 receptor affinity (Neve et al., 1991; Neve et al., 2001; Ericksen et al., 2009). However, despite these molecularly-based observations, it is difficult to determine if increased levels of intracellular sodium had a role in the manifestation of tardive dyskinesia in the two case reports from Wang and colleagues (2009) since no evidence was presented for a cardiovascular or metabolic disorder. If the homeostatic balance of sodium was indeed abnormal in these cases, a possible

mechanism may be that increased intracellular sodium shifted aripiprazole potency causing it to behave as an antagonist instead of a partial agonist. In this hypothesis, D2 receptor hypersensitivity would persist or worsen and dopaminergic neurotransmission would remain as the primary instigator of the dyskinesia. Despite these adverse reactions, aripiprazole is easily tolerated, has an extremely low incidence of tardive dyskinesia, and remains a useful treatment for patients experiencing adverse reactions to other neuroleptics.

Agonism of dopamine receptors is the primary modality to alleviate the symptoms of Parkinson's disease. A pathological hallmark of Parkinson's disease is the loss of dopamine producing neurons from the substantia nigra which results in a greatly diminished capacity to signal striatal motor neurons. Dopaminergic agonism replenishes the dopaminergic signals to the striatal neurons which alleviates the bradykinesia associated with the disease. While the most common antiparkinsonian treatment is L-Dopa, a dopamine precursor, agonists of D1, D2, and D3 receptors such as pramipexole, ropinirole, bromocriptine, pergolide, and dinapsoline are showing some promise as alternative therapies (Schetz and Sibley 2007). In particular, pramipexole, a D3/D2 receptor agonist with D3>D2 selectivity, may have the advantage of additional anti-neurodegenerative capabilities due to the ability block mitochondrial transition pores and act as an antioxidant (Cassarino et al., 1998; Gu et al., 2004; Johnston and Brotchie, 2004). However, the primary neuroprotective effect of pramipexole appears to be mediated through stimulation of the D3 receptor (Joyce and Millan 2007; LeWitt and Taylor 2008). Dihydrexidine, a D1 selective full agonist, was initially found to have anti-Parkinsonian potential in MPTP treated monkeys and no agonist activity at D2 or adrenergic receptors (Schneider et al., 1994; Kohli et al., 1993). However, the therapeutic potential of dihydrexidine and other D1 agonists for Parkinson's and cognitive disorders has diminished since they appear to rapidly

desensitize D1 receptors and attenuate hippocampal acetylcholine release (Lin et al., 1996; Giardina and Williams, 2001; Wade and Nomikos 2005). However, dinapsoline, a sparingly selective D1 agonist, has diminished capability for D1 receptor desensitization and may therefore be a useful mixed selectivity drug for the treatment of Parkinson's (Gulwadi et al., 2001; Ghosh et al., 1996).

As mentioned in Chapter I, the related D4 receptor was once targeted as a potential vector for the treatment of schizophrenia (Van Tol et al., 1991, Seeman et al., 1993; Helmeste et al., 1996; Tang et al., 1997, Oak et al., 2000). However, the failure of multiple clinical trials proves that D4 antagonism is not an efficacious treatment for schizophrenic symptoms (Kramer et al., 1997; Truffinet et al., 1999; Corrigan et al., 2004). Additionally, while reports on the role of D4 dopamine receptors in cognitive attention processes had initially suggested that polymorphic variants of the third intracellular loop were associated with attention deficit hyperactivity disorder (ADHD), more recent studies have challenged both the hypo-responsiveness and genetic linkage of these variants (for review see Schetz and Sibley 2007). Current reports suggest that, while there is no genetic linkage between the diagnosis of ADHD and the D4.7 variant, at least for children, inviduals expressing the D4.7 variant have better clinical outcomes (Shaw et al., 2007; Johansson et al., 2008). However, D4 receptors have been associated with other important physiological effects such as centrally mediated arousal and circadian rhythm. As briefly mentioned in Chapter I, partial agonism of the D4 receptor is associated with centrally mediated penile erection. A well documented side effect of the non-selective but D2-prefering dopamine agonist apomorphine is centrally mediated penile tumescence (Lal et al., 1987; Dula et al., 2000; Melis et al., 1987). Ironically, prior to our modern understanding of sexual preference, the centrally mediated sexual side effects of apomorphine were historically used as an off-label

method to ‘increase’ heterosexual libido in homosexual individuals (Smith et al., 2004). However, in a large clinical survey of patients prescribed Uprima (Abbott Laboratories Ltd, UK), apomorphine was largely ineffective as a pro-erectile drug in elderly male patients (Maclennan et al., 2006). In studies focusing on the D4 receptor, erectile effects similar to apomorphine were observed for rats dosed with the 1,4-DAP partial agonists PD168,077, CP226,269 or ABT-274, suggesting that D4 receptors were the primary instigators of centrally mediated erectogenesis (Brioni et al., 2004; Hsieh et al., 2004; Melis et al., 2005). More importantly, an additional study has shown that PD168,077 stimulates rat penile erection by stimulating oxytocin release in the ventral tegmental area, an area associated with the perception and expectation of reward in humans (Succu et al., 2007; D'Ardenne et al., 2008). This role in arousal has parallels in retinal D4 receptor mediated contributions to circadian rhythm (for review see Iuvone 2005). Light stimulates the release of dopamine from retinal amacrine cells which activates intracellular pathways in photoreceptors bearing D4 receptors. This dopaminergic activity has a complex and not well understood relationship with melatonin (Ivanova et al., 2008; Pozdeyev et al., 2008; Jackson et al., 2009). While melatonin appears to be the central zeitgeber, dopaminergic release in response to retinal detection of light appears to play an important role in synchronization of circadian rhythm. For example, in humans, the absence of light detection in blind patients is associated with free-running circadian rhythms and sleep disorders (Waller et al., 2008). While a D4 receptor agonist might be efficacious in circadian disorders of arousal, resetting the circadian rhythm with appropriately timed melatonin is the current therapeutic of choice.

Sodium induced G Protein Independent Inactivation of G Protein Coupled Receptors is Linked to Movements in Transmembrane Segments Two and Three

A common mechanism of allosteric inhibition in Class A GPCRs is receptor modulation in the presence of sodium. This allosteric interaction is believed to have either positive or negative interactions on receptor function depending upon the receptor type and subtype. Therefore, in order to understand how sodium modulation may be related to physiological processes, an understanding GPCR classification may be necessary. GPCRs are classified based on >20% amino acid sequence homology, endogenous ligand, gene clustering, and the appearance of defining characteristics such as special N terminus domains. The largest division, Family or Class A GPCRs includes rhodopsin, dopamine, adrenergics and many other GPCRs (see Kristiansen 2004). However, this organizational scheme limits options for subdivision by placing many different receptors into one massive category without further subdivision. Instead, the GRAFS system which subdivides G proteins based on phylogenetic analysis into glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin receptor families may be better suited for subdivision of sodium modulatory effects (Fredriksson et al., 2003; Bjarnadottir et al., 2006). In this classification scheme, rhodopsin receptors are further subdivided into α, β, γ, and δ groups based on the four main phylogenetic branches of the rhodopsin family cluster. In terms of sodium sensitive receptor classifications, the α-group of rhodopsin receptors contains the adrenergic, dopamine, histamine, muscarinic, serotonin, trace amine receptors, adenosine, opsin, melatonin, cannabinoid, and prostanoid receptors. The β-group of contains neurotensin, oxytocin, arginine vasopressin (AVP), and tachykinin receptors. The γ-group contains chemokine, angiotensin, and somatostatin receptors. The δ-group contains the opioids. For the majority of these receptors, modulation by sodium occurs when sodium binds to an allosteric

intracellular binding pocket formed by amino acids located within transmembrane segments two, three, and seven (see page 125).

As discussed in Chapter IV, allosteric binding of sodium induces a conformation change in both dopaminergic and adrenergic receptors that possesses low affinity for agonists independent of coupled or uncoupled G proteins. Other receptors share striking similarities with dopaminergic and adrenergic receptors with respect to the conserved aspartate at position 2.50 and sodium sensitive conformational change independent of G protein coupling. In a study on the Leutinizing Hormone/Choriogonadotropin (LH/CG) receptor, a D2.50N mutation eliminates the sodium binding site rendering the mutant receptor insensitive to sodium (Quintana et al., 1993). Additionally, as expected for this mutant (see Chapter IV), the affinity of LH for the mutant receptor was similar to the affinity of LH at the wild type receptor in the presence of sodium bound to the allosteric site. Similarly, potency for both LH and hCG, as tested by cAMP accumulation, was decreased by one order of magnitude from the potency at the wild type. In contrast, a D2.50A mutation in the Adenosine 1 receptor (A1) exhibits no change in the affinity of the agonist cyclopendyladenosine (CPA) when competing against the antagonist [3H]8-cyclopentyl-1,3-dipropylxanthine (DPCPX) in the presence of high concentrations of sodium, while the wild type A1 receptor has an approximately 10 fold decrease in affinity (0-400 mM NaCl; Barbhaiya et al., 1996). Later studies observed that wild type A1 receptors had a 3-fold decrease in affinity, but, more importantly, observed a greater decrease in affinity for the constitutively active A1-G1.36(14)T mutant receptor (5-fold, de Ligt et al., 2005). Adenosine 2a (A2a) receptor dissociation rate experiments using the antagonist [3H]ZM241385 in competition with the allosteric modulators 5-(N,N-hexamethylene)amiloride (HMA) and sodium illustrate that 100 mM sodium decreases the dissociation rate of [3H]ZM241385 in rat striatal membrane

preparations while not affecting the rate of [3H]ZM241385 association (Gao and Ijzerman 2000). This is similar to previous evidence in solubilized α2a adrenergic receptors which showed that sodium bound to the allosteric site not only increased the dissociation rate of [3H]yohimbine, a potent inverse agonist, but, more importantly, greatly increased the association of this ligand with the wild type α2a receptor (Horstmann et al., 1990; Wade et al., 2001). While there is no evidence for the inverse agonism of ZM241385 in the A2a receptor, in a constitutively active Adenosine 2b (A2b) mutant receptors, ZM241385, similar to DPCPX, is only able to fully reverse the activity of mutant receptors with low constitutive activity (Li et al., 2007). These results suggest that distinct features within the different receptor binding pockets may account for the subtle differences in rates of ligand association and dissociation, and that these microdomains, when modulated by sodium, produce the observed increases or decreases in ligand affinity.

M1 muscarinic acetylcholine receptors with a D2.50N mutation exhibit a small 5-fold decrease in affinity for the agonist carbachol but greatly decreased potency (24-fold) in measurements of IP3 accumulation (Fraser et al., 1989). Similarly, mutant NTR1 and NTR2 receptors in the presence of sodium were impaired in both binding affinity and PI production (Martin et al., 1999). NTR1-D2.50(113)A mutant receptors become insensitive to modulation by sodium and experience an loss in potency of about two orders of magnitude ($EC_{50\ NTR1\text{-}D2.50(113)A}$ = 10.3 ± 2.3 nM; $EC_{50\ NTR1\text{-}WT}$ = 0.09 ± 0.01 nM). These losses of affinity are, however, independent of G-protein coupling as observed by GTPγS binding to NTR1 wild type and NTR1-D2.50(113)A mutant receptors. NTR2 receptors are unique in that an alanine occupies position 2.50 instead of the highly conserved aspartate which renders the wild type receptor insensitive to sodium. Interestingly, in NTR2-A2.50(79)D mutants, the receptor gains sodium

sensitivity allosteric binding site mediated inhibition of orthosterically bound [^{125}I]Neurotensin ($IC_{50\ NTR2\text{-}A2.50(79)D}$ = 55 ± 5 mM; $IC_{50\ NTR2\text{-}A2.50(79)D}$ = 225 ± 17 mM). Unfortunately, the mutant NTR2 receptor was unable to gain the ability to activate phospholipase C, as the authors had hoped. These results are a contrast to the sodium induced G-protein independent receptor inactivation of somatostatin receptor type 2 (SST2) (Kong et al., 1993). In this study, somatostatin affinity at the mutant SST2-D2.50N receptors was unchanged from SST2 wild type receptors. However, both GTPγS and PTX were able to significantly decrease somatostatin binding to the mutant somatostatin receptor indicating that G protein coupling was not significantly inhibited by the substitution of an asparagine at this position. These studies suggest that, at least for neurotensin and somatostatin receptors, position 2.50 is not always a determinant for G protein coupling, but rather an allosteric site through which sodium may modulate the states of the receptor.

Early studies in opioid receptors noted that opioid agonist binding and activity were decreased in the presence of sodium (Pert and Snyder, 1974). Later experiments using membrane preparations showed that antagonist mediated inhibition of opioid receptor activity has a distinct GTPase component which may be modified by sodium (Costa et al., 1990). More specifically, NG108-15 cell membrane fragments containing δ-opioid receptors were tested for [^{32}P]GTP hydrolysis in the presence of sodium, lithium, rubidium, and potassium. Basal levels of GTPase activity were ranked as sodium<lithium<rubidium<potassium, in order of increasing activity (range 12-18 pmol/(mg*min)), however, minimal inhibition of GTPase activity was observed in this experiment for either the agonist [D-Ala^2-D-Leu^5]-enkephalin (DADLE) or the peptide antagonist [N,N'-diallyl-Tyr1 ,Aib23]Leu-enkephalin (ICI 174864) in the presence of monovalent cations. Intrigued, the authors examined the effect that modification of the

$[Na^+]/[K^+]$ ratio would have on various opioid ligands. Based on the ability to inhibit GTPase activity below basal levels the antagonists were broken into three classes: "full negative" which included ICI 174864 and the analogue [N,N'-diallyl-Tyr1, ψ (CH_2S)-Phe^4]Leu-enkephalin (ICI 154129); "partial negative" which included naloxone; and "neutral" which included naltrexone and MR 2266. These "negative" antagonists are inverse agonists. Similarly, based on the ability to stimulate GTPase activity, DADLE and [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) were classified as full agonists while diprenorphine and morphine were classified as partial agonists. Interestingly, full agonists and full inverse antagonists were unaffected by sodium-induced inhibition of GTPase activity (relative intrinsic activities of 1 and -1 respectively) while partial inverse agonists, partial agonists, and neutral agonists had sodium-sensitive decreases in GTPase activity. Furthermore, in rabbit and guinea pig cerebellar membrane preparations, both mu and kappa opioid receptors were found to be inactivated in the presence of both sodium and guanyl-5′-yl imidodiphosphate (Gpp(NH)p) a non-hydrolyzable GTP analog (Frances et al., 1985). This opioid receptor inactivation may serve as a feedback mechanism to regulate sodium intake into neuronal populations. For example, a substantial increase in acute salt craving was observed for rodents in rat lateral parabrachial nuclei treated with the opioid receptor agonist β-endorphin, (de Oliviera et al., 2008). When these endorphin challenged rats were given the opioid receptor antagonist naloxone, these cravings ceased.

In COS1 cell membrane preparations, competition studies between bradykinin and [3H]Phe5HOE140 for bradykinin 2 (BK2) receptors revealed a 100-fold loss in bradykinin binding in the presence of a little as 10 mM NaCl (Quitterer et al., 1996). The high sensitivity of the BK2 receptor to sodium was mirrored in the three-fold decreases in both constitutive (basal) and bradykinin stimulated IP3 production in the presence of 140 mM NaCl. Despite the loss in

bradykinin efficacy, the potency of this agonist at the BK2 receptor was unaffected by sodium. Interestingly, knockout mice with homozygous deletion of the BK2 receptor, have significant increases in blood pressure (20-25 mmHg) when fed a high sodium diet for eight weeks (Alfie et al., 1999). Taken together these results suggest that allosteric regulation of the BK2 receptor, similar to the opioid receptors, may be important for homeostatic regulation of sodium.

While some small controversy has emerged regarding the role of sodium sensitive modulation of receptor function, sodium is generally thought to stabilize a state of receptor inactivity i.e. the agonist low affinity state. The main argument against this hypothesis is the quote from Neve and colleagues (2001) which states "Thus, binding of sodium to this residue may be important for stabilizing an active receptor conformation" in relation to several different data sets that showed mutations of D2.50 decrease or eliminate receptor function. However, for this to be true, sodium would need to stabilize the active state of the wild type receptor. Functional data for GTPase activity in membrane fragments containing wild type δ-opioid receptors (Costa et al., 1990) or wild type D2 receptors (Lin et al., 2006) suggest that, when ionic gradients are maintained, the potency of agonist response, and in some cases the efficacy of partial agonist response, is increased in the absence of sodium. Additionally, data I gathered in D2 receptors suggests that the TM2/3 microdomain, which moves in response to sodium bound at the intracellular allosteric site, may be important for the ability of certain ligands to activate the receptor (see Chapter II). This not only suggests that activation of sodium sensitive GPCRs is hindered in the presence of sodium, but also that, when sodium is removed from the experiment in a controlled method, sodium sensitive GPCRs are primed for activation.

Consistent with the literature, sodium induced GPCR states appear to be primarily associated with transmembrane segment two with contributions from the other transmembrane

segments. However, the data presented here (Chapter IV) illustrates an outward rotation in extracellular portions of transmembrane segments one and two coupled to an inward rotation of transmembrane segments two and three when sodium is bound to the intracellular allosteric site. By using D4-selective 1,4-DAPs as molecular probes, I was able detect the large changes in affinity resulting from the re-positioning of transmembrane segment two and three in the D2-V2.61F receptor by sodium bound to the intracellular allosteric site. These results, when coupled with the modeling provided by Dr. Spencer Ericksen (Cornell University), revealed for the first time the sodium induced movement of transmembrane segments in a sodium sensitive GPCR. These results will help to further define the role sodium modulation in sodium sensitive GPCR systems by contributing data which illustrates the specific movements induced by sodium on transmembrane segments two and three. It is my hope that these results will eventually contribute to our understanding of how sodium fluctuations across cell membranes affect, not only the binding and function of endogenous and exogenous ligands, but also the molecular mechanisms that are the foundations of cellular homeostasis.

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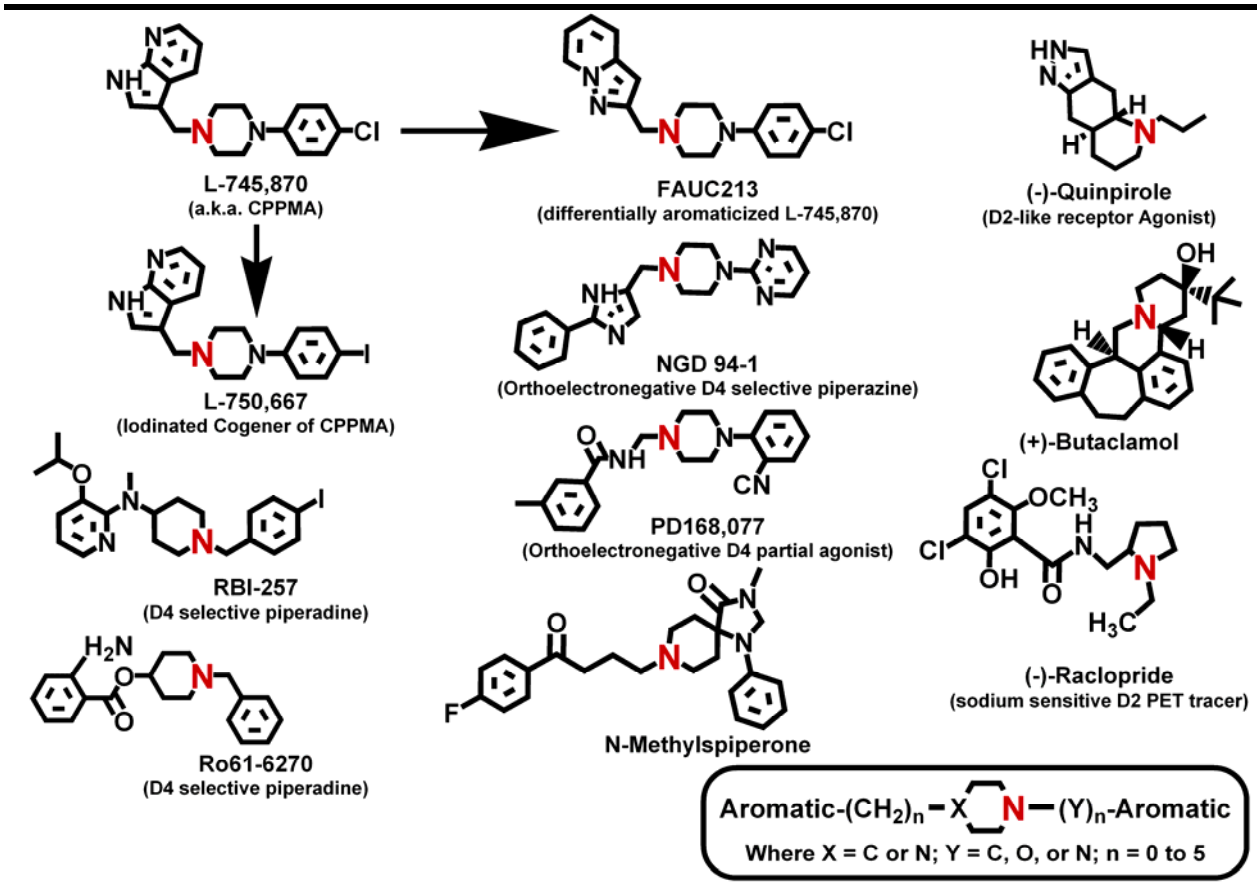
APPENDIX A

THE MOLECULAR STRUCTURES OF 1,4-DISUBSTITUTED AROMATIC PIPERADINE/PIPERAZINES AND OTHER PERTINENT MOLECULES

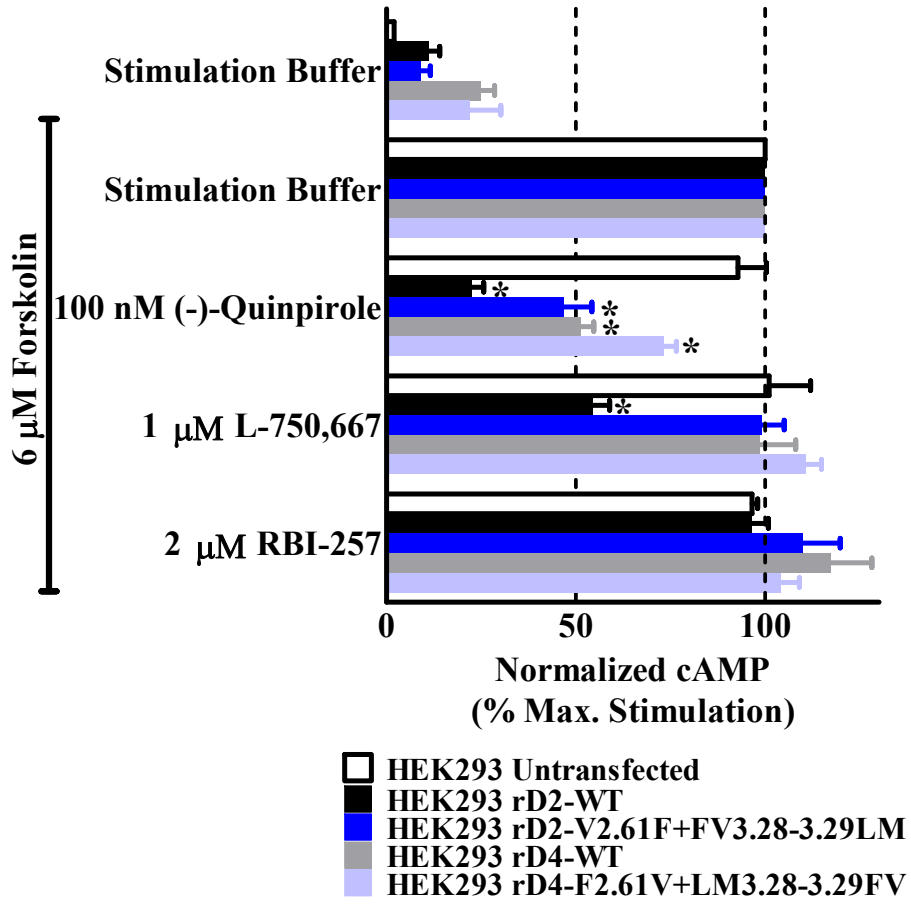
Appendix A. The Molecular Structures of 1,4-Disubstituted Aromatic Piperadine/ Piperazines and Other Pertinent Molecules. The molecular structures of the 1,4-DAPs are presented here and arranged with a red protonatable amine, a left facing Arm A, and a right facing Arm B. With the exception of N-Methylspiperone, all of the illustrated 1,4-DAPs putatively interface with TM2/3 through Arm B aromatics. The red protonatable amine is protonated at physiological pH by the acidic conserved aspartic acid at 3.32. In the lower right hand corner, a simplified molecular scheme for 1,4-DAPs is shown. Quinpirole, Butaclamol, and Raclopride are provided for reference. See Kortagere et al., 2004 for further information.



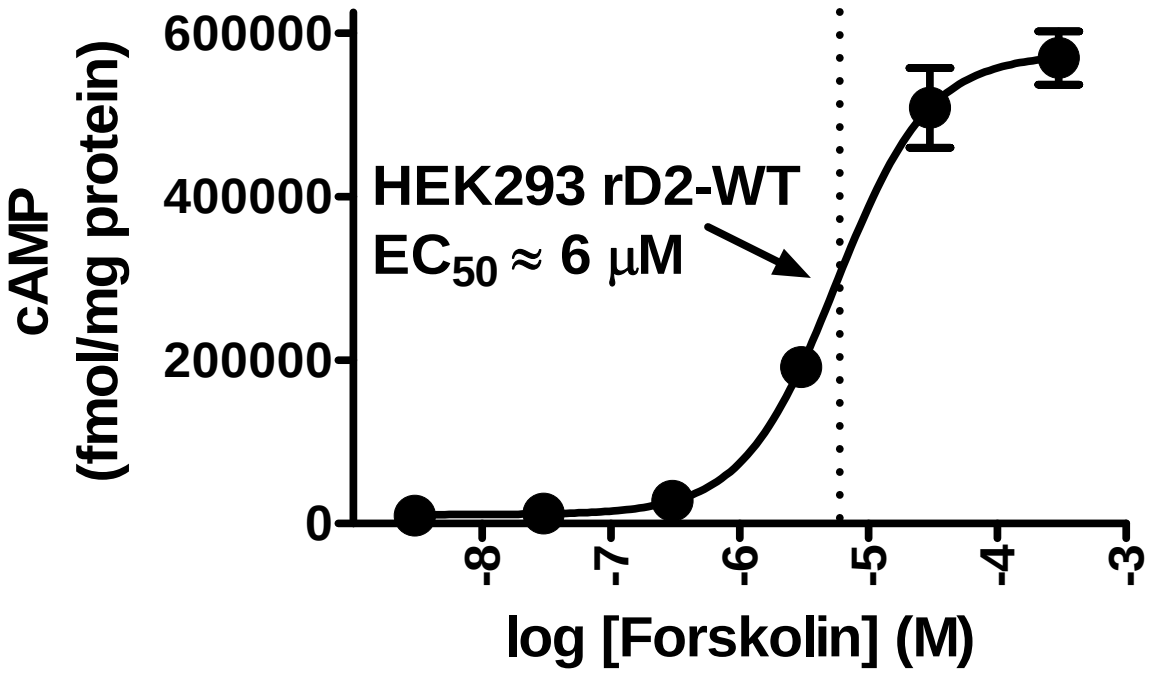
APPENDIX B

CYCLIC ADENOSINE MONOPHOSPHATE CONTROL EXPERIMENTS

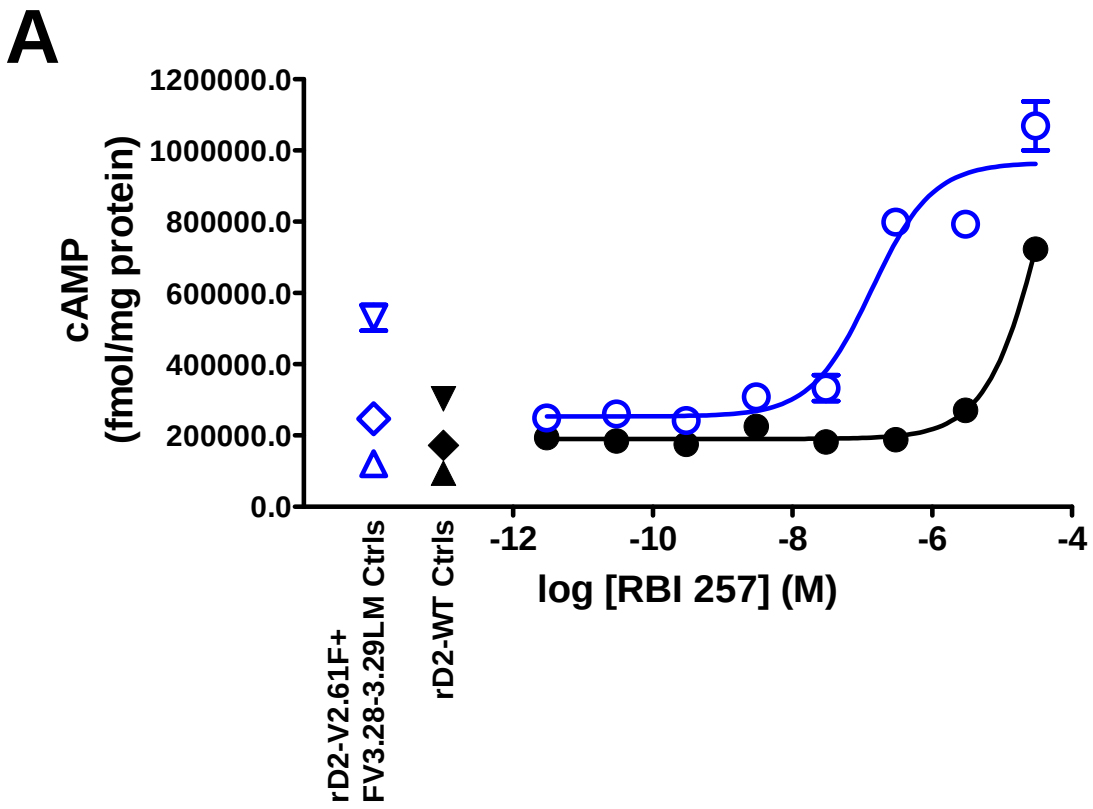
Appendix B-1. (-)-Quinpirole, L-750,667, and RBI257 do not produce an endogenous cAMP response in untransfected HEK293 cells. Untransfected HEK293 cells were challenged, in triplicate, with single concentrations of (-)-Quinpirole, L-750,667, and RBI257. 100 nM (-)-Quinpirole was sufficient to establish a significant ($p \leq 0.05$, Dunnett's post hoc analysis) full agonist response in all the transfected HEK293 cell lines while demonstrating no significant effect in the untransfected cells. Similarly, while concentrations up to 10 µM can be used (Figure 2-4), 1 µM L-750,667 was sufficient to induce a significant partial agonist effect in only rD2-WT expressing HEK293 cells. No significant agonist response was generated for 2 µM RBI257. The results are shown as the average ± SEM for three or more experiments. Data was analyzed via one way ANOVA with Dunnett's pos hoc analysis ($p \leq 0.05$).

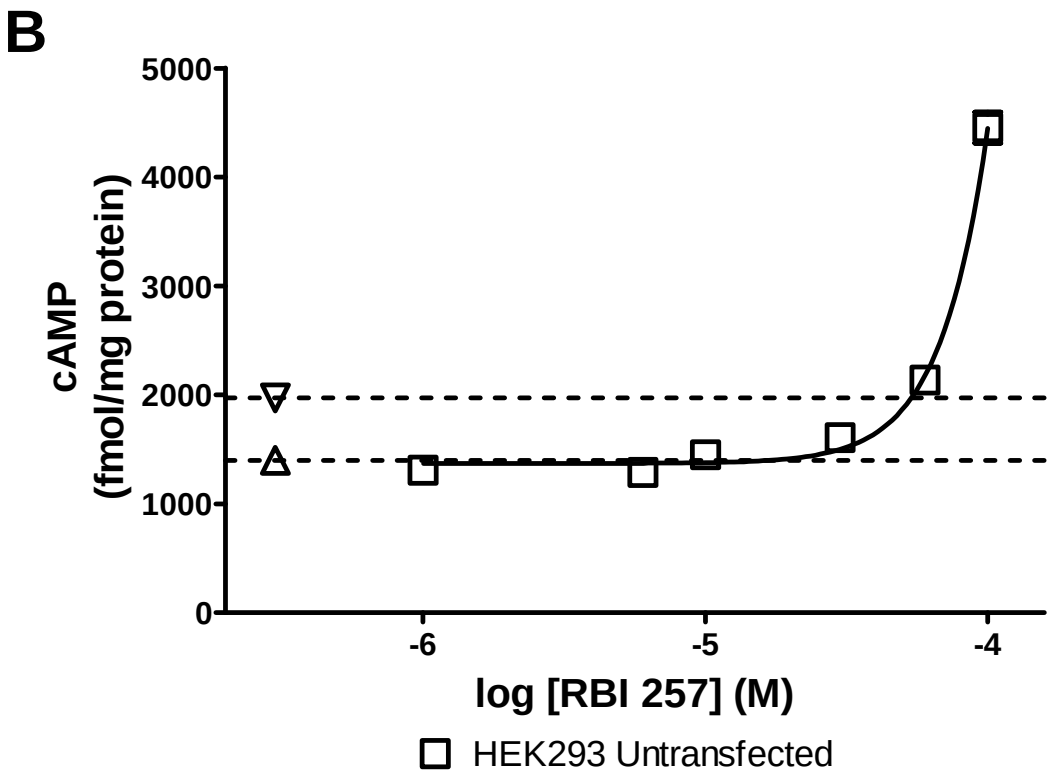


Appendix B-2. Forskolin concentration response in HEK293 rD2-WT cells. This is a representative example (n=1) of forskolin induced concentration-dependent cAMP elevation in HEK293 rD2-WT (●) cells assessed in triplicate. Based on this and other similar assessments in untransfected and transfected HEK293 cells, 6 μM Forskolin was chosen for subsequent assays (n>3).

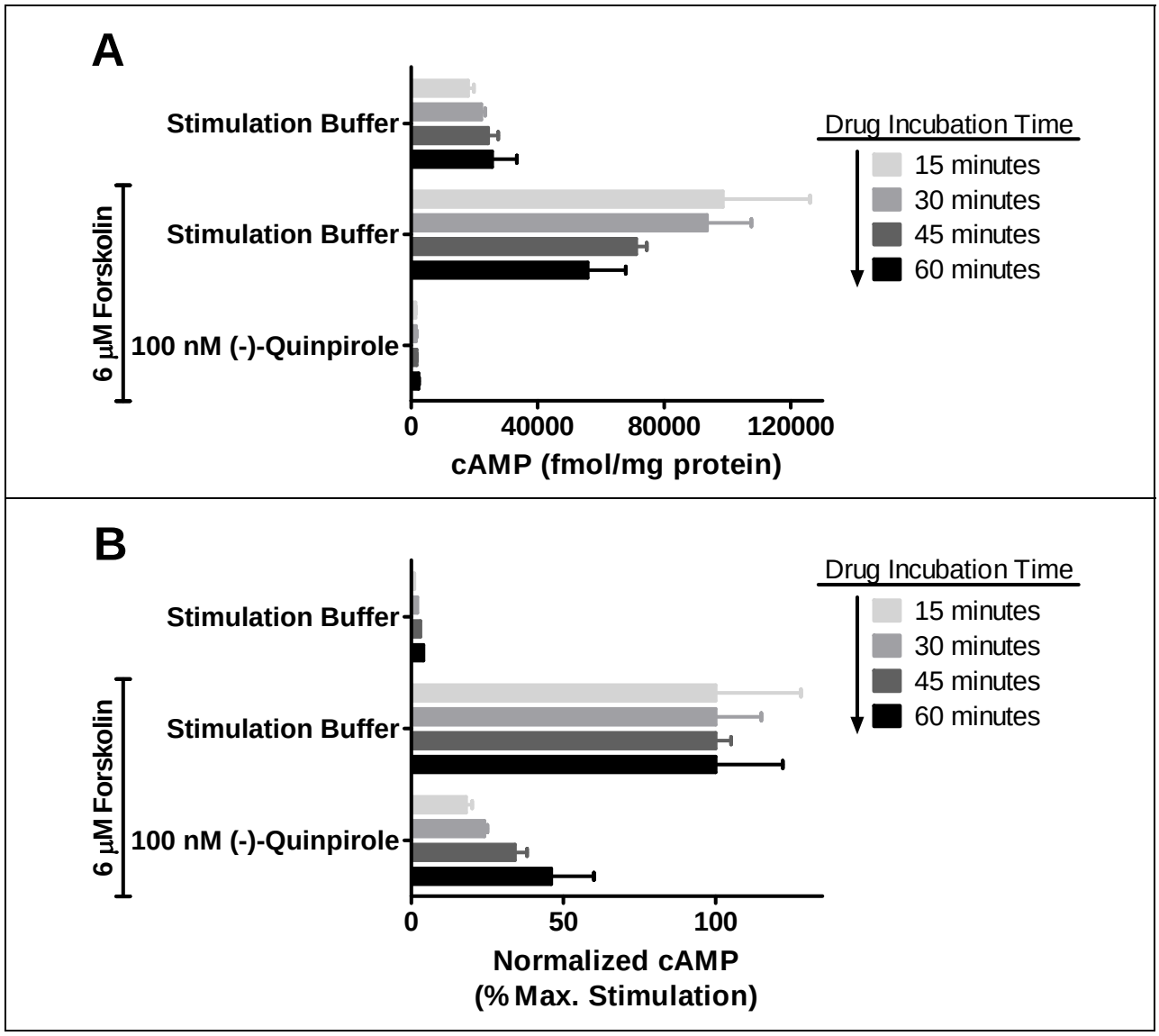


Appendix B-3. Limitations to the concentration response of RBI257. A) The initial functional testing of RBI257 in transfected HEK293 cells revealed a non-specific increase cAMP elevation at concentrations of RBI257 greater then 10 µM. A. Micromolar concentrations of RBI257, while able to concentration-dependently reverse 60 nM (-)-quinpirole (◆,◇), elevated cAMP levels beyond the respective forskolin controls (▼,▽) of rD2-WT (●) and rD2-V2.61F+FV3.28-3.29LM (○). Also shown is the respective stimulation buffer control (▲,△). B) Untransfected HEK293 cells (□) were challenged with increasing concentrations of RBI257. Concentrations of RBI257 greater then 1 µM were tested versus a 6 µM forskolin control (▽) for cAMP elevation beyond basal (stimulation buffer,△). Based on these results, RBI257 concentrations were kept below 10 µM in future assays to avoid cell line dependent increases in cAMP.

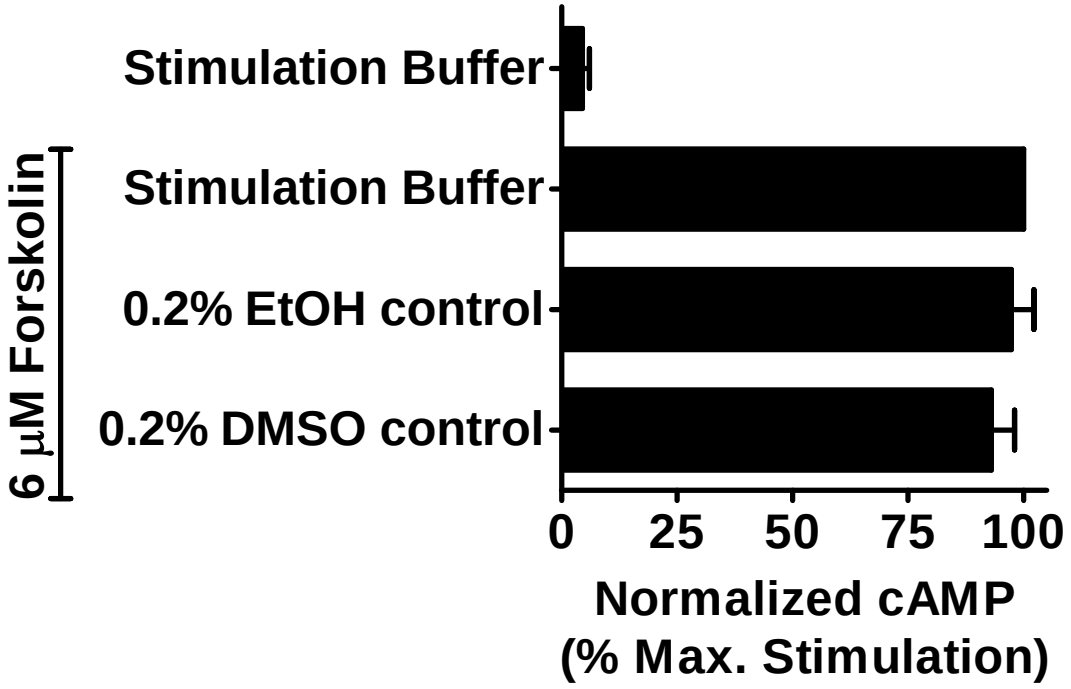



B
5000
4000
3000
2000
1000
0
cAMP
(fmol/mg protein)
-6
-5
-4
log [RBI 257] (M)
HEK293 Untransfected

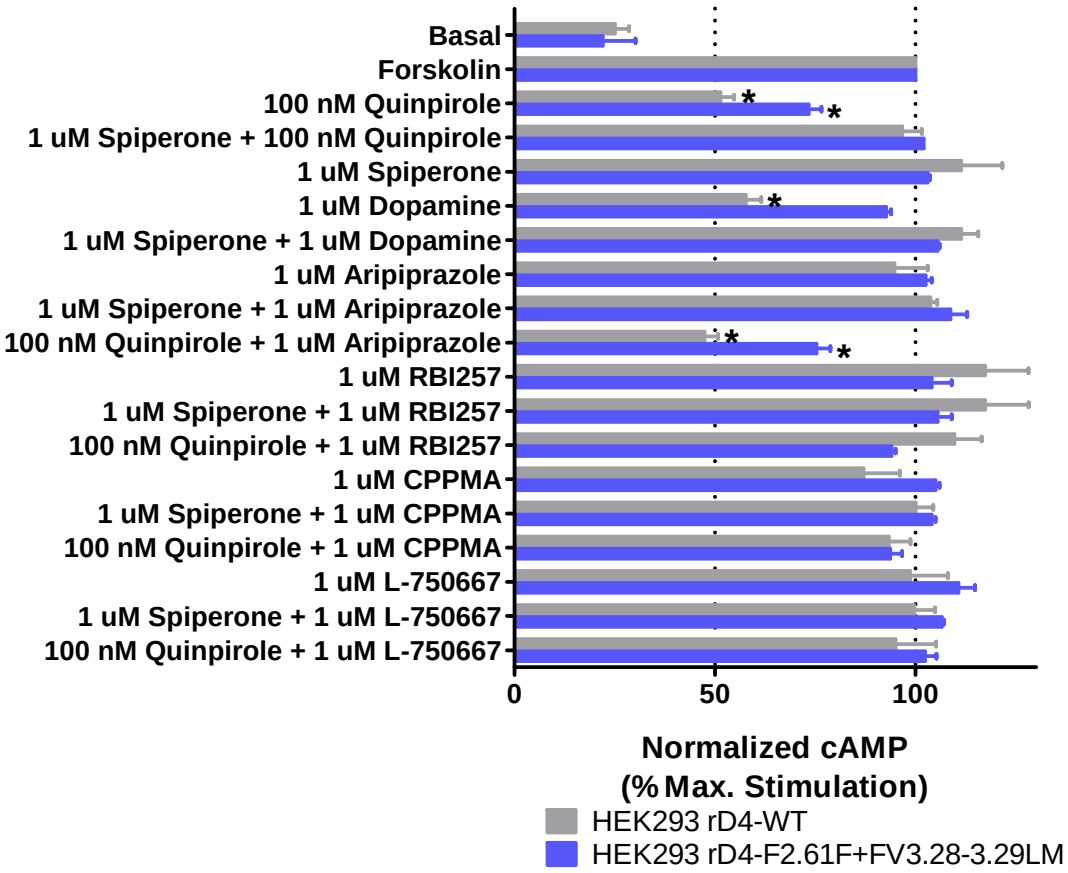
Appendix B-4. Maximal generation of Forskolin induced intracellular cAMP is limited by drug challenge duration. HEK293 rD2-WT cells were challenged for 15 to 60 minutes with the same concentrations of 6 μM forskolin and 100 nM (-)-quinpirole. Similar to other cAMP assays, cells were seeded at a density of 50,000 cells/well on the day prior to assay. On the day of the assay, temperature equilibrated drugs (30 minute equilibration) were added every 15 minutes to the appropriate wells of the same plate. This methodology staggered the durations of the drug challenges while maintaining the cells on the same microtiter plate, however, samples in the longer drug challenges were subjected to brief (<1 minute) lapses of time outside the incubator. This short time gap had minimal impact on the variance of the assay. After the appropriate intervals of time had passed for all samples, the plates were centrifuged at 3500 rpm for 5 minutes to minimize the loss of the weakly adherent HEK293 cells, and then lysed according to protocol (see Chapter II – Materials and Methods) **A.** Efficacy was measured as the amount of cAMP generated (fmol) per average concentration of cell lysate protein (mg). This data analysis illustrates a progressive decline in forskolin response for incubation times exceeding 30 minutes. **B.** Efficacy measured as a percentage of total forskolin response is shown to illustrate that the magnitude of agonist response, when normalized to percent maximal forskolin, is only appropriate at similar durations of drug incubation. For this graph, all data is normalized against forskolin induced cAMP mobilization at the appropriate time interval, however, this graph now fails to illustrate that a significant change in forskolin induced cAMP has occurred. This is shown as an example of when normalization is inappropriate for this assay. Normalization is only appropriate when the duration of the drug challenge remain constant.


A
Stimulation Buffer
Stimulation Buffer
6 μM Forskolin
100 nM (-)-Quinpirole
Drug Incubation Time
15 minutes
30 minutes
45 minutes
60 minutes
0
40000
80000
120000
cAMP (fmol/mg protein)
B
Stimulation Buffer
Stimulation Buffer
6 μM Forskolin
100 nM (-)-Quinpirole
Drug Incubation Time
15 minutes
30 minutes
45 minutes
60 minutes
0
50
100
Normalized cAMP
(% Max. Stimulation)

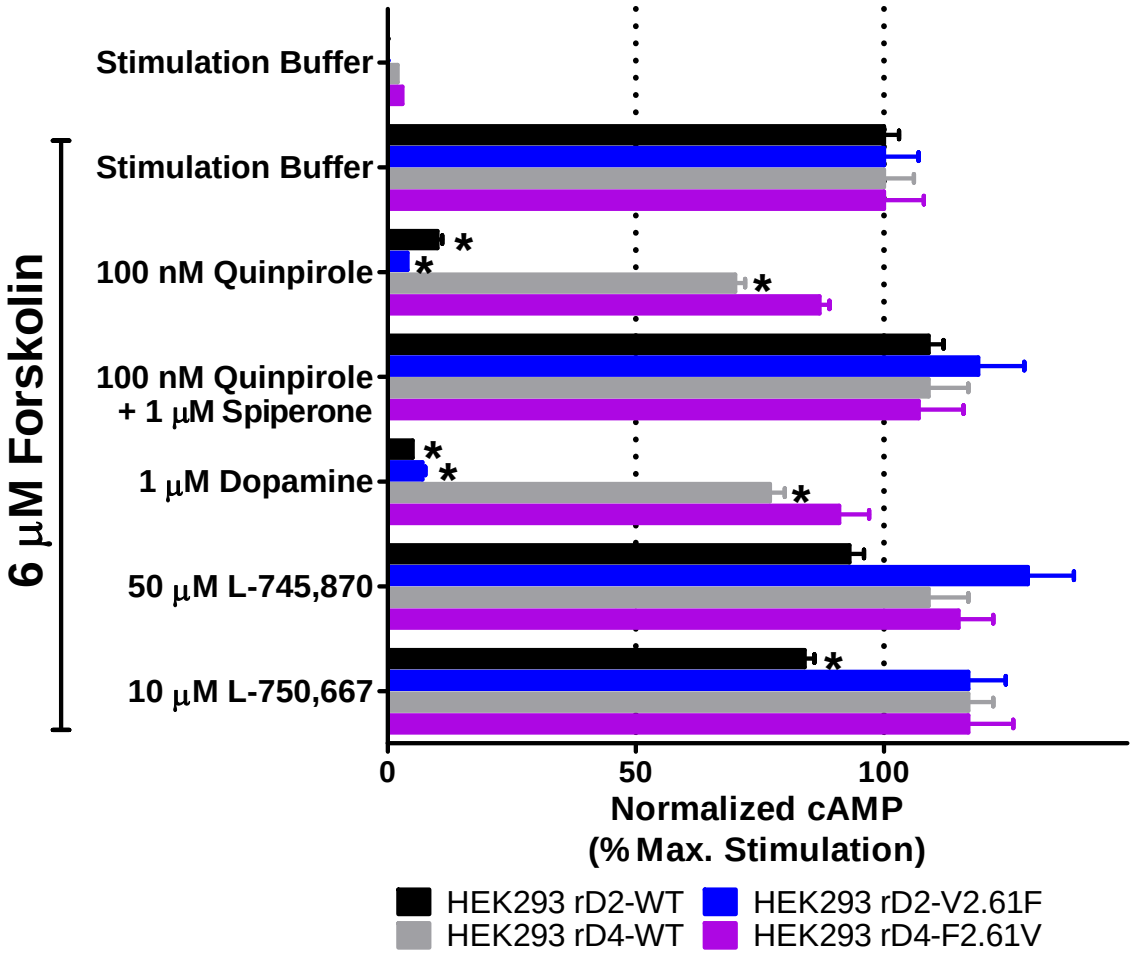
Appendix B-5. Dimethyl sulfoxide and ethanol at have no significant effect on the cAMP response of HEK293 cells. High concentrations of drug solvents (dimethyl sulfoxide and ethanol) failed to produce significant deviations in the cAMP response of untransfected HEK293 cells when compared to forskolin controls. For all functional assays, the drug solvent concentrations were less then a tenth (<0.02%) of the solvent concentrations tested here. Testing higher concentrations of drug solvents allowed for a margin of error in the event that higher drug concentrations might be needed. Three assays were done with three replicates per sample. Statistics were analyzed using a one-way ANOVA and Dunnett's post hoc analysis.



Appendix B-6. L-750,667 is an antagonist at the D4-F2.61V+FV3.28-3.29LM receptor. Multiple drugs were tested for agonist, partial agonist, and antagonist activity at HEK293 cells expressing D4-WT and D4-V2.61F+FV3.28-3.29LM dopamine receptors. While the D2 receptor mutant D2-V2.61F+FV3.28-3.29LM changed the functional properties of L-750,667 from partial agonist at the wild type D2 receptor to antagonist at the mutant (see Chapter II), triple mutation of the same positions in the D4 receptor (D4-F2.61V+LM3.28-3.29FV) does not change the functional properties of L-750,667 or related compounds. Quinpirole has significant functional efficacy at both the wild type and mutant D4 receptors. Interestingly, 1 μM dopamine does not have significant function at the D4 mutant while only exhibiting a 5 fold loss in affinity relative to the D4-WT (Schetz et al., 1999). Aripiprazole, despite having nM affinity for both the D4-WT and D4 mutant (144 nM and 207 nM in respectively in COS7 cells), is unable to hinder (-)-quinpirole function and therefore may be a very weak partial agonist. In more recent functional assessments in a CHO10001 rD4-WT cell line, aripiprazole is shown to have weak partial agonist activity at the wild type receptor (data not shown). No significant decrease cAMP response was generated for 2 μM RBI257 suggesting that this ligand is an antagonist. The results are shown as the average ± SEM for three experiments. Data was analyzed via one way ANOVA with Tukey's pos hoc analysis (*$p \leq 0.05$).


Basal
Forskolin
100 nM Quinpirole
1 uM Spiperone + 100 nM Quinpirole
1 uM Spiperone
1 uM Dopamine
1 uM Spiperone + 1 uM Dopamine
1 uM Aripiprazole
1 uM Spiperone + 1 uM Aripiprazole
100 nM Quinpirole + 1 uM Aripiprazole
1 uM RBI257
1 uM Spiperone + 1 uM RBI257
100 nM Quinpirole + 1 uM RBI257
1 uM CPPMA
1 uM Spiperone + 1 uM CPPMA
100 nM Quinpirole + 1 uM CPPMA
1 uM L-750667
1 uM Spiperone + 1 uM L-750667
100 nM Quinpirole + 1 uM L-750667
*
*
*
*
*
0
50
100
Normalized cAMP
(% Max. Stimulation)
HEK293 rD4-WT
HEK293 rD4-F2.61F+FV3.28-3.29LM

Appendix B7 – L-745,870 is an antagonist of the D2-WT receptor. 50 μM L-745,870 was tested at wild type and single reciprocal mutations of D2 and D4 dopamine receptors. No significant decrease in forskolin generated cAMP was observed for L-745,870 at the highest concentration of ligand (50 μM) that remained soluble in the stimulation buffer. It is interesting to note that the rD4-F2.61V receptor, despite having excellent receptor density (B_{max} = 4650 ± 1421 fmol/mg protein) and methylspiperone affinity (0.79 ± 0.18 nM) does not appear to have a significant response to the full agonists (-)-quinpirole and dopamine. Significance for all replicates was analyzed at *p<0.05 by two way ANOVA with Dunnett's post hoc analysis.



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