MECHANISMS OF LIGAND-RECEPTOR INTERACTIONS OF

THE DOPAMINE D_{2L} RECEPTOR AND THEIR RELATION

TO FUNCTIONAL SELECTIVITY

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ABSTRACT

**JUSTIN COREY FOWLER: Mechanisms of ligand-receptor interactions of the
dopamine D_{2L} receptor and their relation to functional selectivity**
(Under the direction of Richard B. Mailman, Ph.D.)

Binding and functional studies indicate that some agonists are capable of
differentially activating pathways linked to a single receptor isoform. In cases where known
mechanisms like other receptors or receptor reserve are ruled out, this phenomenon is
referred to as "functional selectivity," "agonist directed trafficking," and a variety of other
terms. Rather than viewing receptors in a traditional way as digital entities (i.e., one or more
active or inactive states), functional selectivity posits that a receptor can be induced into
unique conformations that may lead to different patterns of activation of functional responses
linked to a single receptor. This dissertation elucidates the molecular mechanisms that
explain why some ligands can differentially activate the effector pathways coupled to the
human D_{2L} receptor. The focus will be to discern the structural interactions of the hD_{2L}
receptor with such functionally selective ligands, and how these interactions result in
selective activation of effector pathways. Computationally-selected point mutations of the
D_{2L} receptor were made, and detailed analysis of both binding and function made for a series
of rationally selected ligands, emphasizing compounds that were relatively rigid and had
fewer degrees of freedom in possible docking poses. Functional assays tested the intrinsic
activity and potency for GTPγS binding, inhibition of forskolin-stimulated cAMP,
phosphorylation of MAPK, and release of [^{3}H]-arachidonic acid. Results from this

dissertation provide evidence to support the hypothesis that ligand-selective interactions with specific residues of the receptor can induce conformational changes that lead to a characteristic pattern of activation of one of more signaling pathways. Developing a structure-based understanding of functional selectivity will provide general insight into the mechanisms of GPCR activation, and may also open the door to design of small molecules with desired patterns of functional effects at a single receptor.

DEDICATION

The work in this dissertation is dedicated to two brilliant men. These men are my uncle Johnny Paul "JP" Rigsby, who is no longer with us, and my high school biology teacher Ken Murphy. This dedication is in recognition of their strength and continued encouragement, which have tremendously influenced the scientist whom I have become.

PREFACE

I have prepared my dissertation in accordance with the guidelines set forth by the University of North Carolina Department of Graduate Studies. The dissertation consists of a general introduction, five chapters of original data, a conclusion chapter, and an appendix that contains the experimental methods. A complete list of the literature cited throughout the dissertation has been appended to the end of the dissertation. References are listed in alphabetical order and follow the format of The Journal of Pharmacology and Experimental Therapeutics.

Available publications from this work

Fowler, JC, Gay, EA, Nichols DE, Mailman, RB. Mechanisms of ligand-induced functional selectivity at the hD_{2L} dopamine receptor. Program # 163.11 Society for Neuroscience, San Diego, CA, 2004.

Fowler, JC, Filizola, M, Weinstein, H, Javitch, JA, Nichols, DE, Mailman, RB. Receptor-ligand interactions related to dopamine D_{2L} receptor functional selectivity. Abstract # 318.9 Experimental Biology, San Diego, CA, 2005.

Fowler, JC, Filizola, M, Weinstein, H, Javitch, JA, Nichols, DE, Mailman, RB. Receptor-ligand interactions related to dopamine D_{2L} receptor functional selectivity. Poster # 25 Molecular Pharmacology Gordon Research Conference, Barga, Italy, 2005.

Fowler, JC, Filizola, M, Weinstein, H, Javitch, JA, Nichols, DE, Mailman, RB. Stereochemical effects on functional selectivity at the dopamine D_{2L} receptor. Abstract # 126. Keystone Conference on G protein-coupled receptors: Emerging Concepts and New Techniques, Keystone, CO, 2006.

Fowler, JC, Filizola, M, Gilliam, M, Weinstein, H, Javitch, JA, Nichols, DE, Mailman, RB. Transmembrane five effects on functional selectivity at the dopamine D_{2L} receptor. Experimental Biology, San Francisco, CA, 2006.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
AC	adenylate cyclase
MAPK	Mitogen activated protein kinase
AA	Arachidonic acid
cAMP	cyclic AMP; adenosine 3',5'-cyclic monophosphate
DA	dopamine
DHX	dihydrexidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexa-hydrobenzo[*a*]phenanthridine)
DNS	dinapsoline
DNX	Dinoxyline
DHX	dihydrexidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexa-hydrobenzo[*a*]phenanthridine)
RNPA	R(+)propyl norapomorphine
SNPA	S(-)propyl norapomorphine
QP	quinpirole
EC50	Concentration required for 50% effect
EEDQ	1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline
G_i	inhibitory guanine nucleotide binding protein
G_q	member of inhibitory guanine nucleotide protein family
G_s	stimulatory guanine nucleotide binding protein
G_z	member of inhibitory guanine nucleotide protein family
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IBMX	isobutylmethylxanthine
$K_{0.5}$	apparent affinity constant ($n_H < 1.0$)
MAO	monoamine oxidase
NE	norepinephrine
n_H	Hill coefficient
VTA	ventral tegmental area

CHAPTER 1. INTRODUCTION

DOPAMINE RECEPTORS: THE MODEL SYSTEM FOR MY STUDIES

History of dopamine systems

For the first half of the 20th century, dopamine was considered to function solely as an intermediate in the synthesis of epinephrine and norepinephrine. It was not until the late 1950s that Arvid Carlsson discovered the important role of dopamine itself (Carlsson et al., 1957). This seminal work showed that reserpine could cause depletion of dopamine and a concomitant loss of sympathetic nerve stimulation. These effects were reversed by administration of 3,4-dihydroxyphenylalanine (DOPA). Most importantly, DOPA restored the levels of dopamine, but not norepinephrine levels, that had been depleted by reserpine, suggesting an independent role for dopamine. This was confirmed by subsequent experiments with monoamine oxidase inhibitors (MAOIs). The corollary to this work was discovering that dopamine distribution in the brain was localized in the basal ganglia, a region critical for motor control. Carlsson's work was thus the first to suggest that dopamine depletion was responsible for Parkinsonism and that L-DOPA could be used to restore normal behavior (Carlsson et al., 1958). Additional studies were conducted on deceased Parkinson's patients, where Hornykiewicz noted marked reduction in dopamine levels (Ehringer and Hornykiewicz, 1960). Follow up studies were then conducted with living patients, noting that L-DOPA treatment temporarily improved akinesia in Parkinson's patients (Birkmayer and Hornykiewicz, 1961).

Dopamine neurotransmission

Using newly developed histochemical methods, it was soon reported that dopamine occupied discrete neuronal tracts (Anden et al., 1964; Anden et al., 1965; Anden et al., 1966) as summarized in Figure 1.3. There are four major dopamine pathways, three of which innervate the forebrain (the mesolimbic, nigrostriatal, and mesocortical pathways). The dopaminergic neurons arising from the substantia nigra (SN) and ventral tegmental (VT) area project to the caudate and putamen (striatum) via the nigrostriatal pathway, the nucleus accumbens, amygdala, and olfactory tubercle via the mesolimbic pathway, and the prefrontal cortex via the mesocortical pathway. The fourth pathway, the tuberoinfundibular system, is intrinsic to the hypothalamus (Figure 3).

Dopamine neurotransmission in the basal ganglia plays a vital role in initiation and control of movement, and is an important center of integration. The mesocortical system is believed to be responsible for the negative symptoms of schizophrenia and is also thought to be involved in motivation and emotional response. The mesolimbic system, known as the reward center is associated with feelings of reward and desire. This system is responsible for the positive symptoms of schizophrenia and is the major target of antipsychotic drugs. Conversely, nigrostriatal pathway is responsible for many of the neurological side effects associated with antipsychotic drugs, and also is the pathway that degenerates in Parkinson's disease. Lastly, the tuberoinfundibular pathway is responsible for controlling prolactin levels regulated by the anterior pituitary gland (Cooper et al., 2006).
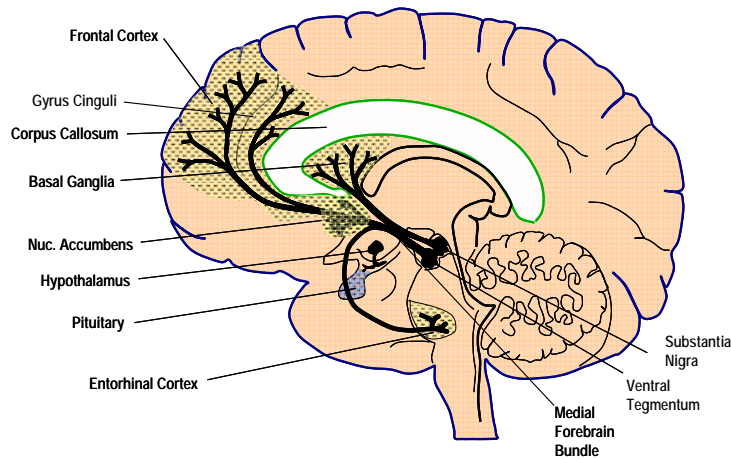
Figure 1.3. Dopamine pathways in human brain

ANTIPSYCHOTIC DRUGS AND THEIR IMPACT ON UNDERSTANDING OF DOPAMINE RECEPTOR FUNCTION

Schizophrenia and the serendipitous discovery of chlorpromazine

The psychiatric condition schizophrenia (split-mind) was described by Bleuler as a lack of interaction between the thought processes and perception. Schizophrenics experience delusions and hallucinations, exhibit disorganized speech and behavior, and exhibit negative symptoms such as declined emotional response, speech, and motor response (American Psychiatric Association, 2000). The first drug effectively used in the treatment of schizophrenia was chlorpromazine, a drug whose antipsychotic properties were found serendipitously during the search for better sedative agents. Laborit and Huguenard described the behavioral profiles of patients administered chlorpromazine as being in a state of "artificial hibernation" (Laborit and Huguenard, 1951). These observations lead to subsequent testing of chlorpromazine on psychiatric patients, the first of whose "erratic, uncontrollable behavior" significantly decreased within days after administration of chlorpromazine (Hamon et al., 1952). After three weeks of treatment the patient was released, appearing largely normal. The fact that one could achieve control of psychotic

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symptoms that was dissociated from gross sedation was a major breakthrough, and these early studies with chlorpromazine opened the door for both biological psychiatry and the advent of the era of neuropsychopharmacology.

Proliferation of typical antipsychotic drugs, recognition of dopamine importance, and recognition of side effects

Within two years of introducing chlorpromazine to the clinic, the first studies reporting significant side effects were released (Steck, 1954). Chlorpromazine and the follow-up antipsychotics (e.g., other phenothiazines and haloperidol) all cause extrapyramidal side effects (EPS) such as parkinsonism, dystonias, and akathesia, as well as tardive dyskinesia (Meltzer and Stahl, 1976). It became clear that it would be desirable if the next generation of antipsychotic drugs were free of these neurological side effects. The accepted term for these next generation drugs that are essentially free of both EPS and tardive dyskinesia is now "atypical antipsychotics," with chlorpromazine and its cousins being termed "typical."

The search for atypical agents would obviously be aided by understanding of the mechanisms of both therapeutic and side effects. Biochemical experiments soon demonstrated that the primary mechanisms were antagonist action of antipsychotics on the dopamine D_2 receptors in the mesolimbic nuclei (Carlsson and Lindqvist, 1963; Meltzer and Stahl, 1976; Creese et al., 1976; Davis et al., 1991). Meltzer however described the cause for EPS as blockade of dopaminergic inhibition of the cholinergic neurons of the striatum. This blockade caused increased cholinergic activity in the basal ganglia which increased the side effects observed (Meltzer and Stahl, 1976). Positron emission tomography (PET) studies also provided quantitative evidence to suggest that occupancy of striatal D_2 receptors greater than

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80% caused significant EPS (Farde et al., 1992). Interestingly, the Germans were skeptical of the EPS profiles, and this led to research resulting in the development of clozapine and related atypical antipsychotics.

The discovery of atypical antipsychotic drugs focuses attention on dopamine receptor mechanisms

Clozapine spurs the search for atypicals

Clozapine was the first drug developed that exhibited atypical properties, that is, antipsychotic activity with little or no EPS. Introduced in the late 1960s, clozapine was initially met with skepticism because of the high degree of efficacy coupled with an excellent neurological profile. Because clozapine caused a very high degree of life-threatening agranulocytosis (Idanpaan-Heikkila et al., 1997), the drug was shelved for more than a decade. Later, it was realized that despite the high toxicity of this compound, it was apparently the most effective antipsychotic in terms of efficacy coupled with low neurological side effects (Kane et al., 1988). This led to its reintroduction in the US in 1990, with the requirement of a concomitant blood monitoring program. Those early reports about the unsurpassed efficacy of clozapine against both positive and negative symptoms of schizophrenia (American Psychiatric Association, 2000) have been confirmed by numerous studies.

The efficacy of clozapine spurred attempts to develop structurally and pharmacologically similar drugs having the useful properties of clozapine without its toxicity. This led to the development of atypical antipsychotics that now include risperidone, olanzapine, ziprasidone, quetiapine, and aripiprazole (the latter to be addressed later). Although only olanzapine is similar structurally to clozapine, all of these compounds have

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complex pharmacology (Rauser et al., 2001). An obvious question then is what mechanism(s) differentiated clozapine and the other atypicals different from chlorpromazine and haloperidol. Meltzer (1989) has argued that atypicality is defined by drugs that exhibit more potent serotonin 5-HT$_{2A}$ receptor antagonism than D$_2$ receptor antagonism. This hypothesis would explain the atypicality of risperidone, olanzapine, quetiapine, ziprasidone, and quetiapine, yet risperidone has greater selectivity for the D$_2$ and 5-HT$_{2A}$ receptors, yet has a somewhat greater propensity to induce EPS at high does than the other drugs. Indeed, olanzapine, quetiapine, ziprasidone, and quetiapine have relatively similar affinity to a dozen or more receptors, making such simple hypotheses unlikely. Although PET studies has led one hypothesis that high levels (greater than 70%) of D$_2$ receptor antagonist occupancy may correlate with increased EPS (Kapur et al., 1998; Remington et al., 1998; Kapur et al., 1999), little is crystal clear. Indeed, as of the writing of this dissertation, the mechanisms for both therapeutic and side effects of these clozapine-like drugs remains a source of controversy and investigation.

Non-traditional dopamine receptor actions as a mechanism for atypicality

In parallel with the development of drugs with dopamine and serotonin antagonist properties, the suggestion was made that D$_2$ partial agonists might have utility in treating schizophrenia. Originally, this was based on the "dopamine hypothesis of schizophrenia" that proposed that excess dopamine synaptic transmission (either basal or in response to external perturbation) was a major cause of the disorder. Because presynaptic and autoreceptors were known to be much greater in sensitivity than postsynaptic receptors, it was felt that a partial agonist could decrease dopamine release markedly without causing changes in postsynaptic receptor activation, thereby improving symptoms. This intriguing idea, however, was not

supported by clinical studies with 3-PPP (Tamminga et al., 1992), one of the compounds I shall study.

In 2004, Bristol-Myers Squibb marketed the drug aripiprazole (Abilify), a compound currently approved by the FDA for treatment of both schizophrenia and bipolar disorder. Although the drug may be slightly less effective than the compounds discussed above, it has a superior side effect profile. Aripiprazole clearly is differentiated from the other atypicals in terms of clinical action and mechanism. The prevailing view of thought-leaders in the field is that aripiprazole works via the partial agonist mechanism summarized above.

Conversely, an alternate hypothesis was first proposed by Lawler et al. (1999) that suggested that the intrinsic activity of aripiprazole is markedly affected by the location of the target D_2 receptor in a way that can not be explained by traditional notions such as receptor reserve. Later studies confirmed this milieu-dependent signaling of aripiprazole (Shapiro et al., 2003; Urban et al., 2006b), providing a clear example of how functional selectivity may actually impact on the physiological actions of drugs. It is the mechanisms behind such observations that are the focus of this dissertation. It should be noted, that even with aripiprazole, the picture is clouded by other receptors. The unique actions of this drug (and similar compounds) may include not only its functionally selective or partial agonist D_2 effects, but also partial agonist actions at 5-HT_{1A} or other serotonin receptors (Lawler et al., 1999; Corbin et al., 2000; Shapiro et al., 2003). Nonetheless, a more in-depth understanding of D_2 signaling with ligands such as aripiprazole is of heuristic interest, and may well impact on the discovery of even more novel agents.

BIOLOGY AND PHARMACOLOGY OF DOPAMINE RECEPTORS

The dopamine receptors belong to the G protein-coupled receptor (GPCR) superfamily that is of great scientific and commercial interest. GPCRs may be the largest superfamily of biological signaling molecules and play a critical role in the function of essentially all cells. Thus, it is not surprising that studies of the structure and function of GPCRs has been a major scientific research front covering all major disciplines. Moreover, nearly half of the drugs on the market target GPCRs (Robas et al., 2003). Thus, scientific breakthroughs may have effects on the discovery of more effective clinical agents that target GPCRs.

Dopamine receptors are in the rhodopsin-like Class A family of GPCRs. Such GPCRs are characterized by their seven transmembrane spanning domains linked to three intra- and three extracellular loops. Signaling through GPCRs occurs by agonist activation of the receptor, and subsequent conformational changes of the receptor. This affects the interaction of the receptor with heterotrimeric G protein complexes, leading to the exchange of GDP for GTP via a conformational change of the $\alpha 5$ helix of the α subunit. This movement leads to subunit dissociation of the heterotrimeric complex, such that the α and $\beta\gamma$ subunits can affect second messenger and other functions within the cell (Neve et al., 2004; Oldham et al., 2006). First classified into the D_1 or D_2 pharmacological families (Garau et al., 1978; Kebabian and Calne, 1979), it is now known that five genes code for dopamine receptors. The D_1 dopamine receptors stimulate adenylate cyclase through $G\alpha_s$ and $G\alpha_{OLF}$, whereas D_2 receptors are pertussin toxin-sensitive, inhibiting adenylate cyclase activity through $G\alpha_{i/o}$. The five subtypes are classified as being either D_1-like (D_1 or D_5) or D_2-like (D_2, D_3, D_4). In addition, the D_2 receptor has splice variants, D_{2L} and D_{2S}, that differ by 29 amino acids in the

third intracellular loop of D_{2L} (Missale et al., 1998; Neve et al., 2004). All of the experiments in this dissertation will focus on characterization of the hD_{2L} receptor. Activation of the D_{2L} receptor is known to regulate several pathways including inhibition of cAMP accumulation, phosphorylation of mitogen activating protein kinases p44/42, potentiation of arachidonic acid release, and activation of GIRK channels (Figure 4).

Figure 1.4. Signaling pathways of the dopamine D_2 receptor. *Pathways that will be the focus of this dissertation include $G\alpha_{i/o}$ mediated inhibition of adenylate cyclase, $G\beta\gamma$ mediated phosphorylation of p42/p44 MAP Kinase, and $G\beta\gamma$, PLA_2 mediated release of arachidonic acid. Figure adapted from Neve et al 2004.*

Signal transduction of dopamine receptors

Before the myriad of signal transduction pathways was discovered, iontophoretic experiments conducted on the substantia nigra provided evidence to support a biphasic action of dopamine, suggesting that dopamine signaling occurred through two different receptors (York, 1970). Greengard's group described dopaminergic activation of adenylate cyclase in bovine superior cervical ganglion and later in caudate nucleus homogenates, providing the first evidence for a biochemical consequence of dopamine receptor activation (Kebabian and Greengard, 1971; Kebabian et al., 1972). Studies with neuroleptic drugs were also conducted. Strikingly, these drugs did not activate adenylate cyclase but instead antagonized

dopamine-stimulated adenylate cyclase activity (Kebabian et al., 1972). Further behavioral experiments classified the dopamine receptors as stimulatory or inhibitory (Cools and Van Rossum, 1976). The human dopamine receptor (hD_{2L}) studied in this dissertation exhibits inhibitory properties through the following signal transduction pathways:

Adenylate Cyclase

Adenylate cyclases (ACs) play an important role in signal transduction of the CNS including physiological responses such as learning and memory, synaptic plasticity, and circadian rhythms (Chern, 2000). Disruption of the expression of ACs has been linked to a variety of physiological and pathological conditions including circadian rhythms (Tzavara et al., 1996; Chern et al., 1996; Cagampang et al., 1998), alcoholism (Hashimoto et al., 1998; Ikeda et al., 1999), and Alzheimer's disease (Yamamoto et al., 1996). Ten adenylate cyclases have been identified to date, and each is responsible for stimulating the production of cAMP. All mammalian isozymes of AC have been shown to be activated by $G\alpha_s$ and subsequently synthesize cAMP. In addition, AC isozymes have been shown to be inhibited by $G\alpha_i$ (Taussig et al., 1994), but there is also evidence that $G\beta\gamma$ can play a role in regulating cyclase (Tang et al., 1991). The dopamine D_{2L} receptors that are the focus of this dissertation signal through $G\alpha_{i/o}$ and have been shown to inhibit AC types I, V, VI, and VIII (Robinson and Caron, 1997; Nevo et al., 1998)

MAP Kinase

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases whose activation is directly linked to control of gene expression and downstream cell growth and differentiation. Three MAPK subfamilies are known in mammalian cells. They are: 1) extracellularly-responsive kinases ERK1/ERK2 ($p44^{mapk}$ and $p42^{mapk}$ respectively); 2) c-Jun

N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs); and 3) p38-MAPKs. Several GPCRs have been shown to activate MAPK via signaling through PTX-sensitive G proteins $G\alpha_{i/o,}$ activating ERK1/2 (Welsh et al., 1998; Choi et al., 1999). Although the exact nature of D_2 signaling through ERK1/2 is still incompletely understood, it is known that GPCR signaling through MAP kinase in general involves membrane bound $G\beta\gamma$ promoting tyrosine phosphorylation of Shc. The latter then complexes with Grb/Sos, recruiting GTP-Ras → Raf → MEK → ERK1/2 (Sugden and Clerk, 1997; Lopez-Ilasaca, 1998).

Arachidonic Acid

Release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2) has generally been associated with inflammatory response (Needleman et al., 1986), but it also serves important second messenger functions such as long-term potentiation and synaptic plasticity (Williams et al., 1989). In addition, AA release may be involved in the pathophysiology of several neurodegenerative disorders including cerebral ischemia, stroke, Alzheimer's, and Parkinson's (Lynch, 1998; Kramer et al., 2004). PTX sensitive G protein-coupled receptors (i.e. dopamine D_2) have been shown to stimulate release of AA through PLA_2. The exact mechanism has yet to be elucidated, but evidence exists to support GPCR activation of PLA_2, mediated through ATP binding and subsequent activation of the P_2-purinergic receptor (Felder et al., 1991). There is also some data that is interpreted as showing that D_2 receptors can activate PLA_2 directly without ATP mediation (Nilsson et al., 1998), but the latter findings have not been replicated in our lab.

GIRK channels

For some time now, dopaminergic neurotransmission has been associated with regulating G protein-regulated inward-rectifier potassium channels (GIRK). In mammals,

four subunits of GIRK (GIRK1-4, also designated $K_{ir}3.1$-4) have been identified. Data suggests that these channels are modulated through a direct interaction between the N- and C- termini of the channel and a $G\beta\gamma$ subunit (Mark and Herlitze, 2000). In addition, regulators of G protein signaling (RGS) proteins have been found to accelerate both the activation and deactivation of GIRK currents in a CHO cell line (Doupnik et al., 1997). D_2 receptor activation of GIRK channels has been evidenced in several models including rat striatum, lactotrophs, and CHO cell lines expressing hD_{2L} (Einhorn et al., 1991; Liu et al., 1996; Kuzhikandathil et al., 1998; Liu et al., 1999).

Dopamine receptor agonists

Several agonists of the hD_{2L} receptor were chosen for studying the mechanisms of functional selectivity within this dissertation (Figure 1.5). The endogenous ligand dopamine (DA) and the prototypical agonist quinpirole (QP) were chosen to serve as reference full agonists. Both compounds were used because dopamine is more sensitive to loss of affinity and/or functional activity by several of the mutations we planned to study (Mansour et al., 1992; Cox et al., 1992). Dihydrexidine (DHX), a rigid analog, was chosen because it has been shown to display functional selectivity at the D_{2L} receptor in the brain and in MN9D (Kilts et al., 2002) and CHO cell lines (Gay et al., 2004). In addition, the rigid analogs dinapsoline (DNS) and dinoxyline (DNS), whose structures differ merely by the substitution of an ether (DNX) for a methylene bridge (DNS) at a site believed to not be critical for binding, were chosen as test compounds because they are functionally selective at activating GIRK channels (Gay et al., 2004).

S(-)-3-(3-Hydroxyphenyl)-N-propylpiperidine, S (-) 3-PPP, was chosen because it will serve as a representative D_2 partial agonist. The propylnorapomorphine compounds,

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R(+) and S(-)propylnorapomorphine (R/S NPA), were chosen because of their different functional profiles in the CHO expressing D_{2L} receptor at phosphorylation of MAP kinase and activation of GIRK channels (Kilts et al., 2002; Gay et al., 2004). These compounds are the most interesting of all the test compounds because they differ merely by the orientation of the hydrogen atom at their chiral center, yet have such vastly different functional profiles. The diversity of these rigid test compounds will broaden our knowledge of the ligand-receptor residue interactions of the hD_{2L} receptor and provide insight regarding how specific structural interactions relate to function.
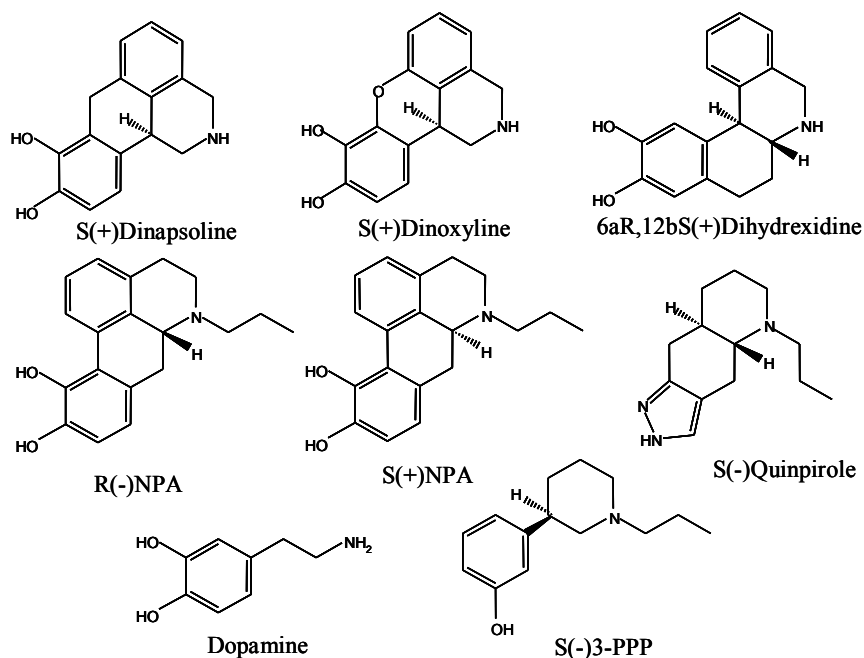
Figure 1.5. Test compounds. *S(+)Dinapsoline (DNS), S(+)Dinoxyline, 6aR,12bS(+)Dihydrexidine (DHX), R(-)propylnorapomorphine (R(-)NPA), S(+)propylnorapomorphine (S(+)NPA), (-)Quinpirole (QP), Dopamine (DA), and S(-)3-PPP.*

I should also explain the nomenclature that will be used when referring to the hydroxyl groups attached to these test compounds. I will refer to each of these groups based upon the alpha-rotamer of dopamine. Consider DNS for example: the hydroxyl group

opposite the ethyl amine of dopamine will be referred to as para-OH and the remaining hydroxyl will be referred to as meta-OH. This rule will be applied to all of my test compounds and should further clarify subsequent binding hypotheses.

Background on catecholamine receptor mutagenesis experiments

Nomenclature and conserved residues

Throughout this dissertation I shall refer to specific amino acid residues within the receptor using universal indexing nomenclature (Ballesteros and Weinstein, 1995). The most conserved residue of each transmembrane (TM) domain (Table 1) is given an integer value referring to its TM domain, and a decimal number, 0.50 referring to the most conserved residue in that helix. The decimal values are indexed positively (toward the carboxy-terminus) or negatively (toward the amino terminus). Thus, the conserved aspartate in TM 2 is designated 2.50. When referring to a specific amino acid residue, I will refer to it by the one letter code, followed by the universal index. When referring to an amino acid mutated to another residue, I will refer to the residue using the original residue one letter code followed by the universal index, and the one letter code of the mutated residue (e.g. threonine 3.37 mutated to alanine: T3.37A). The following Table shows the most conserved residue in each TM helix, as well as the residues that will be targeted by this research.

Table 1.1. Universal Index. Universal Indexing of most conservative residues and targeted residues and their position in dopamine D$_{2L}$ receptor.

Universal Index	Absolute position	TM#	Residue
1.50	52	1	N
2.50	80	2	D
3.37	*119*	*3*	*T*
3.50	132	3	R
4.50	160	5	W
5.42	*193*	*5*	*S*
5.43	*194*	*5*	*S*
5.46	*197*	*5*	*S*
5.50	201	5	P
6.50	388	6	P
6.55	*393*	*6*	*H*
7.50	4.23	7	P

The role of TM5 serine residues in ligand-receptor interaction

The serine residues in TM5 have been implicated in ligand binding interactions necessary for activation and receptor/G protein coupling for catecholamine type GPCRs. Strader was the first to provide evidence supporting bonding interactions with the serine hydroxyl groups (serines 5.43 and 5.46) of the β$_2$-adrenergic receptor and its endogenous ligand (Strader et al., 1989). Studies that followed provide evidence for all three serines 5.42, 5.43, and 5.46 in ligand-residue interactions of the β$_2$-adrenergic receptor necessary for receptor/G protein coupling (Liapakis et al., 2000). In addition to the β$_2$-adrenergic receptor,

these serines play essential roles in many other catecholamine receptors (Cox et al., 1992; Pollock et al., 1992; Woodward et al., 1996; Cavalli et al., 1996; Hwa and Perez, 1996; Wiens et al., 1998; Rudling et al., 1999).

Earlier studies of the D_2 receptor have provided evidence that led to the conclusion of important bonding interactions between dopamine agonists and serine residues 5.42, 5.43, and 5.46 (Mansour et al., 1992; Cox et al., 1992; Wiens et al., 1998; Lee et al., 2000). Although these studies showed the importance of such interactions, the data did not show conclusively which aspects of various ligands interacted with specific serines. For example, some of these previous studies suggest that serine 5.43 interactions with dopamine and the related non-catechol p-tyramine. Because dopamine and p-tyramine are both molecules with a flexible, rotatable side chain there are many possible conformations by which serine 5.43 interactions could occur. This residue not shown to interact with any other compounds (Cox et al., 1992), including rigid ligands that might have resolved some of this uncertainty. This issue will be addressed in this dissertation.

Background on molecular modeling of GPCRs

Despite a great deal of effort, only one GPCR crystal structure has been solved to date at high resolution. Solving the three dimensional (3D) crystallographic structure of bovine rhodopsin at 2.8 Å (Palczewski et al., 2000) led to numerous modeling studies on other rhodopsin-like class A GPCRs, using the transmembrane region of the bovine rhodopsin crystal structure as a template (for recent reviews, see Ballesteros et al., 2001 and Visiers et al., 2002). Detailed analyses of the rhodopsin structure, together with the results of both sequence analysis and molecular modeling, have served to probe the accuracy of these 3D models created by homology modeling (Ballesteros et al., 2001b; Visiers et al., 2002). In

the absence of experimental structural information, these 3D molecular representations of rhodopsin-like GPCRs are used to interpret experimental results in a structural context, incorporating directly and consistently the many types of function-related information (for comprehensive review see Visiers et al., 2002). Such molecular models are also used as hypothesis-generators for experimental probing of functional inferences, and are continuously refined by the data obtained from such experiments. Refined models can therefore be used in computer simulations to generate and/or probe novel aspects of the receptor mechanisms, based on the dynamic properties of the proteins, both wild type and mutant constructs. Together, all the inferences from both homology modeling and computer simulation serve as mechanistic working hypotheses for new and more focused experiments. Given the success reported for several receptors (for some recent examples and reviews, see (Shi and Javitch, 2002; Visiers et al., 2002; Shapiro et al., 2002; Chalmers and Behan, 2002; Klabunde and Hessler, 2002; Singh et al., 2002; Shi et al., 2002; Ebersole et al., 2003), interdisciplinary studies involving collaborations between computational and experimental teams have become a sustained characteristic of current research on structure-function relations of GPCRs.

THE CHANGING FACE OF RECEPTOR PHARMACOLOGY

Classical receptor theory: agonists, antagonists, and partial agonists

The field of pharmacology is based in large measure on a body of information that might be called "receptor theory." The primary use of receptor theory is to provide both a conceptual and quantitative framework that can aid in the understanding of how ligands may interact with, and modulate, the function of receptors. It has been nearly a century since A.V.

Hill first described "receptive substances" as the vehicle by which nicotine and curare induce contractions of the frog *rectus abdominis* muscle (Hill, 1909). Hill and A.J. Clark were pioneers of this early theory that led to quantitative relationships to describe the concentration effects of drug action on receptors. It was not until the 1950s that Stephenson would use the term "affinity" to describe binding to the target, and "efficacy" to describe the production of response (Stephenson, 1956).

The term "agonist" was coined to describe drug action whereby binding and activation of the receptor caused a 100% biological response as defined by the endogenous ligand for said receptor species. Drug action upon receptor that produces no measurable response, or that could block the response of the endogenous agonist was coined "antagonism." Stephenson, however, erred in assuming that efficacy was always directly proportional to drug concentration, such that maximal occupation of receptors with drug would result in maximal effect (or full agonist activity). Intrinsic activity was first described by Ariens as a means to describe the relationship between the effect elicited by a drug and the concentration of drug-receptor complexes (Ariens, 1954; Ariens and Simonis, 1954; Ariens and De Groot, 1954). Ariens also formulated the term "partial agonist," describing when certain compounds, even at receptor-saturating concentrations, do not elicit maximal response.

Implicit within this framework is that characterization of drug action on a single receptor isoform is independent of the pathway measured (e.g. a full agonist exhibits the same intrinsic activity at all functional endpoints measured through a single receptor isoform). Unlike intrinsic efficacy, where ligand mediated effects on a single receptor are independent of pathway measured (Furchgott, 1966), intrinsic activity is dependent on the

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measurement of individual pathways mediated through a single receptor isoform. These concepts, and the underlying concentration dependent effects, have been the foundation for the study of drug-receptor interactions.

My research deals with one such situation, the notion of "intrinsic efficacy," a fundamental part of pharmacological dogma. Intrinsic efficacy embodies the idea that a ligand that interacts with one specific receptor always has the same type of functional effect on that receptor. Thus, the ligand may be called a "full agonist" (activates the receptor to the same degree as the endogenous ligand), "partial agonist" (causes only sub-maximal effects even when occupying all of the receptor), and "antagonist" (causes no functional effects, and by occupying the receptor, blocks the effects of agonists or partial agonists). More recently, antagonists have been divided into neutral antagonists and inverse agonists, the latter also decreasing the basal level of the function being studied. I shall review the history and basis for these ideas below, and describe the new concept of "functional selectivity" that my research investigates.

Digital nature of classical models of GPCR signaling

Since the inception of quantitative pharmacology, mathematical models have been utilized to evaluate and hypothesize the physiological states of receptor activation/inactivation (Figure 1). Monod, Wyman, and Changeux applied the idea of a two-state model to the interaction between oxygen and hemoglobin, where the target hemoglobin existed in an unbound, inactive R state or a bound, active R^* (Monod et al., 1965). Later, De Lean et al. (De et al., 1980) described the activation of G protein coupled receptors as a ternary complex, where the ligand bound active state (DR^*) requires an additional kinetic step before it can couple to G protein and activate downstream effector pathways (DR^*G)

(De et al., 1980). Several years later, however, GPCRs were found to exhibit constitutive activity (basal activity in the absence of agonist), the ternary complex model was expanded to what has been termed the extended ternary complex model (ETC) (Samama et al., 1993). When it was suggested that one must consider the possibility of a ligand-receptor-G protein complex that is inactive (DR_iG), Kenakin et al. developed an extension of the ETC by incorporating this inactive state, calling their model the cubic ternary complex model (CTC) (Weiss et al., 1996).
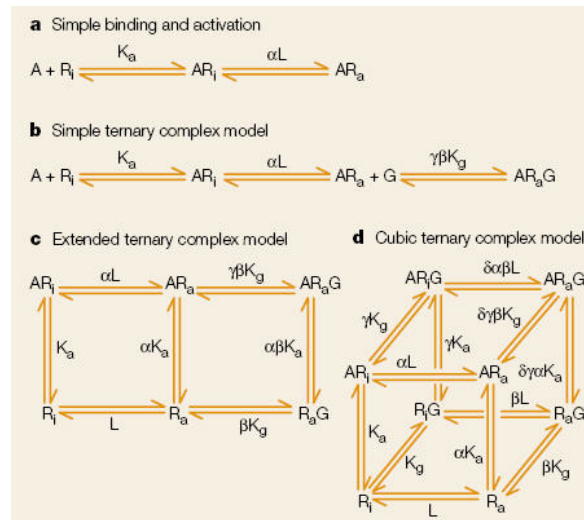
Figure 1.1. Models of ligand activation of GPCRs. *a. Two-state model, b. ternary complex, c. extended ternary complex (ETC), and d. cubic ternary complex (CTC). Figure adapted from Kenakin 2002.*

Each of the previously described models intrinsically suggests that receptor activation of downstream effector pathways involves rigid, digital receptor states induced by ligand binding and conformational change. The first evidence to support agonist induced conformational changes to target receptors was provided by Del Castillo and Katz in 1957 when they showed that acetylcholine binding to nicotinic acetylcholine receptors lead to channel opening (Del Castillo and Katz, 1957). Since that time, Kenakin and others have taken these mathematical models of ligand activation and applied them to graphical

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representations, hypothesized as rationale to explain observed signaling profiles (Figure 1.2).
It has been argued that agonists exhibit a frequency of specific receptor conformations and
that preferential activation of one conformational state over another (termed "conformational
selection") could potentially explain the differential signaling observed with functionally
selective ligands (Kenakin, 1997). The hypothesis continues by arguing that a multitude of
conformational states exist in the absence of ligand binding, and that when ligand binds, it
selects the conformational state to which it has the highest affinity. Binding removes that
specific conformational state from the available pool of receptors and via mass action is
replaced by a conformation with lower affinity for the ligand (Kenakin, 1997; Kenakin,
2002; Clarke, 2005).
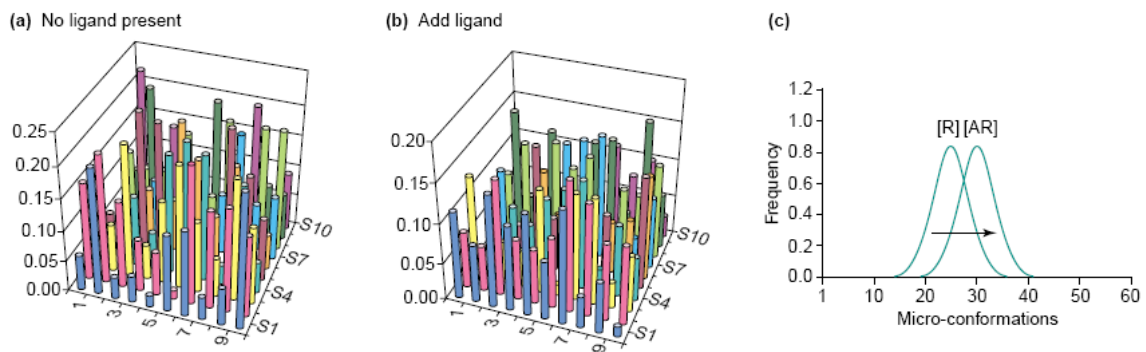
Figure 1.2. Ligand-specific receptor conformations. *a. absence of ligand, b. presence of ligand, c. frequency distribution in presence and absence of ligand. Figure adapted from Kenakin 2004.*

Functional Selectivity: an analog view of receptor action

During the past two decades, pharmacological research has benefited from technical
and research advances that have permitted the measurement of numerous effector pathways
modulated by a single type of receptor. As the breadth of effector pathways measured has
increased, a plethora of observations have been made that are difficult to rationalize with
some of the concepts discussed above. Specifically, some ligands were found to cause

patterns of effects that were inconsistent with the idea of intrinsic efficacy. At the extreme, compounds were found that were full agonists at one function yet antagonists at another function mediated by a single receptor in a single cell type.

Our laboratory was one of the first to recognize that there could be differential signaling of a single ligand working through a single receptor. We coined the term "functional selectivity" (Mailman et al., 1997; Mailman et al., 1998) to describe the ability of a ligand to activate in a differential fashion individual receptor pathways linked to a single receptor isoform. A plethora of recent evidence shows such differential signaling through single receptor isoforms of a variety of different GPCRs (Kenakin, 1995a; Kenakin, 1995b; Berg et al., 1998; Whistler et al., 1999; Lawler et al., 1999; Watson et al., 2000; Dutertre and Smith, 2000; Mottola et al., 2002; Kilts et al., 2002; Gazi et al., 2003; Mukhopadhyay and Howlett, 2005; Nickolls et al., 2005; McLaughlin et al., 2005). Although "functional selectivity" seems to be the term coming into most common use (Simmons, 2005; Urban et al., 2006b), the same phenomenon has been called agonist directed trafficking (Berg et al., 1998; Brink et al., 2000; Kukkonen et al., 2001), differential engagement (Manning, 2002), biased agonism (Jarpe et al., 1998), and a variety of other terms.

Functional selectivity versus intrinsic efficacy

It is important to define a few terms that shall be used throughout this dissertation. The term "intrinsic activity" will be used in a purely operational sense to define the observed functional changes caused by a ligand in a signal system relative to a reference compound (e.g., the endogenous ligand or some other compound defined *a priori*). Conversely, the term "intrinsic efficacy" reflects the concept put forth by Stephenson (1956) describing functional effects of a ligand after binding to a specific receptor. Thus, in general use, when one labels a

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compound a "full agonist" or "partial agonist" or "antagonist," it means that the compound will always have these actions at the target receptor although the degree of activity can be affected by mechanisms such as receptor reserve (Limbird, 1996). The formation of these concepts of quantitative pharmacology by Stephenson, Ariens, and many others have been extremely useful for the field for decades. Unfortunately, in almost every pharmacology textbook, and possibly in the minds of most pharmacologists, these concepts are considered fundamental principles rather than useful guideposts. It is possibly for this reason that examples of functional selectivity were met with great (possibly undue) skepticism.

As an example, the stoichiometry of receptor:G protein in *in vitro* systems suggests that receptor reserve might potentially explain the observed signaling profiles otherwise construed as representing functional selectivity. Receptor reserve (also known as spare receptors) is defined as a system in which the stoichiometry of the receptor is of greater molar excess compared to G protein effector subunits. Within such systems, it is possible for a ligand of low affinity to bind and produce maximal response (full intrinsic activity) without occupying all receptors available. This phenomenon was demonstrated with the dopamine D_1 receptor, where three different cell lines of varying expression levels produced variable responses to effector pathways measured (SKF38393 and SKF82958 exhibited partial agonist activity in a low expressing system, but full agonist activity in a high expressing system (Watts et al., 1995)). Although receptor reserve does exist in the system studied within this dissertation, a variety of lines of evidence suggest this cannot explain the many instances of functional selectivity. First, several of the test compounds used in my work have been shown to exhibit functional selectivity in a manner not related to receptor reserve (Mottola et al., 2002). Moreover, even in the earliest reports of this phenomenon, ligands

were found that caused opposite changes than predicted by receptor reserve (Berg et al., 1998; Mottola et al., 2002; Kilts et al., 2002).

It has also been suggested that the observations termed functional selectivity can be explained by differences in "strength of signal." Strength of signaling is best described as follows: upon activation of a receptor, cascading events take place, where one pathway is activated, followed by activation of additional pathways. It is conceivable that agonist activation of one pathway could be partially activated whereas another pathway by the same agonist could be fully activated. One potential argument is that some pathways being measured are downstream of earlier pathways, and that the earlier pathways would essentially be partially activated by agonists and the more downstream pathways could therefore exhibit full agonist activation. Kenakin suggests that strength of signaling can be ruled out by demonstrating the ability of one agonist to activate a specific effector pathway over another. This same agonist would also not activate a second pathway tested. Secondly, another agonist, capable of activating only the second pathway would be tested. This same agonist would likewise fail to activate the first pathway, thus ruling out strength of signaling (Kenakin, 1995a).

A non-linear view of receptor theory

The quantitative models that have dominated receptor pharmacology are based on discrete receptor states. Although they have been of great utility, they can only be made consistent with functional selectivity by the addition of so many additional states that the models lose their quantitative utility. One could generate a model with a discrete active state for every unique ligand, but the resultant complexity in the model loses heuristic value as was illustrated above (e.g., see Figure 1.1).

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One way to conceptualize functional selectivity is to envision a nearly unlimited possibility for the conformational states induced by different ligands that interact with a single receptor. Thus, rather than thinking of ligand interaction with receptor as a digital phenomenon (i.e., a few active states), one can hypothesize a dynamical systems. A dynamical system is defined as "any process or set of processes that evolves in time and in which the evolution is governed by some set of physical laws" (http://amsglossary.allenpress.com/glossary).

Through the use of ultrahigh x-ray crystallography at atomic resolutions < 1 Å under cryogenic conditions, dynamical sub-states can be frozen, allowing visualization of individual sub-states on electron density maps. Recent evidence with aldose reductase and parvalbumin at ultrahigh resolution x-ray crystallography has made possible the visualization of such dynamical sub-states (Declercq et al., 1999; Howard et al., 2004). Howard et al. found significantly different conformational states for a number of residues at 0.66 Å, including residues that involved intermolecular contacts in one conformation and none in the other. It was shown that water contacts were involved in stabilizing some conformations of aldose reductace in its ternary complex form, but in other areas of the structure, water contacts were in more disarray than necessary for contacts. Crystals comparing different inhibitor interactions with aldose reductase also provided evidence for distinctly different conformational states necessary for interaction and subsequent physiological response. Studies conducted by DeClercq et al. also found significantly different conformational sub-states for specific amino acid residues at 0.91 Å. This study provided evidence for differential interconversions between sub-states of amino acid residues, specifically addressing differences in χ_1 torsional angles. Each of these studies provides evidence, with

frozen sub-states of protein crystals, that suggests the possibility of unlimited unique receptor conformational states.

It is my view that the structurally subtle differences in individual ligand interactions with target receptors can induce a unique conformational profile. This hypothesis is consistent with data from high resolution x-ray crystallographic studies such as those mentioned previously. Further evidence to support not only ligand-specific receptor conformational states, but also its relevance to functional selectivity, comes from work with selective estrogen receptor modulators (SERMs). Crystallographic studies of estrogen receptors in different states have provided significant evidence to suggest that indeed ligand-specific receptor conformational states do exist (Shiau et al., 1998; Pike et al., 1999). Structural studies conducted with both ERα (2.03 Å) and ERβ (0.93 Å) show the induction of distinctly different conformational states by ligands of various classes including full agonists, partial agonists, and antagonists. In addition, these studies provide evidence to support a physiological role for ligand-induced conformational states.

What currently is not well understood, however, is how these unique ligand-receptor sub-states affect functional measurements with G protein-coupled receptors. This question will be the focus of this dissertation within the framework of studying mechanisms related to ligand-induced functional selectivity. Although I have provided my conceptual overview of this arena, the work proposed below had much more discrete and circumscribed foci. It is my hope that the results might influence others to think about the broader mechanisms involved in this phenomenon.

GOALS OF THIS DISSERTATION

The functional selectivity hypothesis is now being widely recognized (Simmons, 2005; Urban et al., 2006b) if not universally accepted. Despite this, relatively little is known about underlying mechanisms despite robust demonstrations of this phenomenon in many GPCR systems. To achieve my primary goal of investigating some of the mechanisms of functional selectivity, I chose the dopamine D_{2L} receptor as a model system for several reasons. First, although more structural studies have been done with the β_2 adrenergic receptor, there actually is a larger group of interesting functionally selective ligands that have been identified for the D_2 receptor. Moreover, many of these D_2 ligands are relatively rigid, somewhat simplifying data analysis and hypothesis testing. Finally, this receptor is of great clinical importance (already a target for some of the most serious and prevalent neurological and psychiatric disorders). It is therefore important to understand the role of this receptor in neural function.

The global hypothesis for my research is that functional selectivity is influenced by the particular range of conformational changes induced by a given ligand interacting with its target receptor. My goals were two-fold. First, I wanted to develop data that provided some understanding of how ligand-receptor interactions affect the structural basis of functional selectivity. Secondly, as an overall test of this idea, I hypothesize that the functional profile of some ligands can be altered by mutation of the receptor (i.e., the mutation can engender functional selectivity). These goals were addressed by examining how specific ligand-receptor residue interactions affected the signaling of a set of model compounds, some of which are functionally selective ligands at the dopamine D_{2L} receptor. This was accomplished by the following aims:

Aim 1: Determine the role of serine residues 5.42, 5.43, and 5.46 in the binding and subsequent activation of the hD$_{2L}$ receptor by functionally selective ligands.

Using rigid analogs that are known to exhibit functional selectivity at the hD$_{2L}$ receptor, this aim critically evaluated several hypotheses regarding the role of serines located in TM 5.

Aim 2: Determine the role of a novel threonine residue 3.37 in the binding and subsequent activation of the hD$_{2L}$ receptor by functionally selective ligands.

Previous studies including extensive cysteine scanning accessibility methods (SCAM) failed to suggest a role for T3.37 in agonist binding or function. Using rigid agonists hypothesized to interact via hydrogen bonding with T3.37, this aim tested the hypothesis that T3.37 played an important role in ligand interaction and receptor/G protein coupling to downstream effectors.

Aim 3: Determine the role of histidine 6.55 in the binding and subsequent activation of the hD$_{2L}$ receptor by functionally selective ligands.

Previous studies conducted at the 6.55 residue of the β$_2$-adrenergic receptor provided evidence for receptor interaction at asparagine 6.55 with the β-OH of some β$_2$ ligands (Wieland et al., 1996). In addition, using SCAM methods Javitch found H6.55 of D$_{2L}$ receptor water-accessible (Javitch et al., 1998). These experiments will seek to determine the importance of H6.55 in the interaction of ligands with the D$_{2L}$ receptor.

Aim 4: Determine the role of the Na$^+$/pH sensitive aspartate 2.50 in the allosteric modulation and subsequent activation of the hD$_{2L}$ receptor by functionally selective ligands.

This aim will focus on D2.50, a known Na$^+$/pH sensitive amino acid whose location is not within the active (orthosteric) binding site, yet is the most conserved residue of TM II (Neve et al., 1991; Neve, 1991). Previous studies have shown, however, that when mutated

to alanine or asparagine, this site allosterically regulates receptor activation and coupling to specific downstream effector pathways. Thus, this aim will focus on elucidating the role this residue plays in modulating ligand-induced receptor activation with our functionally selective test compounds.

CHAPTER 2. (R)-PROPYLNORAPOMORPHINE IS CONVERTED FROM A TYPICAL TO FUNCTIONALLY SELECTIVE LIGAND BY MUTATION OF THE DOPAMINE D_{2L} RECEPTOR.

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PREFACE

My first studies of functional selectivity with the dopamine D_{2L} receptor were founded on hypotheses that were generated from an existing body of data concerning the classic dopamine agonist enantiomeric ligands (R)- and (S)-norpropylapomorphine. These data suggested that these semi-rigid agonists might be particularly interesting probe ligands. The experiments in this chapter used these two enantiomers to probe specific ligand-receptor interactions occurring with TM5 serines, residues known to be critical for binding and activation of catecholamine receptors. The goals were to determine if these specific residues influence the signaling properties of these ligands, and to test the hypothesis that selected receptor mutations could change the signaling profile of a compound, converting from a typical agonist to a functionally selective ligand (or vice versa).

ABSTRACT

The functional selectivity hypothesis states that ligands can cause differential activation of signaling pathways mediated by the same receptor. A corollary is that mutations to the receptor can convert "typical" agonists into functionally selective ligands. These hypotheses were tested with the dopamine D_{2L} receptor, and two relatively conformationally restrained ligands (the enantiomers of propylnorapomorphine, RNPA and SNPA),. The ligands were docked to the hD_{2L} receptor, and resulting hypotheses tested by binding and functional analyses of the point-mutated receptors. Binding studies indicate that the para(10)-OH of RNPA H-bonds with S5.46(197), whereas the m-OH H-bonds with S5.42(193). Conversely, the docking pose of SNPA is altered by 180º such that H-bonding of the p-OH and m-OH occur only with serine 5.42(193). Functional analyses were done for GTPγS binding, adenylate cyclase inhibition (AC), MAPK phosphorylation (MAPK), and AA-release (AA). Relative to quinpirole, RNPA was a full agonist at all functions at the wildtype (WT) receptor. The S5.42A mutation decreased GTPγS binding for RNPA, yet RNPA still stimulated AC, MAPK, and AA fully. RNPA fully inhibited AC with S5.46A, whereas SNPA did not inhibit AC or AA with S5.46A, yet had MAPK activity similar to WT. Conversely, the S5.43A mutation decreased the affinity of all test ligands, yet had minimal effects or actually increased potency vs. AC or MAPK, yet dramatically affected AA signalng. These examples of the data provide direct evidence for the hypothesis that functional selectivity can be induced by changes to specific residues of the receptor.

INTRODUCTION

The dopamine receptors belong to a family of transmembrane receptors known as G protein-coupled receptors (GPCRs). Five genes produce variants of dopamine receptors grouped as D_1-like (D_1/D_5) and D_2-like ($D_2/D_3/D_4$). A splice variant of D_2 (additional 29 amino acids of the third intracellular loop) yields two major expressed isoforms: D_{2L} and D_{2S} (see Missale et al., 1998 for example). The D_1 class generally couples to stimulatory G proteins that activate adenylate cyclase, whereas the D_2 class couples to inhibitory G proteins that often inhibit adenylate cyclase activity.

Ligand interactions with TM3 and TM5 are known to be important in binding and signaling for catecholamine GPCRs (Shi and Javitch, 2002) Strader first showed a role for the TM5 serines of the β_2-adrenergic receptor (Strader et al., 1989), and later work suggested these serines were also critical for D_2 signaling (Mansour et al., 1992; Cox et al., 1992; Woodward et al., 1996; Wiens et al., 1998). Both S5.42A and S5.46A mutations of the rat D_{2S} decrease the potency of dopamine for inhibiting adenylate cyclase, whereas S5.43A abolished all functional responses (Cox et al., 1992). Binding data with the human hD_{2L} suggested a critical role for S5.46 with several compounds including dopamine and (R)-propylnorapomorphine (*vide infra*), two of the ligands to be explored in this Chapter (Mansour et al., 1992). Wiens et al. (1998) later studied coupling of the rat D_{2S} receptor to second messenger pathways after TM 5 serine mutations. They found that the S5.43A mutation caused dopamine to lose efficacy at both adenylate cyclase and GIRK channels, whereas quinpirole was unaffected. The functionally selective D_2 ligand dihydrexidine (Mottola et al., 2002; Kilts et al., 2002), had decreased high affinity binding and concomitant loss of function with S5.42A and S5.43A. Such data can be interpreted to suggest that unique features by which any ligand interacts with its target receptor

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(WT or mutant) can result in differential functional effects, a hypothesis that underlies this dissertation.

This chapter focuses on the R(-)- and S(+)- enantiomers of propylnorapomorphine (NPA; Neumeyer et al., 1983; Neumeyer et al., 1988; Neumeyer et al., 1991), hereafter called RNPA and SNPA. RNPA is described as a high affinity agonist at the dopamine D_2 receptor. RNPA has classical dopamine agonist pharmacological actions *in vivo*, both behaviorally [e.g., causes strong stereotyped sniffing, licking, and gnawing (Campbell et al., 1986)] and physiologically [e.g., inhibits cell firing in the substantia nigra and ventral tegmental area (Cox et al., 1988)], and also has expected characteristics *in vitro* [i.e., fully inhibits cAMP accumulation (Kula et al., 1985)]. Conversely, SNPA, although also described as a high affinity D_2 ligand (Neumeyer et al., 1991), has a completely different profile than RNPA *in vivo* [e.g., selectively inhibits locomotor activity, and does not induce stereotypy or catalepsy (Campbell et al., 1986)] and *in vitro* [e.g., does not inhibit cAMP accumulation (Kula et al., 1985) but does inhibit cell firing in the SN and VTA similar to RNPA) (Cox et al., 1988)]. Later, Kilts et al. (2002) demonstrated that SNPA was functionally selective *in vitro* in MN9D cells, possibly providing a mechanism for the anomalous pharmacological actions of this compound.

Further studies have been conducted with these compounds in heterologous expression systems. In Chinese hamster ovary (CHO) cells expressing the human D_{2L} receptor, RNPA had agonist activity at both adenylate cyclase and in stimulating p44/p42 MAP kinase phosphorylation (MAPK), yet also had antagonist activity at G protein-coupled inward rectifying potassium channels (GIRK; Gay et al., 2004). These studies provided additional evidence that SNPA was functionally selective: it had partial D_{2L} agonist activity at MAPK, full agonist activity at adenylate cyclase, and antagonist activity at GIRK channels (Gay et al., 2004).

Not only do such data with the NPA enantiomers suggest that RNPA and SNPA have different patterns of functional selectivity at the D_{2L} receptor that might explain some of their unusual neuropharmacology, but they lead to the hypothesis that these ligands can be of utility in understanding mechanisms that result in functional selectivity. Specifically, I hypothesized that some mutations to the D_{2L} receptor may turn typical agonists into functionally selective ligands, or that such mutations may alter the functional patterns of ligands that are already functionally selective. The enantiomers of NPA, by nature of their behavior with the WT receptors, and because of their relatively conformationally restrained structures, offered the possibility of being excellent tools to test these hypotheses.

This was addressed by first using computational approaches to hypothesize specific binding interactions between each enantiomer-receptor complex, and then an iterative process of modeling and experimental validation for hypothesis testing. In this study we targeted two hydroxyl-containing amino acids that were predicted to interact with these ligands [S5.42(193) and S5.46(197)], as well as an adjacent residue [S5.43(194)] whose role is less certain. Each residue was mutated to alanine, and then characterized for antagonist radioligand binding. Each probe ligand was then tested for affinity, effects on GTPγS binding, and for three receptor-mediated functional endpoints (cAMP inhibition, MAPK phosphorylation, and [^{3}H]-AA release). These data provide direct evidence for the hypothesis that functional selectivity can be induced by changes to specific residues of the receptor, and provide some insight into how how ligand-specific interactions with certain residues of the receptor may induce such changes in signaling patterns.

METHODS

Most of the Methods described below are common to Chapters 2-6 and to a study described in the Appendix. They are detailed below, and later Chapters only contain experimental details that were not referenced in the methods described below.

Materials

The R(-)- and S(+) isomers of propylnorapomorphine [NPA; 6-propyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline-10,11-diol], isobutylmethylxanthine, dopamine, EDTA, dithiothreitol, sucrose, pepstatin A, leupeptin, PMSF, fetal bovine serum and other standard chemical reagents were purchased from Sigma Chemical Co (St Louis, MO). [^{3}H]-N-methylspiperone, [^{3}H]-arachidonic acid [or [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N)]-AA], and [^{35}S]-GTPγS were purchased from Amersham Biosciences Inc. (Piscataway, NJ). [^{125}I] for cAMP assays was purchased from NEN/Perkin Elmer (Boston, MA). HEPES buffer was purchased from Research Organics (Cleveland, OH). Quinpirole and domperidone were purchased from Sigma/RBI (Natick, MA). Ham's F-12, Opti-Mem, penicillin, streptomycin, primers and lipofectamine were purchased from Invitrogen (Carlsbad, CA). Hygromycin B was purchased from Roche Applied Science (Indianapolis, IN). Primary antibody to phospho-p44/p42 MAPK and secondary antibody, anti-rabbit HRP-conjugated, were purchased from Cell Signaling Technology Inc. (Beverly, MA). cAMP primary antibody was obtained from Dr. Gary Brooker (George Washington University, Washington DC) and secondary antibody, rabbit anti-goat IgG, was purchased from Advanced Magnetics (Cambridge, MA). Dihydrexidine, dinapsoline, and dinoxyline (used in subsequent Chapters of this dissertation were synthesized following published procedures (Brewster et al., 1990; Ghosh et al., 1996; Grubbs et al., 2004).

Molecular biology and cell culture.

CHO hD$_{2L}$ wild type and mutant cells were maintained in Ham's F-12 media supplemented with 10% fetal bovine serum, 100X penicillin-streptomycin, and 100 μg/mL Hygromycin. Mutant cell lines were constructed using the pcDNA5/FRT plasmid obtained from Invitrogen. Following subcloning of the hD$_{2L}$ receptor into the plasmid, point mutations were introduced using PCR techniques. Stable transfections of point mutants into CHO K1 cells were conducted using modifications of a previously published protocol (Milligan, 1999). Throughout this paper, the identification of the mutated amino acid residue will be made using universal notation as proposed by Ballesteros et al. (1995). The residues (listed in both universal notation and absolute position) studied were S5.42A (S193A), S5.43A (S194A), and S5.46A (S197A).

Radioreceptor assays

Membranes for radioreceptor assays were prepared by rinsing cells with phosphate-buffered saline, and then lysing with a solution containing 2 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 1 μg/mL pepstatin A, 0.5 μg/mL leupeptin, and 0.05 μg/mL PMSF. Cell fragments were scraped, homogenized, and centrifuged at 30,000 g for 30 min. Following centrifugation, cell pellets were resuspended, homogenized and placed into storage buffer (50 mM HEPES, 0.32 M sucrose, 1 μg/mL pepstatin A, 0.5 μg/mL leupeptin, and 0.05 μg/mL PMSF) and stored at -80 $^{\circ}$C. Saturation binding assays were conducted using protocols described previously (Gay et al., 2004) but with varying concentrations of [^{3}H]-N-methyl-spiperone to determine the B$_{max}$ and K$_D$ for each membrane preparation (e.g. hD$_{2L}$ WT; hD$_{2L}$ S5.42A, etc.). Domperidone (10 μM) was used to define non-specific binding. Competition binding assays also utilized [^{3}H]-N-methylspiperone.

Molecular Modeling

The ligand-free model of the transmembrane region of hD_{2L} receptor was built using homology modeling techniques based on constraints from the known crystal structure of bovine rhodopsin (Palczewski et al., 2000), and from inferences drawn from experimental probing of the dopamine receptor. Initial structures of all selected ligands were constructed using the BUILDER module of InsightII. Consistent with the parameterization of version 27 of CHARMM force field, atomic partial charges for these ligands that carry a formal charged amine group were generated by fitting the molecular electrostatic potential computed with a 6-31G* basis set using GAUSSIAN03. The ligands then were energy-minimized, and subsequently docked manually inside the putative binding site of the ligand-free model of hD_{2L} receptor. Based on available data for aminergic GPCRs (see Shi and Javitch, 2002 for review), the binding site was initially selected to have the selected ligands interact with S5.46, S5.42, and D3.32, that were hypothesized to be involved in ligand recognition. Specifically, the hD_{2L} D3.32 residue served as an anchoring point for the protonated amine common to all selected ligands. To construct a model suitable for energy minimization of the ligand, the initial conformation of each compounds was then hand-docked in a reasonable binding mode with the protonated amine group directed towards D3.32, and the hydroxyl groups oriented towards S5.46 and S5.42. Next, each ligand was energy-minimized in this binding site using the CHARMM force field with a 2r distance-dependent dielectric constant. Protein backbone atoms were held fixed, whereas all side chain atoms were allowed to move. After minimization, all protein residues within 5 Å of each ligand were identified as the interacting residues in each binding site.

GTPγS assay

Measurement of $[^{35}S]$-GTPγS binding was determined as described previously (Shapiro et al., 2003), with non-specific binding defined by 10 μM cold GTPγS. Assay tubes contained 150-200 pM $[^{35}S]$-GTPγS, binding buffer (50 mM HEPES, 100 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, 0.1% ascorbic acid, pH 7.4 with NaOH), 10 μM GDP, and varying concentrations of agonists and/or antagonist. Membranes (approximately 100 μg protein/mL) were incubated with test compounds for 15 min at 30°C before addition of $[^{35}S]$-GTPγS. After an additional 30 min incubation, the assay was terminated by filtration (Packard Filtermate 190 harvester) with ice cold wash buffer (50 mM HEPES, 4 mM $mgCl_2$, pH 7.4 with KOH), and radioactivity quantified by liquid scintillation spectrometry (Packard TopCount NXT).

cAMP accumulation assay

Measurement of dopamine receptor agonist inhibition of forskolin-stimulated cAMP accumulation was performed in whole cell preparations as described previously (Gay et al., 2004). In brief, CHO cells were seeded in 24-well plates at a density of 2.5×10^6 cells/well, and grown for 48 hr in Ham's F-12 media supplemented with 10% fetal bovine serum and 100X penicillin-streptomycin. Cells were preincubated for 5 min prior in fresh media (serum-free media containing 25 mM HEPES, 500 μM isobutylmethylxanthine, and 0.1 % ascorbic acid) at 37 °C. Assay medium then was aspirated, and fresh assay media containing forskolin and/or various concentrations of the test compounds was added. The plates were incubated for 15 min at 37°C, cells rinsed with fresh assay medium, aspirated, and the reaction halted using 0.1 N HCl. The cAMP was quantified using a modified radioimmunoassay previously described (Harper and Brooker, 1975).

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MAP kinase assay

Measurement of dopamine receptor agonist stimulation of p44/p42 MAPK was performed in whole cell preparations by modifying a previously published protocol (Versteeg et al., 2000). CHO cells were seeded in 96-well plates at a density of 5 x 10^6 cells/well and grown for 48 hr in Ham's F-12 media supplemented with 10% fetal bovine serum at 37 ^{o}C. Cells were serum starved for 6 hr prior to stimulation. Appropriate drug dilutions of the test compounds were added to each well at a volume of 100 μL for 10 min. The reaction then was terminated, and the cells fixed by aspirating the wells and adding 100 μL of 4% formaldehyde PBS solution for 20 min. Cells were washed three times with 100 μL wash buffer (0.1% Triton X-100/PBS solution), followed by a 20 min incubation with 0.6% H_2O_2 Triton/PBS solution to quench endogenous peroxidases. After washing the cells three times again with wash buffer, and after a 1 h incubation with 10% BSA in Triton/PBS solution (to block nonspecific antibody binding), cells were incubated overnight (about 12 hr) with a 1:250 dilution of PhosphoPlus® p44/42 primary antibody in the Triton/PBS solution (100 μL) containing 5% BSA at 4°C. Cells were washed three times with wash buffer for five min, and incubated with 100 μl HRP-conjugated goat anti-rabbit secondary antibody (1:100 dilution) with 5% BSA at room temperature for 1 h. Again, cells were washed three times with wash buffer for five min, and then twice with PBS. Cells were then incubated with 50 μL of an *o*-phenylenediamine (OPD) solution (0.4 mg/mL OPD, 17.8 mg/mL $Na_2HPO_4·7H_2O$, 7.3 mg/mL citric acid and 0.015% H_2O_2) for 15 min at room temperature in the dark. The reaction was terminated by the addition of 25 μL of 1 M H_2SO_4, that causes a light–to-dark orange color change (A_{490} - A_{650}) that is proportional to phosphorylation.

Arachidonic acid assay

Measurement of dopamine receptor agonist potentiation of ATP-stimulated [^{3}H]-arachidonic acid (AA) release was measured in whole cell preparations using modifications of a previously published method (Berg et al., 1998). CHO cells were seeded in 24-well plates at a density of 5 x 10^5 cells/well and grown for 24 hr in Ham's F-12 media supplemented with 10% fetal bovine serum at 37 ^{o}C. Cells are serum starved with 500 μL of serum free Ham's F-12 containing 0.5 μCi/mL [^{3}H]AA for 5 hr at 37 ^{o}C. Ten-μL aliquots were removed to compare with the original tritiated loading media to determine the time course and total cellular uptake of [^{3}H]-AA. Cells were washed three-times for 5 min each with Hank's balanced salt solution (HBSS) containing 0.5 % fatty acid-free BSA and antagonists for respective wells (500 μL/well/wash). Cells then were incubated with agonists for 15 min with or without ATP dissolved in HBSS/BSA (ATP being added last and in timed increments of 5 sec between wells).

Data and Statistical Analysis

Data from all assays were analyzed using Prism 4.0. Saturation analysis was conducted using a one-site binding model. Competition data used non-linear regression and a sigmoidal equation to determine IC50 and E$_{max}$ values. The IC50s were corrected for radioligand concentration, and are reported as corrected affinity values (K$_{0.5}$). Statistical analyses were conducted using SigmaStat 2.03, using algorithms specified with each experiment.

RESULTS

Molecular modeling predictions

RNPA, SNPA, dopamine, and quinpirole were all docked to the hD$_{2L}$ receptor (see Figure 2.1). The proposed RNPA-hD$_{2L}$ receptor complex predicts the involvement of the para-

OH (i.e., C10 on RNPA, Figure 2.1) in H-bonding with S5.46, whereas the ligand meta-OH (C11) interacts with S5.42.
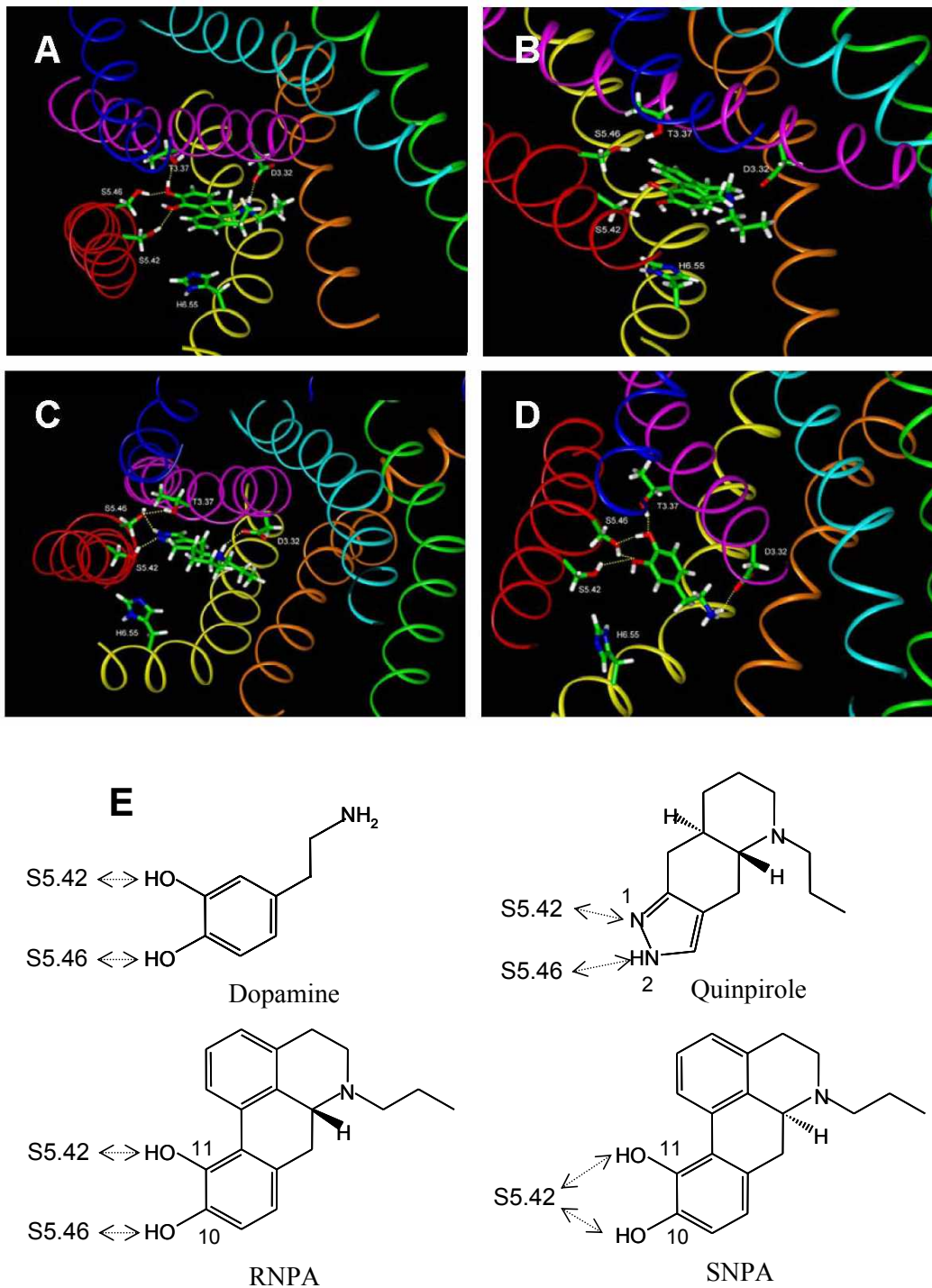
Figure 2.1. Three-dimensional molecular models. *3D molecular models of ligands docked to hD$_{2L}$. View is from the extracellular surface, looking down into the receptor. A) RNPA, B) SNPA, C) quinpirole, D) dopamine E) Schematic of ligand structures and predicted sites of interaction derived from modeling studies.*

In contrast, the opposite orientation of the hydrogen on the protonated amine group of SNPA puts the para-OH (C10) some distance from S5.46, but leads to a predicted H-bond interaction with S5.42, with the meta-OH (C11) also interacting with S5.42. Docking studies conducted with dopamine indicate the involvement of the para-OH with S5.46, and the meta-OH with S5.42. The N2 nitrogen of quinpirole was predicted to donate a proton to the hydroxyl group of the S5.46 side chain, whereas the N1 of quinpirole was hypothesized to hydrogen bond with S5.42. The structures of each ligand and their predicted interaction sites are shown schematically in Figure 2.1A-2.1E.

Effect of S5.42A, S5.43A, and S5.46A on antagonist radioligand binding

The hD_{2L} WT, S5.42A, S5.43A, and S5.46A receptors were expressed stably in CHO K1 cells, and saturation radioreceptor assays performed. The WT receptor was expressed at 4.8 pmol/mg protein with a K_D of 0.58 nM, and the S5.42A (S193A) and S5.46A (S197A) receptors were expressed at a similar density and affinity (Figure 2.2 and Table 2.1). Conversely, the S5.43A (S194A) receptor was expressed at a much higher density (20.7 pmol/mg protein), but with slightly lower affinity (K_D = 1.4 nM). There was no specific [^{3}H]-N-methylspiperone binding in untransfected WT cells.

Effect of S5.42A, S5.43A, and S5.46A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus [^{3}H]-N-methylspiperone in membranes from both WT and mutant receptors. Because the slopes of many of the curves were not of normal steepness, all data (e.g., Table 2.1) are expressed as apparent affinity constants ($K_{0.5}$) determined from experimental IC50 values that were corrected for radioligand concentration using the bimolecular Cheng-Prusoff relationship

(Cheng and Prusoff, 1973). Competition binding studies also were conducted with several structurally different antagonists [domperidone, N-methylspiperone] to rule out gross structural changes induced by the mutation (data not shown). As was seen with [³H]-N-methylspiperone, all of the antagonists had only slightly decreased affinity for each mutant receptor, and retained the same rank order of affinity as was found with the WT receptor. These data suggest that the mutations did not induce profound changes to overall receptor structure.
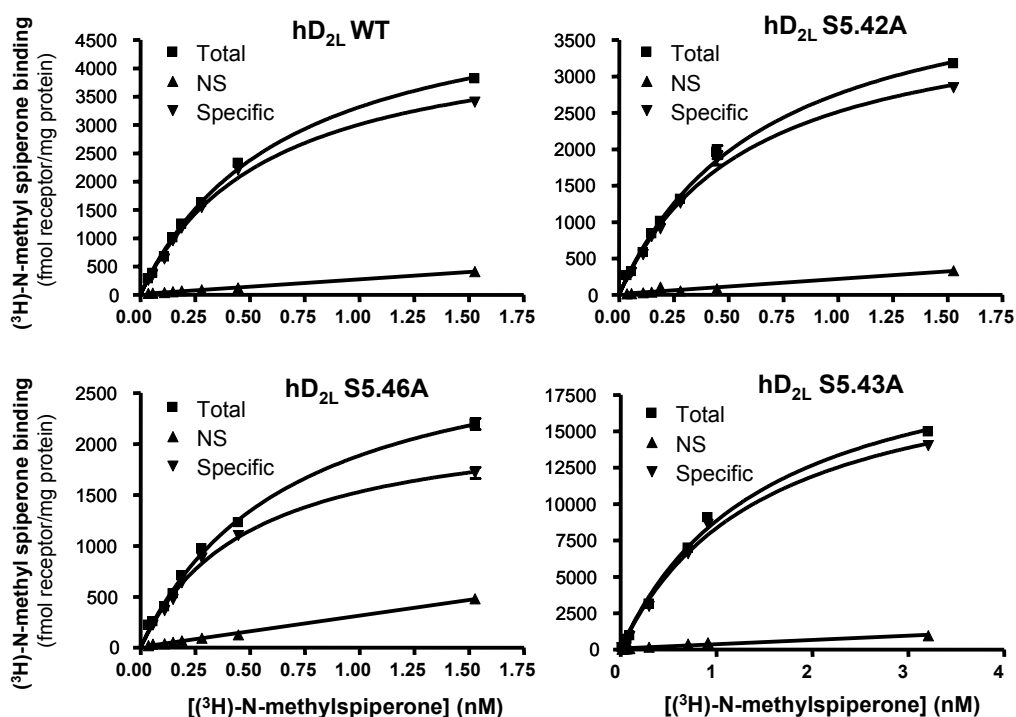
Figure 2.2. Saturation assays for stably expressing mutant and WT hD₂L receptors. *CHO hD₂L membrane fragments were incubated with increasing concentrations of [³H]-N-methylspiperone for 15 min. at 37 °C. Data shown are representative of 3-4 independent experiments conducted in triplicate.*

Representative data for the agonist probe ligands are shown in Figure 2.3, and are summarized in Table 2.1. S5.43A had the largest overall effect of affinity, increasing the $K_{0.5}$ by 50-fold or more for all ligands. The S5.42A mutation caused a marked, but smaller, loss of affinity for all ligands, but also altered rank order of affinity [WT: RNPA >>> SNPA > quinpirole > dopamine; S5.42A: RNPA >>> quinpirole >> SNPA >>> dopamine]. Rank order

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was not affected by the other mutations. The S5.46A mutation caused loss of affinity that was

intermediate between S5.42A and S5.43A.
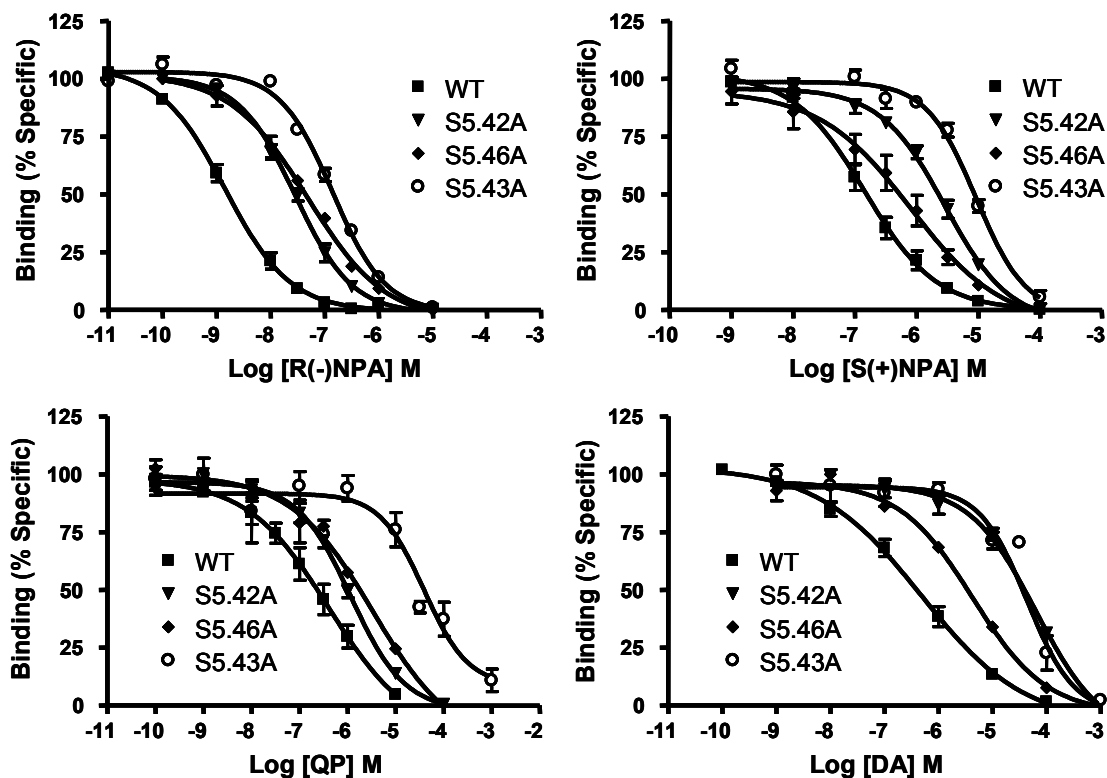
Figure 2.3. Competition binding of test compounds with hD₂L WT and mutant receptors. *Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 2.1. Saturation and competition binding data for RNPA, SNPA, quinpirole, and dopamine for serine mutants.

Mutant	K_D (nM)	B_{max} (pmol/mg)	R(-)NPA	S(+)NPA	Quinpirole	Dopamine
	Saturation Binding		Competition Binding $K_{0.5}$ (nM)			
WT	0.58	4.8	1.3 ± 0.85	129 ± 30	365 ± 66	447 ± 172
S5.42A	0.59	4.0	25 ± 3.7	$2,540 \pm 370$	$1,040 \pm 100$	$80,000 \pm 3,000$
S5.43A	1.4	20.7	98 ± 37	$6,400 \pm 1,100$	$27,000 \pm 10,000$	$31,000 \pm 6,000$
S5.46A	0.50	2.3	42 ± 8.5	698 ± 125	$2,910 \pm 820$	$3,500 \pm 1,500$

Effect of mutations on ligand-induced GTPγS binding

An estimate of G protein turnover was conducted using ligand-induced binding of [^{35}S]-GTPγS. As shown in Figure 2.4 and summarized in Table 2.2, the S5.42A and S5.46A mutations caused a large decrease in potency of RNPA (EC50 of 42 and 78 nM respectively compared to 1.0 nM for WT) whereas S5.43A was minimally affected (EC50 = 3 nM). The S5.42A and S5.46A mutations caused an eight fold decrease in the potency of SNPA (EC50 = 9400 and 8800 nM respectively vs. 1200 nM for WT), but a four-fold increase with S5.43A (EC50 = 312 nM). Neither the S5.42A nor the S5.43A mutations markedly affected the potency of quinpirole (EC50 = 1700 and 203 nM vs. 735 nM for WT), whereas S5.46A caused a notable decrease (EC50 of 7200 nM). Both S5.42A and S5.43A caused some decrease in dopamine potency (EC50 =10700 nM and 4300 nM, respectively vs. 1710 for WT), whereas S5.46A caused a complete loss of GTPγS binding.

It is noteworthy that there was little correlation between the effects of these mutations on functional potency versus their effects on intrinsic activity. Thus, whereas S5.46A caused a marked decrease in RNPA affinity as well as its potency to stimulate GTPγS binding, the E$_{max}$ of RNPA was not significantly altered. Conversely, both the S5.42A and S5.46A mutations caused large effects on GTPγS potency, but only the former mutation affected the E$_{max}$. A similar contrast was seen with quinpirole, whose potency, but not intrinsic activity, was affected differentially by each of the three serine mutations. These data demonstrate that whereas there are some correlations between the effects of these mutations on agonist binding vs. their effects of GTPγS binding, the exceptions are numerous and notable. If these data reflect subtle differences in ligand-induced conformations with each receptor, it should also be reflected in biochemical functions mediated by the D$_{2L}$ receptor.
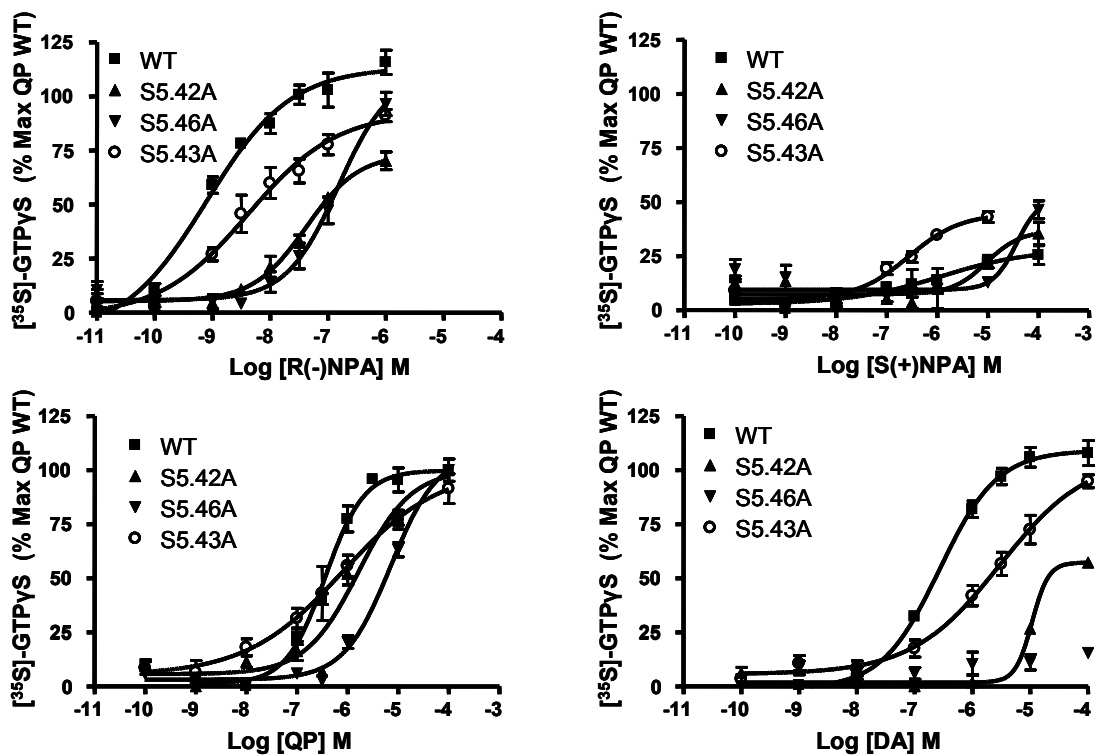
***Figure 2.4. GTPγS turnover experiments of test compounds with hD*2L** *WT and mutant receptors. Analysis was conducted using non-linear regression and a sigmoidal equation (Prism 4.0) to determine EC50s reported in Table 2.2. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 2.2. Potency of probe ligands in affecting GTPγS binding.

Mutant	R(-)NPA [EC50 (nM)]	S(+)NPA [EC50 (nM)]	Quinpirole [EC50 (nM)]	Dopamine [EC50 (nM)]
WT	1.0 ± 1.3	1200 ± 31	740 ± 120	1700 ± 1040
S5.42A	42 ± 14	9400 ± 3900	1700 ± 860	10700 ± 1530
S5.43A	3 ± 1.1	312 ± 102	203 ± 5	4300 ± 1600
S5.46A	78 ± 37	8800 ± 8900	7200 ± 1900	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Functional analysis of the three serine mutants

Three functional assays were used to assess the effects of each serine mutation. Examples of dose-response curves for each ligand, function, and mutant receptor are shown in Figures 2-5 through 2-7. These data from 3-5 replicate experiments (each run with triplicate points) were then analyzed, and the summary data described in the following sections.
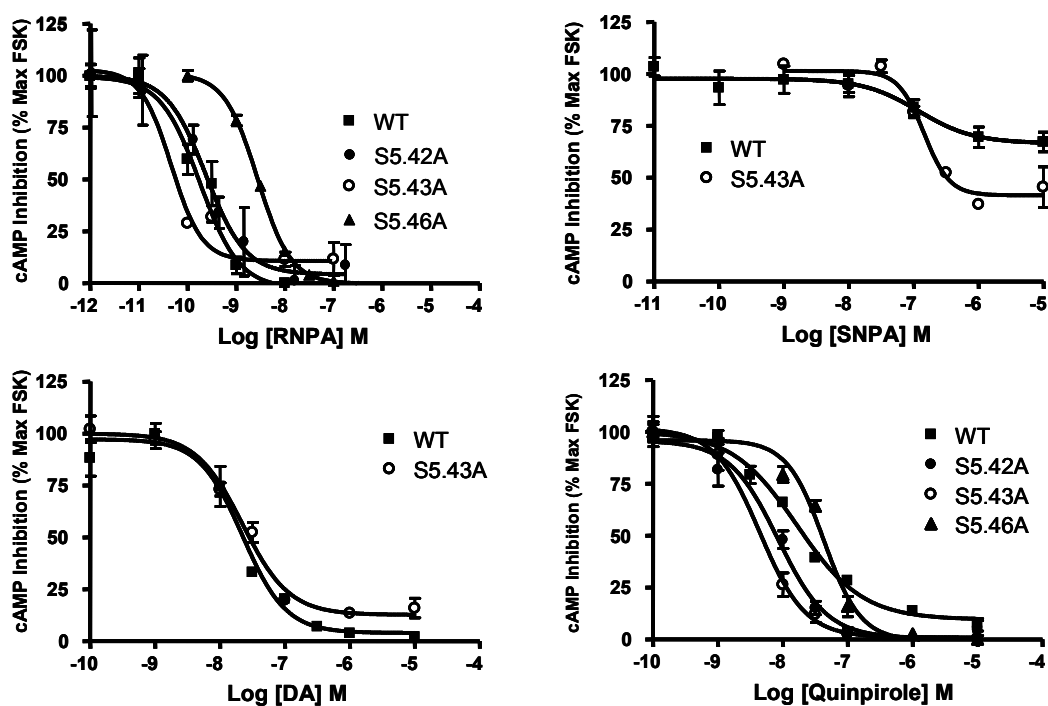
Figure 2.5. Dose response curves for AC inhibition with ligands at S5.42A, S5.43A, and S5.46A. *Data are representative of 3-5 independent experiments conducted in triplicate.*
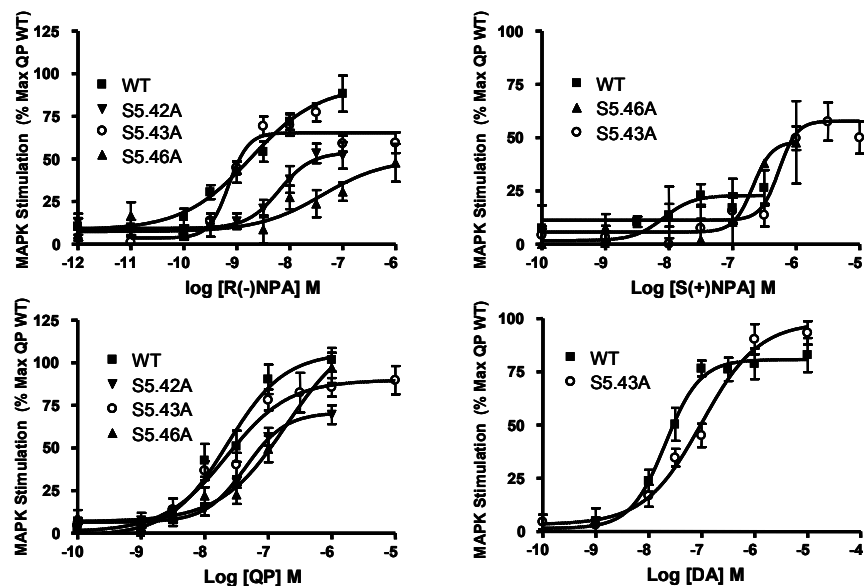
Figure 2.6. Dose response curves for MAPK stimulation with ligands at S5.42A, S5.43A, and S5.46A. *Data are representative of 3-5 independent experiments conducted in triplicate.*
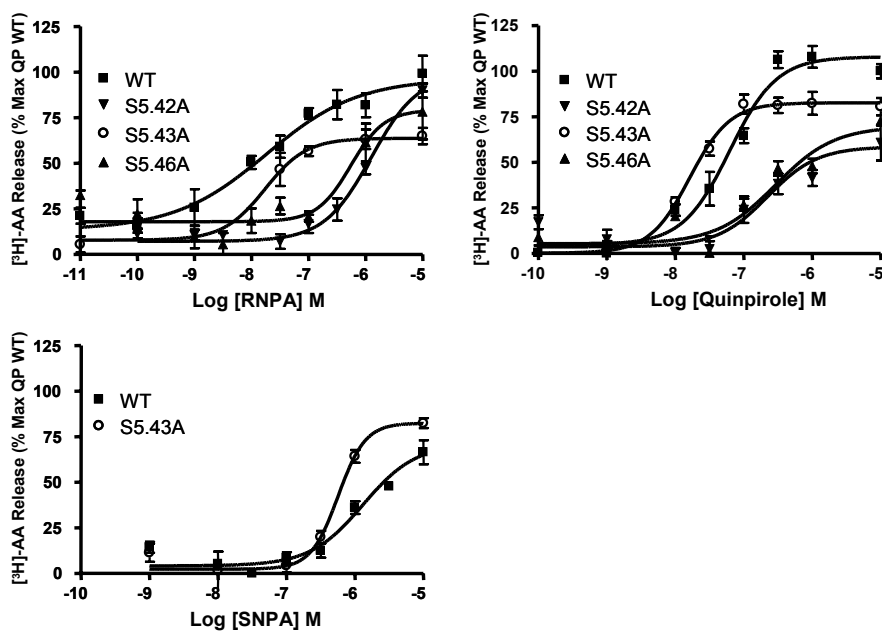
Figure 2.7. Dose response curves for stimulation of AA-release with ligands at S5.42A, S5.43A, and S5.46A. *Curve for DA is not shown because there was no functional activity. Data are representative of 3-5 independent experiments conducted in triplicate.*

Functional differences caused by S5.42A

None of these ligands had effects on forskolin-stimulated adenylate cyclase activity in untransfected CHO K1 cells. With the WT receptor, quinpirole, dopamine, and RNPA all fully inhibited forskolin-stimulated cAMP accumulation (AC), whereas SNPA had partial agonist activity (Figure 2.8, and Table 2.3). The D_2 antagonist domperidone (10 μM) alone had no affect on forskolin-stimulated cAMP accumulation with either WT or S5.42A receptor. Conversely, domperidone completely blocked the effects of quinpirole (1 μM) at the WT receptor, consistent with these effects being mediated via the D_{2L} receptor. The S5.42A mutation caused a complete loss of AC intrinsic activity for all of these ligands except for QP and RNPA.

Table 2.3. Effect of S5.42A mutation on functional potencies.

Ligand	Quinpirole [EC50 (nM)]		Dopamine [EC50 (nM)]		RNPA [EC50 (nM)]		SNPA [EC50 (nM)]	
Function	WT	S5.42A	WT	S5.42A	WT	S5.42A	WT	S5.42A
AC	172 ± 5.5	7.8 ± 1.4	157 ± 22	--	0.18 ± 1.4	0.18 ± 0.01	118 ± 18	--
MAPK	26 ± 5	44 ± 12	28 ± 7	--	1.85 ± 0.9	6.4 ± 1	26 ± 19	--
AA-release	63 ± 12	223 ± 67	49 ± 26	--	18 ± 1.9	1,300 ± 250	1,300 ± 253	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

In the WT receptor, quinpirole, dopamine, and RNPA all fully activated the phosphorylation of the p44/p42 MAP kinase (MAPK), whereas SNPA was a partial agonist (Figure 2.8, and Table 2.3). Similar to the results with AC, the S5.42A mutation caused complete loss of intrinsic activity for dopamine and a dramatic decrease for SNPA. Conversely, this mutation caused only a slight loss of intrinsic activity and potency for quinpirole, and somewhat greater effects in the intrinsic activity of RNPA.

A quite different pattern was seen with D_{2L}-stimulated $[^3H]$-arachidonic acid release (AA). With the WT receptor, SNPA was a near-full agonist, and the other compounds had 100% intrinsic activity. With the S5.42A mutant, quinpirole lost some intrinsic activity, with a slight decrease in potency (223 vs. 63 nM in WT). Interestingly, RNPA remained a full agonist, yet with dramatically lower potency (1,300 vs. 18 nM in WT). Similar to MAPK stimulation (see above), this data suggests that subtle changes in ligand-residue interactions can sometimes cause significant functional effects.

The loss of functional potency for SNPA and dopamine were consistent with the importance of S5.42 predicted by the modeling. On the other hand, both quinpirole and RNPA retained some functional potency at this receptor, although this mutation induced functional selectivity expressed as reversed intrinsic activities at MAPK and AA (Berg et al., 1998).

Functional differences caused by S5.43A

I next examined S5.43, a residue hypothesized as critical because of formation of intrahelical H-bonds that stabilize TM 5. I hypothesized that mutation of S5.43A would have little or no effect on intrinsic activity at all functional endpoints measured with these probe ligands. Consistent with this, RNPA, dopamine, and quinpirole fully, and SNPA partially, inhibited AC in both WT and S5.43A receptors with no difference in rank order of potency [RNPA >> quinpirole > dopamine > SNPA]. The intrinsic activity of SNPA actually was greater in the S5.43A mutant than in WT despite lower potency (Figure 2.8 and Table 2.4). In terms of MAPK function, similar results were seen. There were two noteworthy findings (Table 2.4 and Figure 2.6). First, SNPA had higher intrinsic activity with the S5.43A than WT receptor. Second, the rank order of potency was markedly altered by this mutation (S5.43A: RNPA > quinpirole > dopamine > SNPA vs. WT: RNPA > SNPA = dopamine = quinpirole).

Measurement of a third pathway, agonist-stimulated [³H]-arachidonic acid release (AA), led to a much unexpected finding (Figure 2.6, Table 2.3). Although all ligands were full agonists for AA-release with the WT receptor, only quinpirole and SNPA exhibit full agonist activity similar to WT. RNPA exhibits partial agonist activity whereas DA exhibits loss of activity. These results are a striking contrast to the functional effects of the same ligands in the other two functions we assessed.
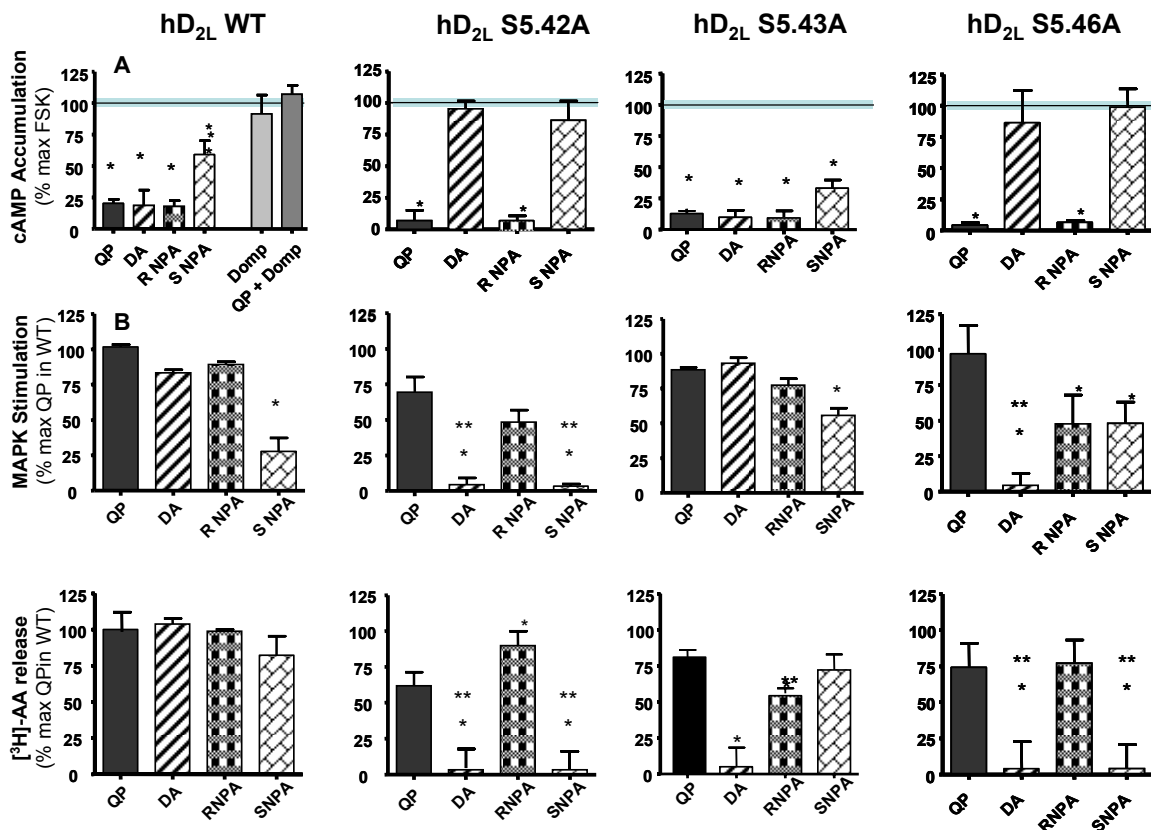
*Figure 2.8. Ligand effects on activation of second messenger pathways of hD$_{2L}$ with mutations at S5.42A, S5.43A, and S5.46A. Top row: Ligand-mediated inhibition of forskolin-stimulated cAMP accumulation (AC); Middle row: Ligand-mediated activation of p44/p42 MAP Kinase (MAPK); Bottom row: Ligand-mediated potentiation of [³H]-arachidonic acid release (AA). Note that agonists inhibit AC, but stimulate MAPK and AA. Data are representative of E$_{max}$ values for 3-5 independent experiments conducted in triplicate. * p < 0.05 (One way ANOVA, post hoc Dunnet's). ** p < 0.05 (Kruskal-Wallace one way ANOVA, post hoc Dunn's).*

Table 2.4. Effect of S5.43A mutation on functional potencies.

Ligand	Quinpirole [EC50 (nM)]		Dopamine [EC50 (nM)]		RNPA [EC50 (nM)]		SNPA [EC50 (nM)]	
Function	WT	S5.43A	WT	S5.43A	WT	S5.43A	WT	S5.43A
AC	172 ± 6	3 ± 1	157 ± 22	19 ± 3	0.18 ± 1.4	0.05 ± 0.01	118 ± 18	143 ± 7
MAPK	26 ± 5	26 ± 11	28 ± 7	114 ± 40	1.9 ± 0.9	0.73 ± 0.12	26 ± 19	340 ± 34
AA-release	63 ± 12	35 ± 27	49 ± 26	--	18 ± 2	7 ± 5	$1,300 \pm 250$	190 ± 150

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Functional differences caused by S5.46A

S5.46A is predicted to be located deepest in the binding pocket of hD_{2L}. The S5.46A mutation caused a complete loss of intrinsic activity for dopamine and SNPA, without affecting that of RNPA or quinpirole (Figure 2.8). Interestingly, the potency of quinpirole was somewhat greater with S5.46A, whereas that of RNPA was somewhat lower (Table 2.3). A quite different pattern was seen when MAP kinase activation was studied. The intrinsic activity of quinpirole was unaltered by the S5.46A mutation, whereas that of dopamine was abolished, and that of RNPA markedly decreased. Interestingly, the intrinsic activity of SNPA was unaffected (Figure 2.7-10 and Table 2.3). The rank order of potency was also changed (WT: RNPA > SNPA = quinpirole vs. S5.46A: RNPA > quinpirole > SNPA). When D_{2L}-mediated [³H]-arachidonic acid release was assessed, a pattern emerged similar to that seen with AC. Neither dopamine nor SNPA had any intrinsic activity, and quinpirole and RNPA were essentially full agonists (see Figure 2.8 and Table 2.3). Both RNPA and quinpirole had significantly lower potency with the S5.46A receptor.

Table 2.5. Effect of S5.46A mutation on functional potencies.

Ligand	Quinpirole [EC50 (nM)]		Dopamine [EC50 (nM)]		RNPA [EC50 (nM)]		SNPA [EC50 (nM)]	
Function	WT	S5.46A	WT	S5.46A	WT	S5.46A	WT	S5.46A
AC	172 ± 6	54 ± 11	157 ± 22	--	0.18 ± 1.4	3.8 ± 1.6	118 ± 18	--
MAPK	26 ± 5	193 ± 28	28 ± 7	--	1.9 ± 0.9	41 ± 5	26 ± 19	334 ± 39
AA-release	63 ± 12	292 ± 16	49 ± 26	--	18 ± 2	550 ± 240	1,300 ± 250	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

DISCUSSION

The approach that was used in this work differs in philosophy from much of the earlier work that studied the structural features involved in ligand-GPCR interactions. The primary endpoint has usually been the relationship of receptor structural features to the binding of ligands, although in some studies (Mansour et al., 1992; Cox et al., 1992; Wieland et al., 1996; Wiens et al., 1998) a single functional effect (usually inhibition of adenylate cyclase, sometimes in combination with GTPγS binding) also was examined. The current approach was based on the hypothesis that whereas mutation-induced changes in affinity and functional properties can be closely related, frequently they will be affected quite independently. This hypothesis leads to several predictions as corollaries to the main functional selectivity hypothesis (Mailman and Gay, 2004; Urban et al., 2006a).

First, in a given assay there is not always a direct relationship between mutation-induced changes in affinity and functional potency. Second, mutation-induced effects on intrinsic activity (i.e., E_{max}) and potency are independent, and do not always occur in a parallel fashion. Third, some mutations may affect the functional properties of a ligand differentially, essentially causing

"typical agonists" to become functionally selective or even functionally selective ligands to become typical. Each of these hypotheses is consistent with the overall notion of functional selectivity, but are not easily reconciled with commonly accepted precepts of quantitative receptor theory (Urban et al., 2006a). The current manuscript provides some novel data that allows testing of these notions. Because on the complex matrix of our data (four receptors by five pharmacological endpoints by four ligands), we have provided in Table 2.6 a summary format to facilitate review of key points.

The key interaction of S5.42 and S5.46 in ligand binding and activation was first shown with the β_2–adrenergic receptor (Strader et al., 1989) and later with other catecholamine receptors including the D_{2L} (Mansour et al., 1992; Cox et al., 1992; Woodward et al., 1996; Shi and Javitch, 2002). Although the role of TM5 serines has been extensively studied, almost all prior work has focused on binding effects and possibly a single function. The current work was based on this foundation, but was directed at testing the hypothesis that such mutations, above and beyond any effects they might have on ligand affinity, could differentially affect one or more of several functions normally mediated by binding of ligands to the D_{2L} receptor. If the resulting data were consistent with this latter hypothesis, it would provide evidence that ligand binding induces ligand-specific states that are manifested as functional selectivity, as opposed to the idea that ligands select from a set of preexisting active states of the receptor.

The current experiments began by using a computationally refined three-dimensional molecular model of the hD_{2L} receptor to predict how the test ligands interacted with the serine residues that were the focus of this work. This model was based on extensive prior work that used both alanine and cysteine scanning mutagenesis in combination with homology based modeling(Javitch et al., 1995a; Javitch et al., 1995b; Shi and Javitch, 2002). As with all

aminergic GPCRs, binding of almost all ligands to the D_{2L} receptor requires the formation of a salt bridge interaction between the aspartate residue of TM3 (D3.32) and the amine group of the ligand(Shi and Javitch, 2002). The reason for selecting the NPA enantiomers as probe ligands were their relatively rigid backbones, and the fact that RNPA has traditionally been characterized as a full agonist at D_{2L} receptor stimulation, whereas SNPA has been characterized as a partial agonist (Kula et al., 1985; Campbell et al., 1986; Cox et al., 1988; Neumeyer et al., 1991) and more recently was shown to be functionally selective (Kilts et al., 2002; Gay et al., 2004).

The modeling data predicted that the asymmetric center of the NPA backbone (Figure 2.1) would give quite different binding poses of RNPA and SNPA at the D_{2L} receptor. The necessity of forming the salt bridge with D3.32 results in the p-OH (C10) of RNPA forming a H-bond with S5.46, whereas the p-OH of SNPA forms a H-bond with S5.42 (see Figure 2.1). The prototypical full agonist quinpirole and the endogenous ligand dopamine were used in this study as positive comparitors as neither compound exhibits functionally selective properties at D_{2L} endpoints in WT. Interestingly, rather than the hydroxyl moieties found in the endogenous monoaminergic agonists, the nitrogens in the quinoline ring of quinpirole function in a similar fashion so that the binding pose of this ligand involves similar aspects of the D_{2L} receptor. Binding data were consistent with the hypothesis that there is an interaction between the N2 atom of quinpirole and S5.46, and between N1 and S5.42. For dopamine itself, the data suggests H-bond interaction between the p-OH and S5.46, and the m-OH with both S5.42 and S5.46. Prior studies using the flexible and relatively low affinity ligands dopamine and p-tyramine gave results consistent with this hypothesis (Cox et al., 1992).

Serine residue S5.43 has been hypothesized to play a significant role in binding (Cox et al., 1992), and consistent with this, SCAM analysis provided evidence for solvent accessibility of S5.43 in the binding site crevice (Javitch et al., 1995a). Conversely, the molecular model we used predicted that S5.43 forms intrahelical H-bonds with TM5 such that the S5.43A mutation could destabilize the intrahelical H-bonds of TM5 compromising the global positioning of this helix. The S5.43A mutation did markedly decrease the affinity of all test ligands, a result that superficially could be interpreted as being due to direct effects on the binding of each ligand. On the other hand, our predicted binding poses do not support the interaction of all ligands with S5.43 (i.e. quinpirole and RNPA are hypothesized to interact deep in the pocket). The elimination of a single intrahelical H-bond could introduce a kink in the TM5 helix, altering the binding interactions of test ligands with the remaining TM5 serines (S5.42 and S5.46). The functional studies seem more consistent with this latter hypothesis.

Table 2.6. Qualitative summary of effects of mutations on probe ligands.

Assay	S5.42A				S5.43A				S5.46A			
	RNPA	SNPA	QP	DA	RNPA	SNPA	QP	DA	RNPA	SNPA	QP	DA
Binding	↓↓	↓↓	↔→↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓↓	→	→↓	→
GTPγS (EC50)	↓↓	→	↔	→	↔→↓	↔↑	↔↑	↔→	↓↓	→	↓↓	–
ACase (EC50)	↔	–	←	–	↔	↔	↑↑↑	←	↓↓	–	↔↑	–
MAPK (EC50)	→	–	↔	–	↔	↓↓	↔	↔→	↓↓	→	→	–
AA-rel. (EC50)	↓↓↓	–	↔→↓	–	↔↑	←	↔↑	–	↓↓	–	→	–
GTPγS (Emax)	↓↓	↔	↔	↓↓	→	←	↔	↔	→	←	↔	↓↓↓
ACase (Emax)	↔	↓↓↓	↔	↓↓↓	↔	←	↔	↔	↔	↓↓	↔	↓↓↓
MAPK (Emax)	↓↓	↓↓↓	→	↓↓↓	↔→↓	←	↔	↔↑	↓↓	↔↑	↔	↓↓↓
AA-rel. (Emax)	↔→↓	↓↓↓	↓↓	↓↓↓	→	↔	→	↓↓↓	→	↓↓	→↓	↓↓↓

←   *Positive effect (i.e., decreased $K_{0.5}$, or decreased EC50 or increased E_{max}).*
↔   *Minimal or no effect*
→   *Detrimental effect (i.e., increased $K_{0.5}$, or increased EC50 or decreased E_{max}).*
↓↔   *Trend downwards*
↔↑   *Trend upwards*
–   *Unable to be determined*
[Note ↓ = ~5-9 fold; ↓↓ = ~10-50 fold; ↓↓↓ = ~> 50 Fold

QP = quinpirole; DA = dopamine

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Despite the marked loss of affinity for all ligands with the S5.43A receptor, the effects on GTPγS binding were minmal, and in some cases (e.g., quinpirole and SNPA) the potency of ligands actually was higher with the mutant receptor. The data from the biochemical functions also show unexpected patterns. For example, despite the markedly lower affinity of quinpirole with S5.43A, its potency at inhibiting AC was greater. Globally, the S5.43A mutation caused relatively subtle changes for GTPγS binding, inhibition of AC, and MAPK stimulation despite the marked effects on binding. Conversely, the S5.43A mutation caused DA to be completely inactive at AA release, with RNPA having somewhat reduced intrinsic activity. This very unusual pattern of effect of the S5.43A mutation seems consistent with our modeling-derived hypothesis that this mutation decreased stability in TM5 helix, and therefore might have altered the spatial orientation of S5.42 via loss of intrahelical H-bonds to S5.43.

As opposed to the results with S5.43, the current data are consistent with the prior hypotheses that S5.42 and S5.46 both interact directly with the probe ligands that were used in this study. In general, the S5.42A mutation not only caused marked loss of affinity, but consistent with the hypothesized role of S5.42 in interacting with these ligands, losses of function (decreased potency and/or decreased E_{max}) were also the most common effect that occurred (Table 2.6). Both mutations tended to cause decreases in potency and intrinsic activity of ligand-induced [^{35}S]-GTPγS binding. All of the ligands but RNPA and QP were incapable of inhibiting forskolin-stimulated cAMP accumulation with the S5.42A receptor. The latter was somewhat unexpected as Cox et al. (1992) reported that in this mutant receptor dopamine mediated inhibition of forskolin-stimulated cAMP accumulation, albeit with significantly decreased potency. Because Cox et al. (1992), studied S5.42A in hD$_{2S}$ the

splice variant, and also used a different cell line (C-6 glioma), it may well be that the data are not irreconcilable. Interestingly, S5.42A resulted in complete loss of function for dopamine and SNPA, yet RNPA and quinpirole maintained signaling with all functional endpoints measured, though with decreased potency (both compounds) and decreased intrinsic activity (RNPA at MAPK only) relative to WT. These data demonstrate that this mutation caused both RNPA and quinpirole to become functionally selective. Essentially, mutation of S5.42 created an artificial receptor whereby activation of some effector pathways are minimally affected, whereas others are abolished. Such data are difficult to reconcile with the notion of rigid receptor active states, but seem to support the hypothesis that each agonist induces a unique set of receptor conformations that can cause differential effects on downstream second messenger pathways.

S5.46 has been reported to have a significant role in binding (Mansour et al., 1992; Cox et al., 1992; Woodward et al., 1996) and function (Cox et al., 1992) of the D_2 receptor, and our data are consistent with this view. Similar to S5.42A, RNPA and quinpirole (but not SNPA or dopamine) fully inhibit forskolin-stimulated cAMP accumulation with S5.46A. S5.46A resulted, however, in differential effects at other D_{2L} mediated pathways. RNPA and quinpirole both stimulate D_{2L} mediated MAPK activation and [^{3}H]-AA release, though with decreased potency (both compounds) and decreased intrinsic activity (RNPA at MAPK). Conversely, dopamine is inactive at both of these functions, whereas SNPA activates MAPK (significantly decreased potency) but does not stimulate D_{2L} mediated [^{3}H]-AA release. This is again evidence that some mutations can create artificial receptors in which the functional profile is modified differentially.

The pattern in our data clearly suggests that each of the signaling pathways we assessed was largely independent of each other, yet the approach we used did not address the many possible mechanisms of how differential activation occurs with the WT or mutant receptors. One such mechanism would be coupling of a different Gα (non Gα$_{i/o}$) with βγ dimers that favor (or not) activation of MAPK and AA-release. Previous studies support βγ mediated activation of MAPK (Choi et al., 1999) through hD$_{2L}$ and AA-release through 5-HT$_{2A}$, bovine rod outer segments, and NPY Y$_1$ (Jelsema and Axelrod, 1987; Selbie et al., 1997; Kurrasch-Orbaugh et al., 2003). The CHO cells used in this work contain three inhibitory Gα subunits (Gerhardt and Neubig, 1991), three Gβ subunits, and ten Gγ subunits (unpublished results) that could contribute to the observed results. Although one major hypothesis is that βγ could significantly affect the differential signaling observed with mutants, we cannot rule out other possible scenarios. Scaffolding proteins (Smith et al., 1999) could be affected by receptor mutants, thereby modifying G protein coupling . D$_{2L}$ is also known to form heterodimers (Lee et al., 2003a; Kearn et al., 2005; O'Dowd et al., 2005) and homodimers (Guo et al., 2003; Lee et al., 2003a; Lee et al., 2003b; Guo et al., 2005) whose relationship and function could be modified by receptor mutation. Together, this makes clear the impact of cellular background on ligand-induced functional changes, even when looking at WT receptors.

In summary, the current data demonstrate clearly that one can change the relative pattern of functional activity of ligands either by modifying the receptor at residues directly involved in ligand binding, or at sites that affect the overall structure of the receptor. A clear example was the effects of the S5.43A. This mutation resulted in a receptor in which typical ligands (including the endogenous neurotransmitter) became functionally selective. The

S5.43A mutation did not affect function at AC or MAPK, but markedly altered select ligand actions at AA-release. These findings have several important consequences. From a terminology point of view, the use of the terms agonist, partial agonist, antagonist, or inverse agonist must clearly be recognized as operational definitions, referring to ligand action in a single system. Although the characteristics of a ligand may well be similar when the receptor is expressed in different cells or even cellular locales, the many exceptions require more restricted use of these terms. It seems to us that the functional effects of the ligand will be dependent on not only the target receptor, but also the immediate signaling environment of the receptor, including all directly associated molecules (G proteins, scaffolding molecules, other regulatory proteins, and the lipid environment). The growing list of examples of functional selectivity of native receptors (Urban et al., 2006a) underscore this complexity. The current study now demonstrates for the first time that not only can some ligands cause functionally selective activation, but also that receptor re-design can result in altered functional profiles.

It is important to weigh these data against traditional receptor modeling in which a ligand causes trafficking of receptors to one of several discrete receptor states (Leff, 1995; Kenakin, 1995b; Kenakin, 1997; Leff et al., 1997). Models that involve finite numbers of discrete active states do not seem capable of accounting for the complexity of current observations, even when "energy landscapes" are created from these discrete states (Kenakin, 1997). We favor the view that these findings are a result of the fact that each ligand induces a unique set of conformational states of its target receptor. Testing of this hypothesis and understanding the involved partners are obvious future directions.

CHAPTER 3. FUNCTIONALLY SELECTIVE SIGNALING OF NOVEL RIGID LIGANDS IS MODIFIED AND INDUCED BY MUTATIONS OF TM5 SERINE RESIDUES OF THE D_{2L} DOPAMINE RECEPTOR

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PREFACE

In the previous Chapter, I demonstrated for the first time that functional selectivity could be induced by mutations of the D_{2L} receptor that either directly affected ligand binding, or altered receptor structure. The probe ligands that were used in the Chapter 2 were chosen because they were well-studied prototypical D_{2L} ligands whose functional properties and structural features made them of particular utility. The current Chapter now extends that work into a series of rigid ligands originally developed as D_1 agonists, but later found to have both D_2 affinity as well as D_2 functional selectivity both *in vitro* and *in vivo*. The structural features of these ligands suggested they would be very useful in testing the hypotheses that were tested in the prior Chapter.

ABSTRACT

We have previously shown that mutation of TM5 serine residues of the dopamine D_{2L} receptor can cause typical agonists to become functionally selective. The current experiments focus on three rigid dopamine agonists (dihydrexidine, dinapsoline, and dinoxyline) that share common catecholamine pharmacophoric elements, but have different functional properties at the WT D_{2L} receptor. Hypotheses were formed by docking of the probe and comparator (quinpirole and dopamine) ligands into the hD_{2L} active site, and tested by binding and functional [adenylate cyclase inhibition (AC), MAPK phosphorylation (MAPK), and AA-release (AA)] analyses of point mutated receptors. Binding data were consistent with the following hypotheses: 1) the p-OH of dihydrexidine (C10) and dinapsoline (C9) interacts with S5.46; 2) the m-OH of dinapsoline (C8) H-bonds with both S5.42 and S5.46, whereas the m-OH of dihydrexidine (C11) interacts only with S5.42; 3) dinoxyline (differing from dinapsoline only by an ether-methylene substitution) forms H-bonds with m-OH and both S5.42 and S5.46, and the ether oxygen with S5.42; 4) dopamine forms H-bonds between p-OH and S5.46 and m-OH with both S5.42 and S5.46; and 5) quinpirole forms H-bonds between its N2 atom and S5.46, and between N1 and S5.42. Functionally, S5.42 and S5.46A mutations abolished all functional activity of dopamine, dinapsoline, and dinoxyline, but not quinpirole. Dihydrexidine retained MAPK function but only with S5.42A. Interestingly, S5.43A caused a loss of AA function with DNS, DHX, and DA, but did not markedly affect other functions. The induction of functional selectivity by receptor mutations support the hypothesis that ligand-specific conformational changes, rather than trafficking between active states, is the underlying mechanism of this phenomenon.

INTRODUCTION

As was summarized in the previous chapter, studies with the β_2-adrenergic receptor (Strader et al., 1989), and later with the D_2 receptor (Mansour et al., 1992; Cox et al., 1992; Woodward et al., 1996; Wiens et al., 1998), elucidated many aspects of the role for TM 5 serines in interaction with ligands (Shi and Javitch, 2002), work that formed the basis for Chapter 2. One of the foundations of Chapter 2, as well as the current study, is that molecules with relatively rigid, conformationally restrained structures can be very useful tools in studying a complex phenomenon such as functional selectivity. Such rigid ligands may decrease the degrees of uncertainty that occur when smaller more flexible ligands are the primary probes (Cox et al., 1992). Thus, in the prior Chapter, I used the enantiomers of propylnorapomorphine (RNPA & SNPA) because these compounds have a large and relatively rigid backbone as well as pharmacological properties justifying their study (Neumeyer et al., 1983; Kula et al., 1985; Campbell et al., 1986; Neumeyer et al., 1988; Cox et al., 1988; Neumeyer et al., 1991; 2002).

The current experiments focus on three rigid analogs, dinapsoline [(+)8,9-dihydroxy-2,3,7,11b-tetrahydro-1H-napth[1,2,3-*de*]isoquinoline], dinoxyline [(+)8,9-dihydroxy-1,2,3,11b-tetrahydrochromeno[4,3,2,-de]isoquinoline], and dihydrexidine [trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine]. Dihydrexidine (DHX) originally was designed to be a novel D_1 agonist in which the accessory phenyl ring of needed to give D_1 affinity (Charifson et al., 1989; Mottola et al., 1996) was tethered to the elements of dopamine locked in the β-rotomer form (Nichols, 1983). This pharmacophore (Mottola et al., 1996) ultimately led to the D_1 agonist, dinapsoline

(DNS; Ghosh et al., 1996), and subsequently dinoxyline, another high affinity D_1 ligand (DNX; Grubbs et al., 2004).

Although DHX was designed as a high affinity full dopamine D_1 agonist, it was soon found also to have D_2 affinity similar to the prototypical agonist quinpirole (Lovenberg et al., 1989; Brewster et al., 1990). More surprisingly was the unexpected D_2 functional profile of DHX (Mottola et al., 1992; Mottola et al., 2002). Like quinpirole, DHX exhibited full agonist activity at inhibition of forskolin-stimulated cAMP accumulation in rat striatum or various cell lines, and inhibited prolactin secretion *in vivo*. On the other hand, DHX failed to affect D_2 mediated release or synthesis of dopamine, or firing of nigral dopamine neurons (all functions known to be modulated by D_2 receptors), and actually had antagonistic effects on the actions of apomorphine in the latter assay (Mottola et al., 2002). Consistent with this, studies in isolated cells showed that DHX had full agonist activity at inhibition of forskolin-stimulated cAMP accumulation, yet little or no intrinsic activity at several other functions (e.g., K^+-stimulated dopamine release or G protein inward-rectifying potassium channels (GIRKs) (Kilts et al., 2002), and antagonized quinpirole and dopamine in such systems (Kilts et al., 2002). This was possibly the first direct experimental evidence for functional selectivity (Mottola et al., 1991), and certainly the first with dopamine receptors. Most importantly for the current work, it provided a relatively rigid molecule likely to have restricted numbers of docking poses that could be a useful tool for mechanistic studies of functional selectivity.

Full characterization of both DNS and DNX has been conducted in heterologous expression systems. In Chinese hamster ovary (CHO) cells expressing hD_{2L} receptor

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(Gay et al., 2004), DNS had dopamine agonist activity [i.e., full inhibiting cAMP accumulation (AC) and fully stimulating p44/p42 MAP kinase phosphorylation (MAPK)], yet also had dopamine partial agonist activity [weakly stimulating G protein-coupled inward rectifying potassium channels [GIRK)]. These studies (Gay et al., 2004) also provided evidence that DNX had full intrinsic activity at all three effector pathways (AC, MAPK, and GIRK). At a fourth functional pathway [receptor-mediated release of [^{3}H]-arachidonic acid (AA)], DNS exhibited partial agonist activity, whereas DNX had full intrinsic activity (*vide infra*). These data are interesting because there are only subtle differences in the predicted three-dimensional structure of DNS and DNX. Together, these data suggest the hypothesis that ligand-specific interactions are responsible for the observed functionally selective activation of pathways.

The current experiments address this hypothesis by studying the role of specific binding interactions that occur between the D$_{2L}$ receptor and DNS, DNX, and DHX. Computational modeling was utilized to hypothesize specific binding interactions between each ligand-receptor complex. We used an iterative process of modeling and experimental validation, with predicted residues being mutated to alanine, and binding studies and functional studies (cAMP inhibition, MAPK phosphorylation, and [^{3}H]-AA release) used to assess each predicted ligand-residue interaction. The resulting data are consistent with the hypothesis that ligand-specific interactions with certain residues of the receptor may induce conformational changes that lead to a characteristic pattern of activation of one or more signaling pathways.

RESULTS

Molecular modeling predictions

DHX, DNS, DNX, dopamine, and quinpirole were all docked to the hD_{2L} receptor (see Figure 3.1). The DHX-hD_{2L} docking predicted interaction of the ligand para-OH (C10) with S5.46, and the ligand meta-OH (C11) with S5.42. Simlarly, the para-OH of DNS (C9) was predicted to H-bond with S5.46, yet its meta-OH (C8) accepts a proton from both S5.42 and S5.46. In contrast, with DNX we predicted that the meta-OH of DNX H-bonds to both S5.42 and S5.46, whereas the ether bridge of DNX H-bonds to S5.42. Docking studies conducted with dopamine indicate the involvement of the ligand para-OH with S5.46, whereas the meta-OH is involved with H-bonding to S5.42. Docking studies conducted with quinpirole predict that the N2 atom donates a proton to the hydroxyl group of the S5.46 side chain, whereas the N1 of quinpirole is hypothesized to hydrogen bond with S5.42.

Effect of S5.42A, S5.43A, and S5.46A on receptor expression and antagonist radioligand binding

The hD_{2L} WT, S5.42A, S5.43A, and S5.46A receptors were expressed stably in CHO K1 cells, and saturation radioreceptor assays with [^{3}H]-N-methylspiperone were used to characterize both receptors. As previously reported (Chapter 2), the WT receptor was expressed at 4.8 pmol/mg protein with a dissociation constant (K_D) of 0.58 nM (Figure 3.2 and Table 3.1). The S5.42A (S193A) mutant receptor was expressed at 4.0 pmol/mg protein with a K_D of 0.59 nM. The S5.43A (S194A) mutant receptor was expressed at a much higher density (20.7 pmol/mg protein), but with a somewhat

decreased affinity (K_D = 1.4 nM). The S5.46A (S197A) mutant receptor was expressed at

2.3 pmol/mg protein with a K_D of 0.5 nM.
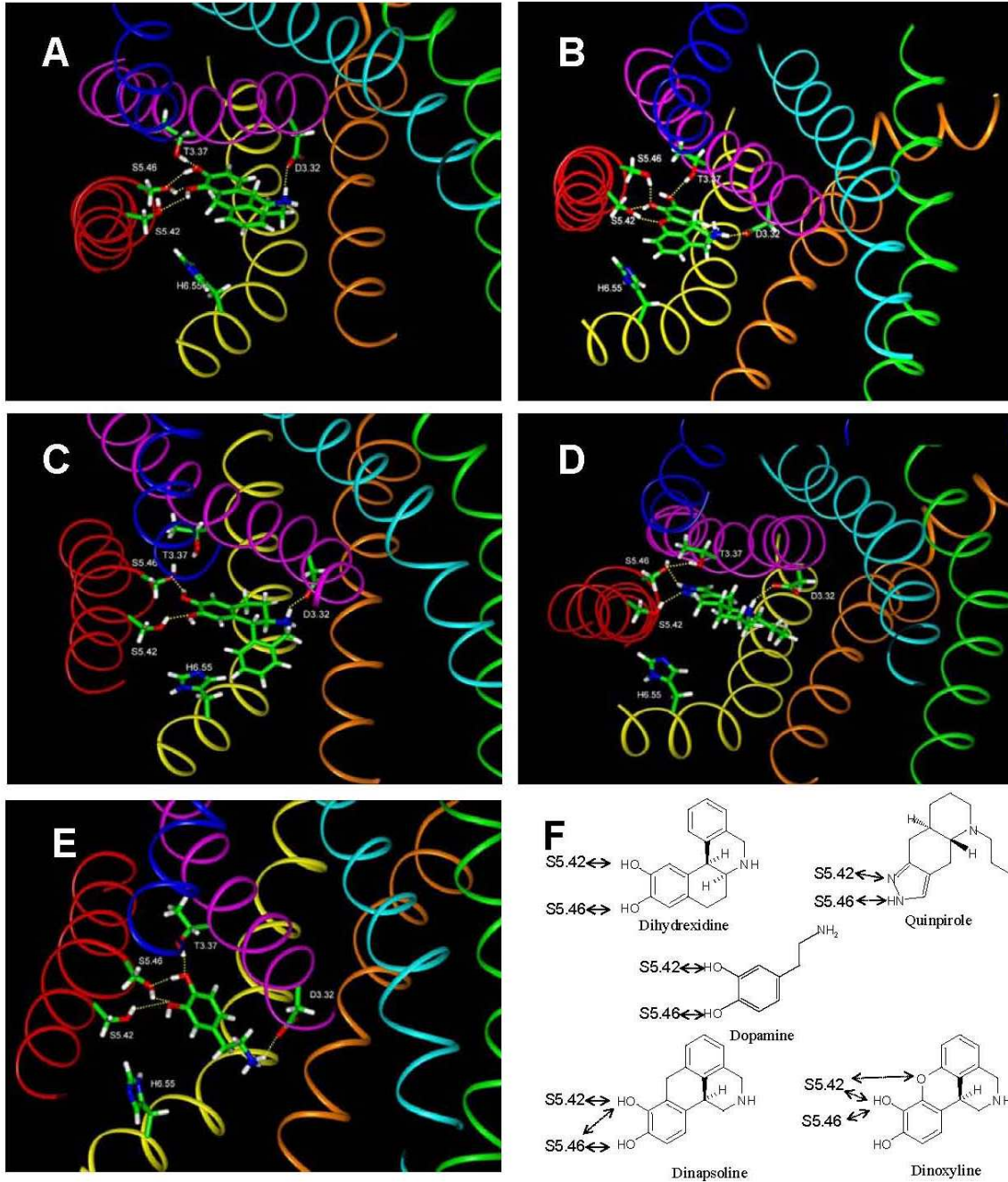
Figure 3.1. Three-dimensional molecular models. *3D molecular models of ligands docked to hD_{2L}. View is from the extracellular surface, looking down into the receptor. A) DNS, B) DNX, C) DHX, D) quinpirole, E) dopamine, F) agonist structures*
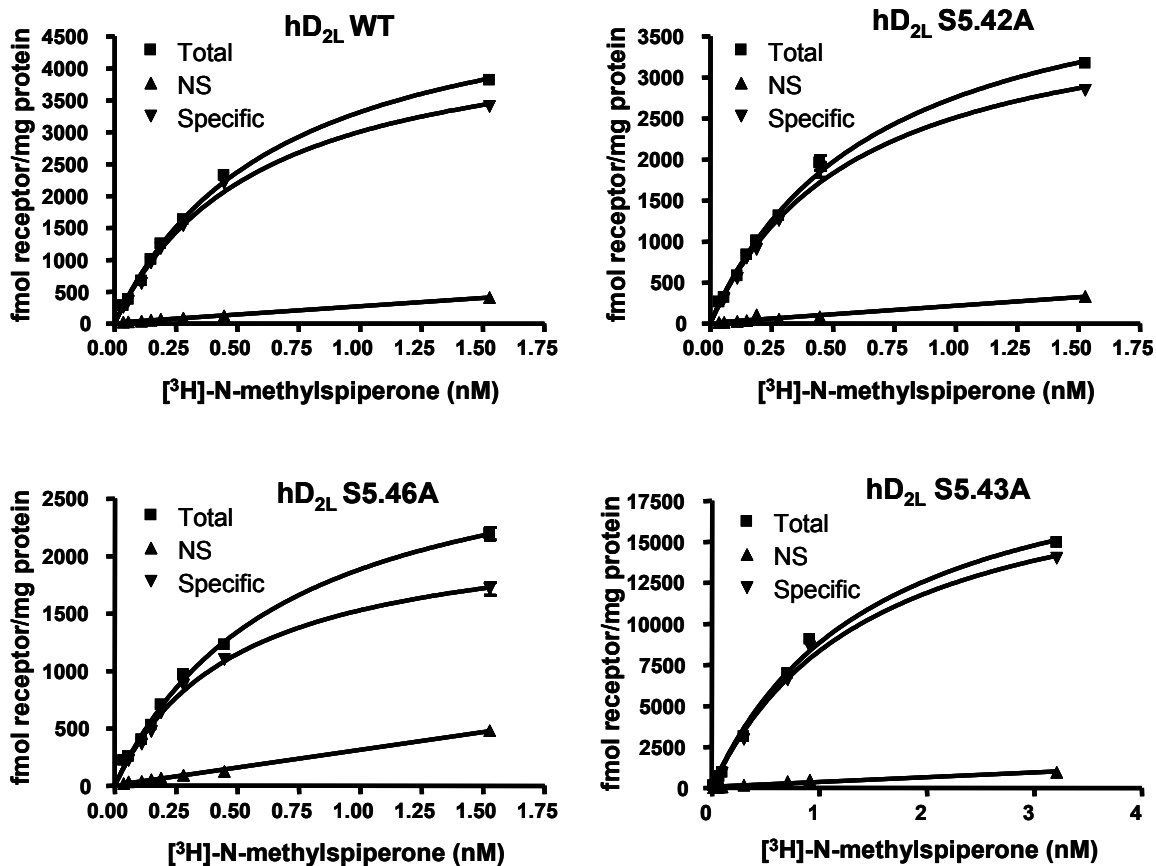
Figure 3.2. Saturation binding for stably expressing mutant and WT hD2L receptors. *CHO hD2L membrane fragments were incubated with increasing concentrations of [3H]-N-methylspiperone for 15 min. at 37 °C. Data shown is representative of 3-4 independent experiments conducted in triplicate.*

Effect of S5.42A, S5.43A, and S5.46A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus [3H]-N-methylspiperone in membranes from both WT and mutant receptors. An apparent affinity constant, $K_{0.5}$ (Table 3.1) was determined from experimental IC50 values corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). Competition binding studies also were conducted with several structurally different antagonists to rule out gross structural changes induced by the receptor. Although all of the antagonists had slightly decreased affinity (as found for N-methylspiperone), their rank order and relative

affinity was unchanged, suggesting that no major changes to overall receptor structure were induced by these mutations.
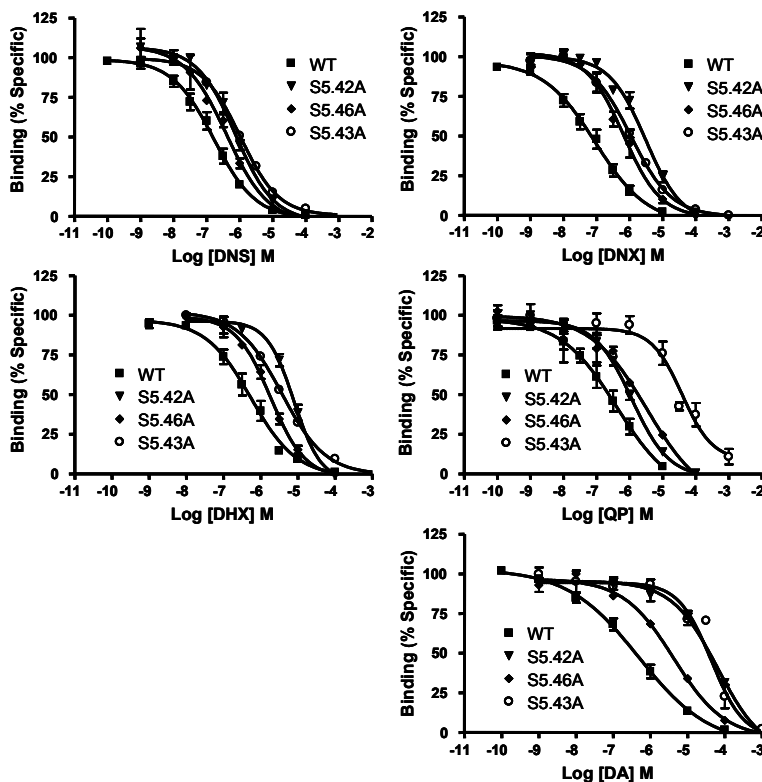
Figure 3.3. Competition binding of test compounds with hD$_{2L}$ WT and mutant receptors. *Membrane fragments were incubated with [^{3}H]-N-methylspiperone at K_D for 15 min. with varying concentrations of test compounds. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC50s, reported as corrected affinity values ($K_{0.5}$) using Prism 4.0. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Representative data for each agonist probe ligand at each receptor are shown in Figure 3.3 and summarized in Table 3.1. The differential effects that these mutations had on the rigid probe ligands are striking. For example, the S5.42A and S5.46A mutations caused a much greater loss of affinity to DNX than to the structurally similar DNS. These differential changes can be seen by comparing the rank orders of affinity: *WT*: DNX > DNS > quinpirole = dopamine = DHX; *S5.42A*: DNS > quinpirole >> DNX >> DHX >>> dopamine; *S5.43A*: DNS = DNX > DHX >> quinpirole = dopamine; and *S5.46A*: DNS > DNX > DHX >> quinpirole = dopamine.

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Table 3.1. Saturation and competition binding data for rigid analogs at serine mutants.

Mutant	WT	S5.42A	S5.43A	S5.46A
Saturation Binding				
K_D (nM)	0.58	0.59	1.4	0.5
B_{max} (pmol/mg)	4.8	4	20.7	2.3
Competition Binding				
DNS	144 ± 17	660 ± 190	750 ± 370	330 ± 160
DNX	83 ± 5	2,700± 990	770 ± 90	580 ± 340
DHX	490 ± 91	7,400 ± 1000	2,600 ± 50	1600 ± 40
quinpirole	365 ± 66	1,040 ± 100	27,000 ± 10,000	2,900 ± 820
dopamine	450 ± 170	80,000 ± 3,000	31,000 ± 6,000	3,500 ± 1,500

Effect of mutations on ligand-induced GTPγS binding

An estimate of G protein turnover was conducted using non-hydrolyzable [^{35}S]-GTPγS (see Figure 3.4 and Table 3.2). The S5.42A mutant decreased DHX potency three-fold (EC50 = 1,300 nM) and intrinsic activity, whereas S5.46A decreased potency eighteen-fold (EC50 = 6,600 nM) relative to WT (EC50 = 373 nM). Conversely, S5.43A increased potency for DHX four-fold (EC50 = 92 nM). Both S5.42A and S5.46A abolished DNS stimulated [^{35}S]-GTPγS, but S5.43A (EC50 = 135 nM) exhibited intrinsic activity greater than WT (EC50 = 286 nM). S5.46A decreased intrinsic activity for DNX (EC50 = 660 nM) compared to WT (550 ± 31 nM), whereas activity was lost with S5.42A. Conversely, S5.43A exhibited an eight-fold increased potency for DNX (EC50 = 69 nM) with intrinsic activity similar to WT. S5.42A and S5.43A minimally affected quinpirole (EC50 = 1,700 nM and 203 nM), whereas S5.46A caused decreased potency

(EC50 = 7,200 nM) compared to WT (EC50 = 735 nM). Dopamine had six- and three-fold decreased potency for S5.42A and S5.43A (EC50 = 10,700 nM and 4,300 nM respectively) versus WT (EC50 = 1,700 nM), whereas activity was nil at S5.46A. These data suggest that specific ligand-residue interactions necessary for binding are not necessarily critical for G protein coupling (e.g. dopamine at S5.43A). A lack of correlation between binding and G protein coupling leads to the hypothesis that activation of downstream second messengers with receptor mutants would also be affected differentially.
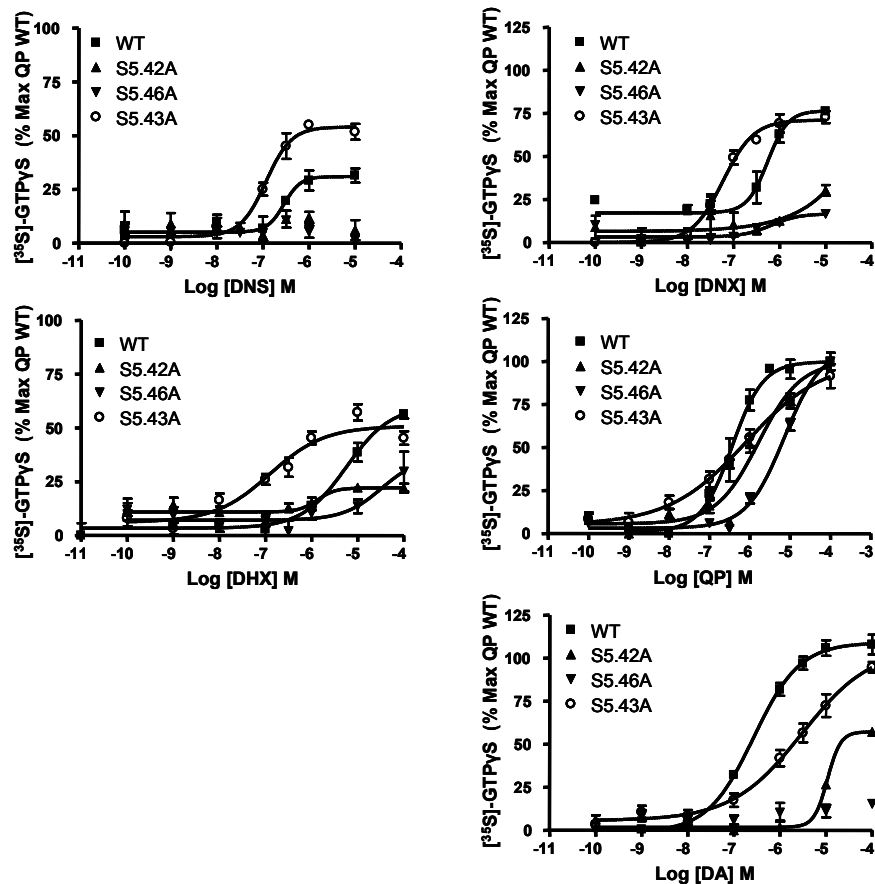
Figure 3.4. GTPγS turnover experiments of test compounds with hD$_{2L}$ WT and mutant receptors. *Membrane fragments were incubated for 15 min. with varying concentrations of test compounds until reaching equilibrium. 0.2 nM [^{35}S]-GTPγS was then added for 30 min. and stimulation was measured. Analysis was conducted using non-linear regression and a sigmoidal equation (Prism 4.0) to determine EC50s reported below in Table 2-2. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 3.2. Potency of probe ligands in affecting GTPγS binding**.**

Mutant	DNS (nM)	DNX (nM)	DHX (nM)	Quinpirole (nM)	Dopamine (nM)
WT	290 ± 70	550 ± 30	370 ± 180	740 ± 120	1,700 ± 1,040
S5.42A	--	--	1,300 ± 410	1,700 ± 860	10,700 ± 1,500
S5.43A	135 ± 25	69 ± 17	92 ± 28	203 ± 5	4,300 ± 1600
S5.46A	--	660 ± 250	6,600 ± 6200	7,200 ± 1900	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Functional differences caused by S5.42A

The hD_{2L} receptor couples to $G\alpha_{i/o}$ subunits intracellularly, inhibiting the adenylate cyclase (AC) mediated conversion of ATP to cAMP. Measurement of agonist inhibition of forskolin-stimulated cAMP accumulation in a whole cell assay system for the WT and S5.42A receptors was conducted (see Figure 3.5-3.8, and Table 3.3). In the WT receptor, all dopamine agonists (DNS, DNX, DHX, quinpirole, and dopamine) robustly inhibited forskolin-stimulated cAMP accumulation. Conversely, inhibition of cAMP accumulation with S5.42A mutant was abolished with all ligands tested except QP. The D_2 antagonist domperidone (10 μM) was tested against WT and all receptor mutants. Loss of function was exhibited at all endpoints tested. Domperidone also blocked quinpirole activation of all effector endpoints with WT and mutant receptors. All of these compounds were tested in non-transfected (CHO K1) cells and were devoid of activity. Together, these data suggest that S5.42A is critical for ligand-receptor mediated conformational changes associated with inhibitory actions of the D_{2L} receptor at AC.

Measurement of a second pathway, agonist stimulation and subsequent phosphorylation of the p44/p42 MAP kinase, then was conducted in a whole cell assay system for the WT and S5.42A receptors (see Figures 3.5-3.8, and Table 3.3). In the WT receptor, all dopamine agonists (DNS, DNX, DHX, quinpirole, and dopamine) fully activated MAPK. S5.42A only minimally affected the actions of quinpirole (EC50 = 44 nM) compared to WT (EC50 = 23 nM). Conversely, the S5.42A mutation had greater effects on DHX (EC50 = 69 nM in mutant versus 213 nM in WT), and caused complete loss of function with all other ligands.

Measurement of a third pathway, agonist stimulated [^{3}H]-arachidonic acid release, was conducted in a whole cell assay system for WT and S5.42A (see Figure 3.5-3.8, and Table 3.3). The potency of quinpirole was decreased by the S5.42A mutation (EC50 = 223 nM vs 63 nM in WT), but intrinsic activity was unaffected. Conversely, all of the other ligands were inactive with the S5.42A receptor (see Table 3.3). The MAPK and AA functional data provide strong evidence that functional effects caused by the S5.42A mutation are ligand dependent, and do not represent a generalized crippling of the receptor.

Functional differences caused by S5.43A

The hypothesis that S5.43A is critical for forming intrahelical H-bonds that stabilize the TM5 alpha helix was tested using a functional profiling as detailed above. (see Figure 3.5-3.8, and Table 3.3). As previously reported for WT receptor (Chapter 2), all of these ligands (DNS, DNX, DHX, quinpirole, and dopamine) fully inhibited cAMP accumulation (AC). We hypothesized that mutation of S5.43A would exhibit minimal affect on intrinsic activity at all endpoints measured with our test ligands. In support of

that hypothesis, S5.43A had no affect on AC inhibition versus WT, although changes in rank order of potency were observed [WT: DNX > quinpirole > dopamine > DHX > DNS; S5.43A: quinpirole > dopamine > DNS > DNX > DHX (see Table 3.3 for EC50s)].

The MAPK data are shown in Figures 3.5-3.8, and Table 3.3). All of the test ligands (DNS, DNX, DHX, quinpirole, and dopamine) fully activated MAPK in both WT and the S5.43A mutant, although changes in rank order of potency were observed [WT: dopamine = quinpirole = DNX > DNS > DHX vs. S5.43: quinpirole > dopamine > DNS > DHX > DNX (see Table 3.3 for EC50s)]. The S5.43A mutation had dramatic effects on agonist stimulated [^{3}H]-arachidonic acid release (see Figures 3.5-3.8, and Table 3.3). With the mutant receptor, QP and DNX were minimally affected, but activity of the other ligands was lost. Of note, the structurally similar ligands DNS and DNX were affected oppositely with S5.43A. These data indicate that the effects of the S5.43 mutation were seen primarily on one function (AA), but not on two others (AC, MAPK) at which the full agonist activity at the WT receptor was maintained.

Functional differences caused by S5.46A

The last residue to be studied in these experiments was S5.46 (see Figures 3.6-3.8, and Table 3.3). The third serine of the critical catecholamine TM5 residues is located deepest in the binding pocket of hD$_{2L}$. With the WT receptor, all dopamine agonists (DNS, DNX, DHX, quinpirole, and dopamine) robustly inhibited forskolin-stimulated cAMP accumulation. Conversely, with S5.46A, whereas the actions of quinpirole were unaffected at AC, none of the other ligands had activity. In a similar fashion, at both MAPK the activity of DNS, DNX, DHX, and dopamine were absent with the S5.46A

mutant. Conversely, quinpirole maintained its intrinsic activity, but had modest loss of potency (5-8-fold) at both functions.
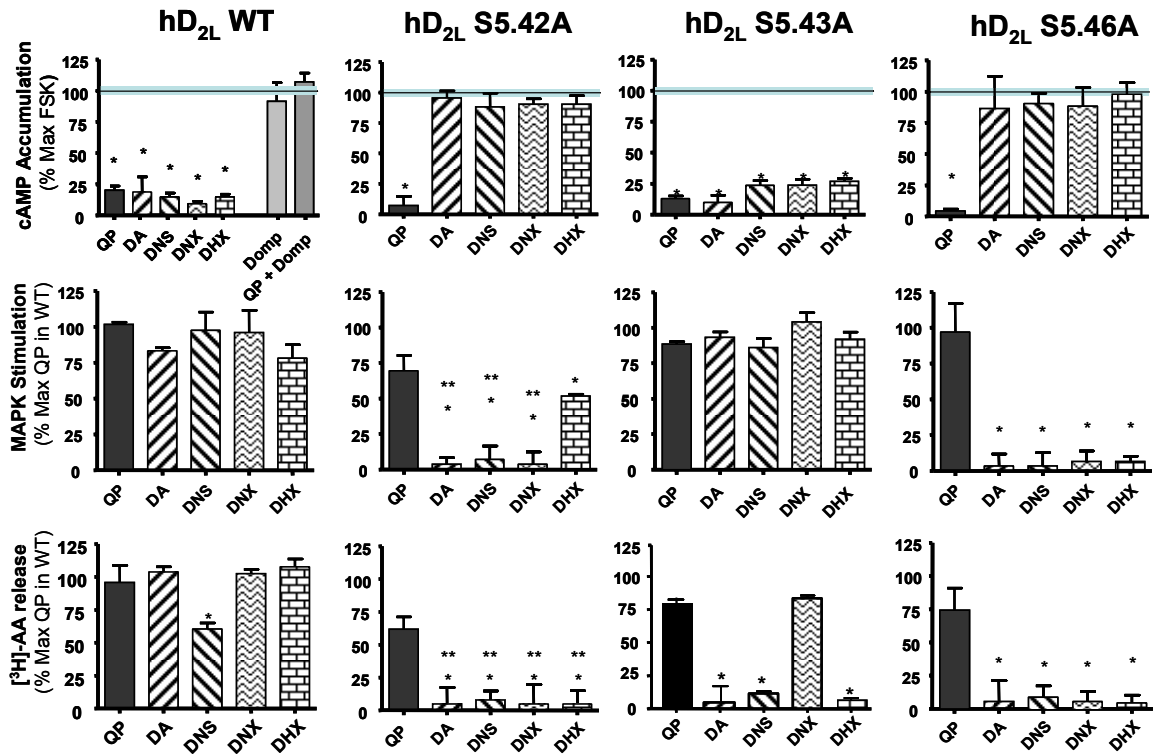
*Figure 3.5. Ligand effects on second messenger pathways of hD$_{2L}$ with mutations at S5.42A, S5.43A, and S5.46A. Top row: Ligand-mediated inhibition of forskolin-stimulated cAMP accumulation (AC); Middle row: Ligand-mediated activation of p44/p42 MAP Kinase (MAPK); Bottom row: Ligand-mediated potentiation of [^{3}H]-arachidonic acid release. Note that agonists inhibit AC, but stimulate MAPK and AA release Data are representative of E$_{max}$ values for 3-5 independent experiments conducted in triplicate. * $p < 0.05$ (One way ANOVA, post hoc Dunnet's). ** $p < 0.05$ (Kruskal-Wallace one way ANOVA, post hoc Dunn's)..*

Table 3.3. Effect of S5.42A mutation on functional potencies.

Ligand	DNS [EC50 (nM)]		DNX [EC50 (nM)]		DHX [EC50 (nM)]		Quinpirole [EC50 (nM)]		Dopamine [EC50 (nM)]	
Function	WT	S5.42A	WT	S5.42A	WT	S5.42A	WT	S5.42A	WT	S5.42A
AC	193 ± 63	--	5.6 ± 0.3	--	93 ± 10	--	172 ± 5.5	7.8 ± 1.4	157 ± 22	--
MAPK	72 ± 68	--	27 ± 17	--	213 ± 105	69 ± 6.1	26 ± 5	44 ± 12	28 ± 7	--
AA-release	340 ± 180	--	101 ± 12	--	395 ± 119	--	63 ± 12	223 ± 67	49 ± 26	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Table 3.4. Effect of S5.43A mutation on functional potencies.

Ligand	DNS [EC50 (nM)]		DNX [EC50 (nM)]		DHX [EC50 (nM)]		Quinpirole [EC50 (nM)]		Dopamine [EC50 (nM)]	
Function	WT	S5.43A	WT	S5.43A	WT	S5.43A	WT	S5.43A	WT	S5.43A
AC	193 ± 63	119 ± 48	5.6 ± 0.3	36 ± 17	93 ± 10	125 ± 28	172 ± 6	3 ± 1	157 ± 22	19 ± 3
MAPK	72 ± 68	155 ± 9	27 ± 17	$2,100 \pm 1,300$	213 ± 105	400 ± 130	26 ± 5	26 ± 11	28 ± 7	114 ± 40
AA-release	340 ± 180	--	101 ± 12	40 ± 18	390 ± 120	--	63 ± 12	35 ± 27	49 ± 26	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Table 3.5. Effect of S5.46A mutation on functional potencies.

Ligand	DNS [EC50 (nM)]		DNX [EC50 (nM)]		DHX [EC50 (nM)]		Quinpirole [EC50 (nM)]		dopamine [EC50 (nM)]	
Function	WT	S5.46A	WT	S5.46A	WT	S5.46A	WT	S5.46A	WT	S5.46A
AC	193 ± 63	--	5.6 ± 0.3	--	93 ± 10	--	172 ± 6	54 ± 11	157 ± 22	--
MAPK	72 ± 68	--	27 ± 17	--	213 ± 105	--	26 ± 5	193 ± 28	28 ± 7	--
AA-release	345 ± 177	--	101 ± 12	--	395 ± 119	--	63 ± 12	292 ± 16	49 ± 26	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.
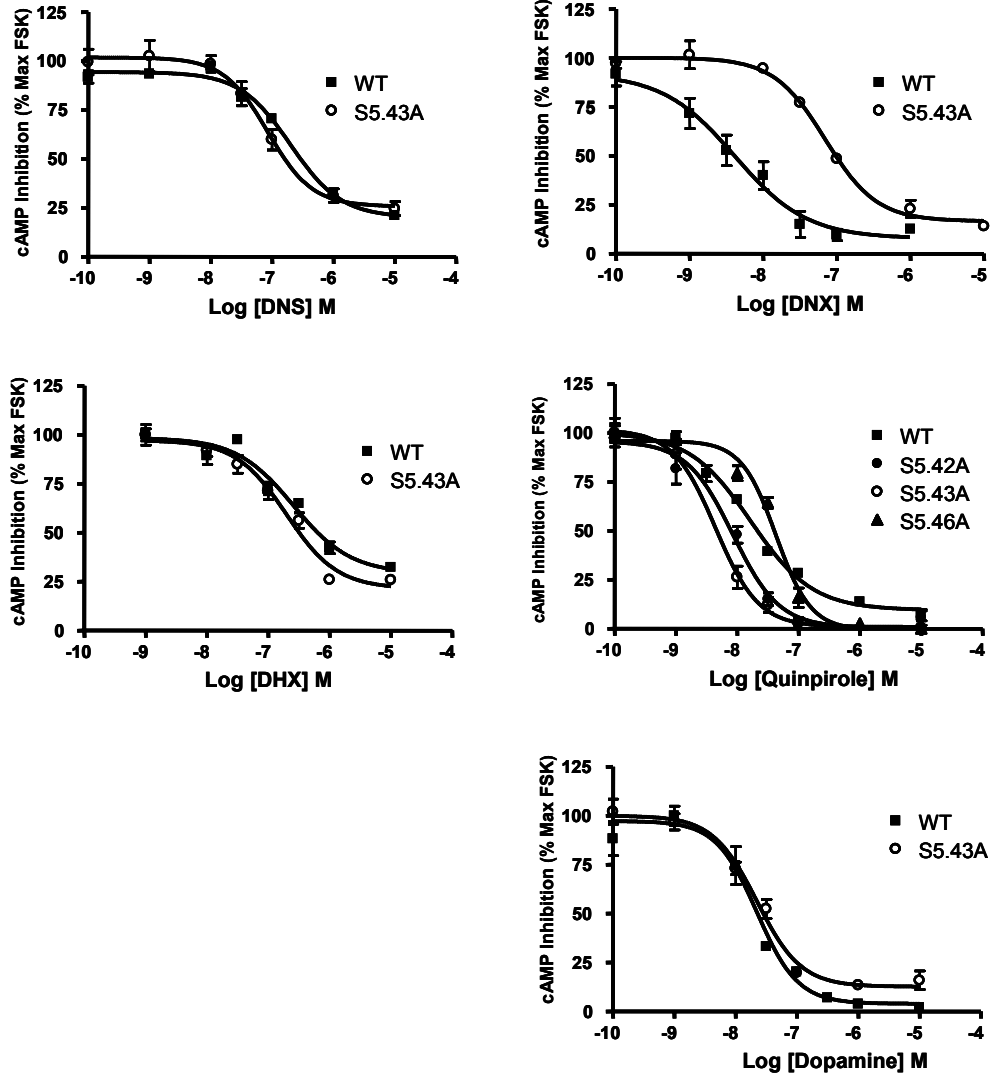
Figure 3.6. Dose response activation of cAMP with ligands at S5.42A, S5.43A, and S5.46A. *Agonist mediated inhibition of forskolin-stimulated cAMP accumulation. Data are representative of 3-5 independent experiments conducted in triplicate.*
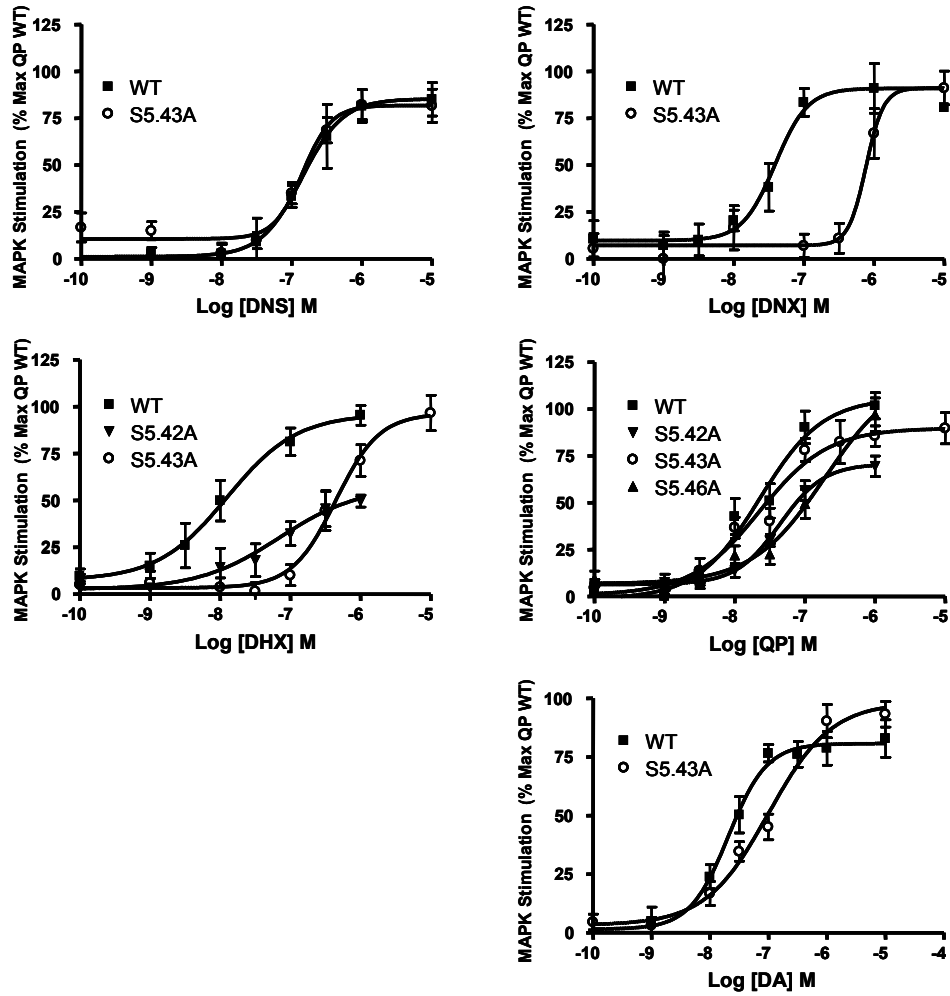
Figure 3.7. Dose response activation of MAPK with ligands at S5.42A, S5.43A, and S5.46A. *Agonist mediated phosphorylation of p44/p42 MAPK. Data are representative of 3-5 independent experiments conducted in triplicate.*
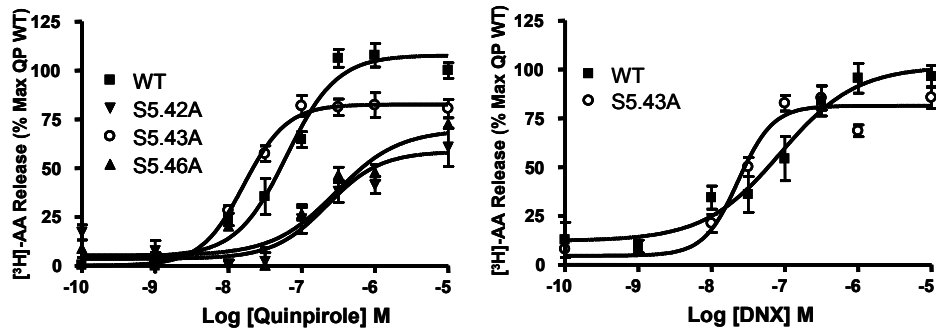
Figure 3.8. Dose response activation of AA-release with ligands at S5.42A, S5.43A, and S5.46A. *Agonist mediated potentiation of [^{3}H]AA-release. Data are representative of 3-5 independent experiments conducted in triplicate.*

DISCUSSION

Functional selectivity is defined as the ability of a ligand to activate differentially, individual receptor pathways linked to a single receptor isoform. Evidence to support this concept has surfaced not only in the GPCR field (Kenakin, 1995a; Kenakin, 1995b; Berg et al., 1998; Whistler et al., 1999; Watson et al., 2000; Mottola et al., 2002; Kilts et al., 2002; Gazi et al., 2003; Shapiro et al., 2003; Gay et al., 2004; Mukhopadhyay and Howlett, 2005; McLaughlin et al., 2005), but also with nuclear receptors such as the estrogen receptors (Dutertre and Smith, 2000). In the prior Chapter, I began to explore how three TM3 serine residues of the D_{2L} receptor affected the functional profile of norpropylapomorphine enantiomers. The current study builds upon this work by studying three rigid dopamine agonists, DNS, DNX, and DHX, two of which were shown previously to be functionally selective at the hD_{2L} *in vitro*, or the rat D_2 receptor *in situ*.

Computational models led to the hypothesis of distinct, but similar binding poses for these rigid agonists, that were first tested by examining the binding of these ligands. As has been shown for many ligands, there is an ionic interaction between an amine of each ligand with a TM3 aspartate (D3.32) (Shi and Javitch, 2002). Both DNS and DNX were hypothesized to form H-bonds to S5.42 and S5.46. Specifically, the p-OH of DNS forms H-bonds with S5.46 and the m-OH of DNS forms H-bonds with both serines S5.42 and S5.46. The methylene-ether change that converts DNS to DNX introduces an additional H-bonding partner to the ligand. The m-OH of DNX is hypothesized to be centrally located between the serines, forming H-bonds with S5.42 and S5.46. The ether of DNX forms an H-bond with S5.42. This may provide opportunity for slightly different docking poses that result in receptor activation, possibly explaining the greater D_{2L}

intrinsic activity for GIRK channels previously reported for DNX vs. DNS (Gay et al., 2004). The effects of each mutation were consistent with these predictions. The next step was to assess the functional actions of these ligands at the mutant receptors. Table 3.6 has been constructed to provide a summary format of the complex matrix of these data (four receptors by five pharmacological endpoints by five ligands).

We examined $[^{35}S]$-GTPγS binding as a functional surrogate, although it is clear that GTPγS binding does not mirror actual functional events. Like previous studies conducted with WT hD$_{2L}$ (Gay et al., 2004), DNS and DHX had partial intrinsic activity for stimulating $[^{35}S]$-GTPγS binding, and were less efficacious than DNX, dopamine, or quinpirole. Our data suggest a critical role for S5.42 and S5.46 with DNS activation of $[^{35}S]$-GTPγS, whereas only S5.42A was critical for DNX. This is consistent with the hypothesis that the ether of DNX creates an additional H-bonding partner that can provide an alternate interaction with the S5.46A mutant. On the other hand, DHX still had intrinsic activity with both S5.42A and S5.46A mutants. Although the affinity for quinpirole was affected slightly by both S5.42 and S5.43, it retained full intrinsic activity at stimulating GTPγS binding.

Table 3.6. Qualitative summary of effects of mutations on probe ligands relative to WT.

Assay	S5.42A					S5.43A					S5.46A				
	DHX	DNS	DNX	QP	DA	DHX	DNS	DNX	QP	DA	DHX	DNS	DNX	QP	DA
Binding	↓↓	→	↓↓	↔↓	↓↓↓	→	→	→	↓↓↓	↓↓↓	↔	↔↓	→	→	→
GTPγS (EC50)	↔↓	-	-	↔	→	↔↑	↔↑	←	↔↑	↔↓	↓↓	-	↔	↓↓	-
ACase (EC50)	-	-	-	←	-	↔	↔	→	↑↑↑	←	-	-	-	↔↑	-
MAPK (EC50)	↔↑	-	-	↔	-	↔	↔	↓↓↓	↔	↔↓	-	-	-	→	-
AA-rel. (EC50)	-	-	-	↔↓	-	-	-	↔↓	↔↓	-	-	-	-	→	-
GTPγS (Emax)	↓↓	↓↓↓	↓↓	↔	↓↓	↔	↑↑	↔	↔	↔	↓↓	↓↓↓	↓↓↓	↔	↓↓↓
ACase (Emax)	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓	↔	↔	↔	↔	↔	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓
MAPK (Emax)	↓↓	↓↓↓	↓↓↓	→	↓↓↓	↔	↔	↔	↔	↔↑	↓↓↓	↓↓↓	↓↓↓	↔	↓↓↓
AA-rel. (Emax)	↓↓↓	↓↓↓	↓↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓	→	→	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↔↓	↓↓↓

↑   Detrimental effect (i.e., decreased K0.5, or decreased EC50 or increased Emax).

↔   Minimal or no effect

→   Positive effect (i.e., increase K0.5, or increased EC50 or decreased Emax).

↓↔   Trend downwards

↔↑   Trend upwards

-    Unable to be determined

Note ↓ = 5-9 fold; ↓↓ = 10-50 fold; ↓↓↓ = > 50 Fold

QP = quinpirole; DA = Dopamine

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S5.42 and S5.46 were shown to be critical for ligand-mediated activation of the β_2-adrenergic receptors (Strader et al., 1989) and later with catecholamine receptors including the D_2 (Mansour et al., 1992; Cox et al., 1992; Woodward et al., 1996; Shi and Javitch, 2002). Although the importance of S5.42 and S5.46 for binding or activation by dopamine had been well-established, it was notable that these rigid ligands, like dopamine, generally required both S5.42 and S5.46 to retain functional activity, consistent with the predicted docking poses. With either of these mutants, DHX was a partial agonist at MAPK, but was inactive at all other functions. DNS and DNX had no activity at any functions with either S5.42 or S5.43 (except DNX at AA-release).

This is in contrast to prior data (Chapter 2) where RNPA and SNPA were shown to be differentially affected by these same mutations, but to retain some activity with each. It is interesting to speculate on whether the N-propyl substituent of the NPA enantiomers was a key factor in the retention of some functional activity. An interesting future experiment would be to compare the effects of the S5.42A and S5.46A mutations on DHX vs. N-n-propylDHX. The latter compound has slightly higher D_{2L} affinity, but like DHX, has been shown to be functionally selective with the WT receptor (Kilts et al., 2002). It is noteworthy that N-propylDHX also has behavioral properties (Smith et al., 1997) not consonant with those expected from a compound that looks like a typical D_2 agonist in many assays (e.g., vs. AC or inhibitng prolactin secretion).

Another interesting result was that the functional profile of quinpirole at the S5.46A mutant was essentially identical to the WT receptor. Conversely, S5.42A mutation turned quinpirole into a functionally selective ligand. Although its intrinsic activity at GTPγS was not affected, quinpirole exhibited nearly full intrinsic activity at AC and MAPK, and was a

good partial agonist at AA. Again, I believe that such data are difficult to reconcile with models that are based on a few discrete receptor active states.

Prior data had suggested that S5.43 was accessible to the binding site crevice (Javitch et al., 1995a), and was directly involved in binding of flexible ligands dopamine and p-tyramine (Cox et al., 1992). Conversely, the current experiments were based on an alternative hypothesis that S5.43 is involved in intrahelical H-bonding and not directly with ligands. The current data, like those from Chapter 2, supported this latter hypothesis. Consistent with an alteration in receptor structure as opposed to a specific effect on ligand interactions, the S5.43A mutation decreased binding affinities of all ligands, yet except for dopamine, actually increased potency for stimulation of GTPγS binding. The S5.43A mutation had no affect on ligand intrinsic activity for AC or MAPK with any ligand tested, yet AA-release was lost for DNS, DA, and DHX but not QP, RNPA, SNPA, and DNX. Thus, the S5.43A mutation has created a receptor in which several of the ligands have dramatic functional selectivity (i.e., full activity at AC and MAPK, but none or minimal at AA). One might dismiss this result as being due to a structure-induced failure of the S5.43A receptor to couple to the appropriate G proteins. This competing hypothesis is unlikely, however, as we have previously shown (Chapter 2) that RNPA has good intrinsic activity at all functions with this receptor.

In summary, the serine mutations studied had modest to large effects on binding, such changes (e.g., in rank order or absolute affinity) being predictable in large measure by existing molecular models. Antagonists were essentially unaffected because the binding is not dependent on specific types of interaction with these serine residues as agonists. Conversely, the mutations caused patterns of effects on the functional activity of the probe

ligands that was not directly predictable from the binding. There are two competing hypotheses that might explain functional selectivity. The first is that ligands can cause receptors to traffic between groups of discrete active states that are linked to specific signaling pathways. The alternative that I favor is that each ligand that binds to a receptor induces a discrete range of conformational changes that affect in a graded fashion whether specific signaling pathways are activated. These two hypotheses differ not only in mechanism, but also in the predicted sequelae. If the former hypothesis is to be meaningful, the number of active states must have a small and finite limit, otherwise it approaches the latter hypothesis. It seems to us that the pattern of functional changes induced by the current mutations cannot be reconciled easily with such a limitation, and that more complex dynamical models are in play.

In the ideal, these experiments would have been performed in reconstituted systems that contain only limited components (e.g., receptor, G proteins, and transducing enzyme) in an appropriate matrix. Unfortunately, such reconstituted systems are rare, and often fail to recapitulate the functional properties of either native or even heterologous systems. Moreover, for the D_{2L} receptor, such systems have not yet been developed. The current data may be evidence for this receptor being an excellent choice for such mechanistic studies. We believe that the rigid ligands that are available and were used in the current studies, coupled with other compounds such as used in Chapter 2, will be particularly powerful probes if reductionist systems can be developed that retain native properties. It is intriguing to think, for example, whether one could detect conformation changes (e.g., Swaminath et al., 2004; Swaminath et al., 2005) in the WT receptor for ligands with clear difference in functional

profile (like quinpirole vs. dinapsoline). Morever, the effects of specific mutations (such as S5.43A) on such systems is equally intriguing.

CHAPTER 4. ANALYSIS OF THE ROLE OF THREONINE 3.37 IN MODULATING FUNCTIONALLY SELECTIVE LIGAND SIGNALING.

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PREFACE

The studies detailed in Chapters 2 and 3 illustrated how the serine residues of TM5, known to be critical for interaction with catechol hydroxyls, can effect subtle conformational changes that can selectively turn on or off effector pathways mediated through the D_{2L} receptor. This chapter focused on T3.37, an amino acid known to be water accessible, but whose role in ligand binding and receptor activation is not well understood.

ABSTRACT

Ligand interactions of T3.37 in the D_{2L} receptor have not been thought to be important in ligand interactions. Molecular modeling studies, however, led to the hypothesis that there were distinct H-bonding interactions of T3.37 with the p-OH of several D_2 ligands including DNS, DNX, RNPA, and dopamine. Quinpirole also was hypothesized to interact with S5.46, but with H-bonding that is stabilized by T3.37. Three other probe compounds (3-PPP, DHX, and SNPA) were hypothesized not to form any bonding interactions with T3.37A. These hypotheses were tested by site-directed mutation of this residue, and detailed binding and functional characterization. The T3.37A receptor had 3-10 fold decreased affinity for both agonists and antagonists, with changes in the rank order of affinity of agonists, but not antagonists. All of the agonists tested caused partial or full activation with the WT receptor, whereas only quinpirole, RNPA, SNPA, and dopamine (not DNX, DNS, DHX, or 3-PPP) stimulated GTPγS binding with T3.37A. Three functional endpoints (inhibition of cAMP synthesis, activation of MAPK, and stimulation of AA-release) were assessed. At all functional endpoints tested, quinpirole and RNPA had full intrinsic activity with the T3.37A receptor relative to WT. Conversely, the other six ligands exhibited no measurable responses to any functional endpoint measured with T3.37A. These data support the critical role of this residue in the D_{2L} receptors, and are consistent with the hypothesis that it affects interhelical interactions that influence conformational changes associated with ligand-induced activation of second messenger pathways.

INTRODUCTION

During the past decade, all of the TM residues of D_{2L} receptor have been studied using the substituted-cysteine accessibility method (SCAM) (Javitch et al., 1994; Javitch et al., 1995a; Fu et al., 1996; Javitch et al., 1998; Javitch et al., 1999; Simpson et al., 1999; Javitch et al., 2000; Shi et al., 2001). The knowledge of which residues are water-accessible has allowed refinement of a molecular model of the hD_{2L} receptor that was used for hypothesis generation of specific ligand-residue interaction(s) in the beginning stages of my dissertation project.

The previous chapters focused on three serine residues common to all catecholamine receptors. Mutagenesis studies with single serine residues demonstrated that alterations to selected aspects of the receptor that were predicted to interact with ligands could cause differential effects on the function of a ligand at the signaling pathways mediated by the D_{2L} receptor. These data underscore the fact that all possible ligand-residue contacts must be characterized if an understanding is to be gained about how subtle structural changes affect ligand-induced signaling. As we integrated the data discussed in Chapters 2 and 3 into the existing molecular model, a novel hypothesis was formulated relating to T3.37, a threonine residue believed to be located in the floor region of the D_{2L} binding pocket. T3.37 was hypothesized to form H-bonds with several of the probe ligands, and also stabilize other residues (e.g., S5.46) through H-bonding. What made this hypothesis of interest was that prior SCAM studies suggested that T3.37 was not an accessible residue (Javitch et al., 1995b).

This chapter used an approach similar to that outlined in Chapters 2 and 3 to test this hypothesis. A set of eight probe ligands were used to examine the affinity and function of

T3.37A and WT D_{2L} receptors. Unlike the differential results obtained from serine studies, this study provides clear evidence to suggest a critical role for T3.37 in ligand-residue mediated functional responses. This study also provides support to suggest that T3.37 is critical for receptor stabilization mediated by TM3-TM5.

RESULTS

Molecular modeling

RNPA, SNPA, DNS, DNX, DHX, dopamine, quinpirole, and (-)3-PPP were all docked to the hD_{2L} receptor (see Figure 4.1), providing hypotheses that relate to the relative roles of TM5 serines and T3.37 (see Chapters 2 and 3 for data on TM5 serines), and how they may affect the different functional profiles previously reported for these ligands (Gay et al., 2004). The modeling predicted H-bonding of the para-OH of RNPA (C10) with T3.37 (see Figure 4.2 for schematic of these interactions). In contrast, with SNPA, the para-OH is distant from T3.37, leading to H-bonding only with S5.42. The proposed interaction for DNS suggests that its para-OH H-bonds with both T3.37 and S5.46, whereas for DNX the para-OH (C9) H-bonds only with T3.37. The docking of DHX and (-)3-PPP predicted no involvement with T3.37, whereas for dopamine the para-OH should interact with both T3.37 and S5.46. The hydroxyl group of (-)3-PPP should hydrogen bond to S5.42 and S5.46. Finally, docking studies conducted with quinpirole suggest no direct interaction with T3.37, although the N2 atom donates a proton to the hydroxyl group of the S5.46 side chain, an interaction stabilized through H-bonding between S5.46 and T3.37.
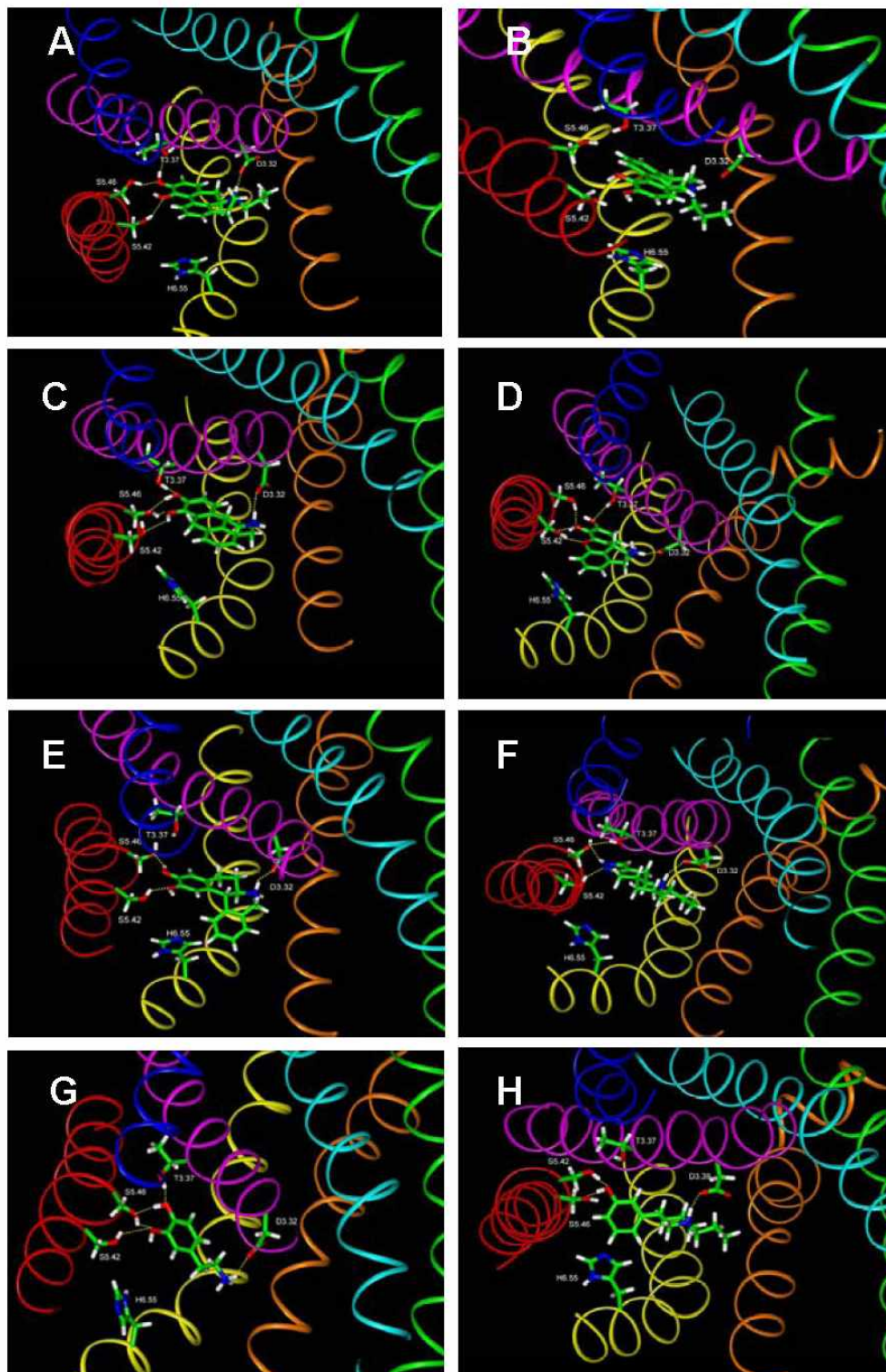
Figure 4.1. Three-dimensional molecular models. *3D molecular models of ligands docked to hD$_{2L}$. View is from the extracellular surface, looking down into the receptor. A) RNPA, B) SNPA, C) DNS, D) DNX, E) DHX, F) quinpirole, G) dopamine, H) 3-PPP.*
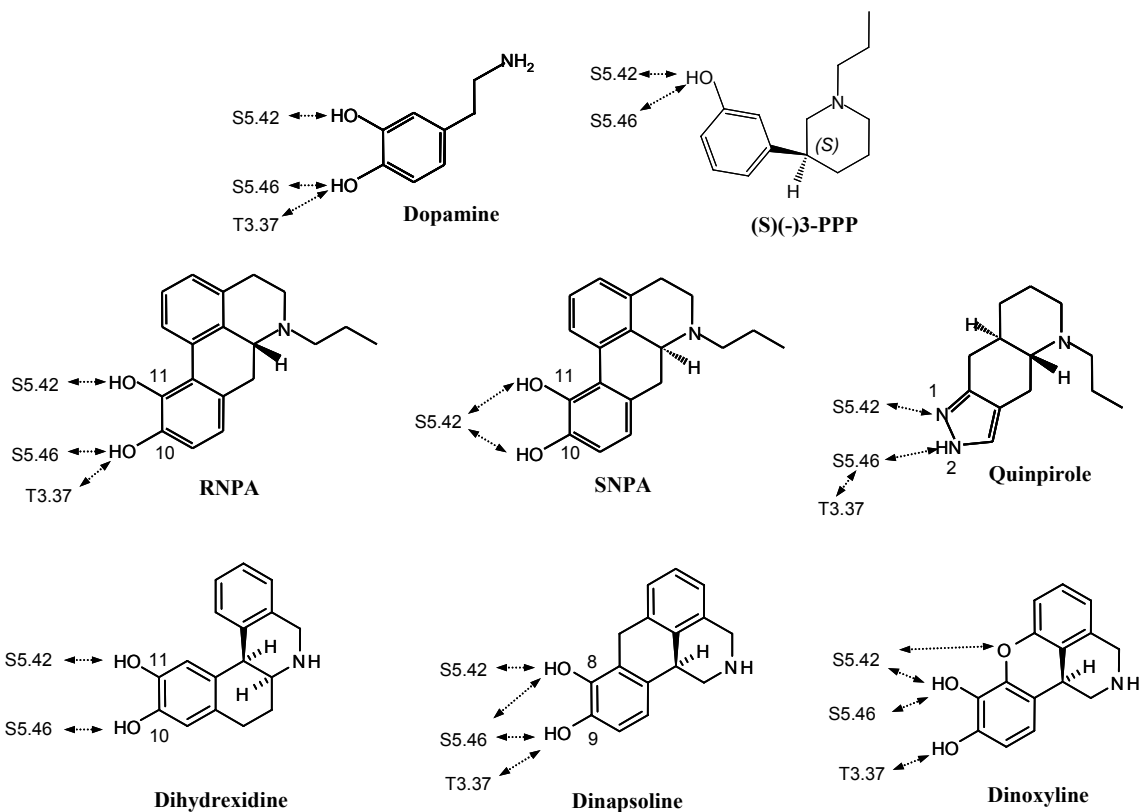
Figure 4.2 Summary of interactions of test ligands with T3.37 (including interactions examined earlier in Chapters 2 and 3).
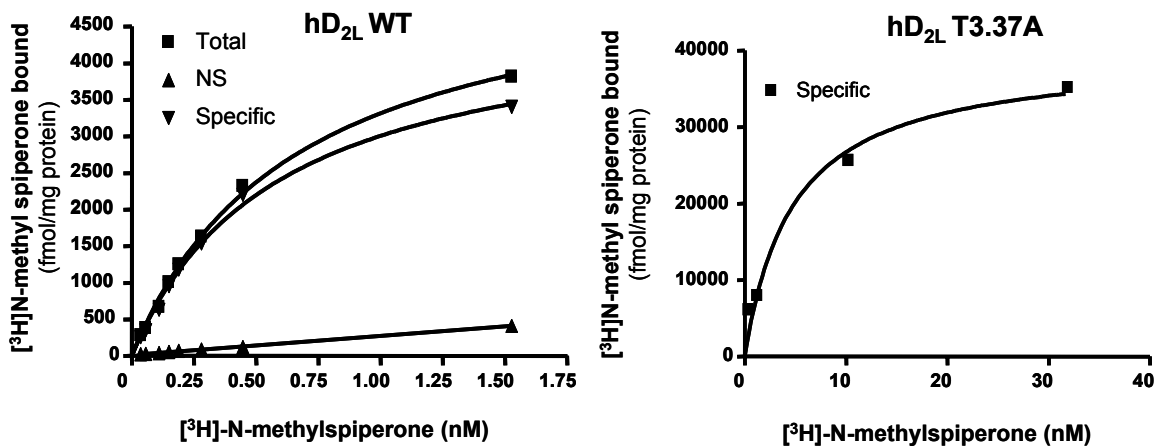
Figure 4.3. Saturation binding assays for stably expressed T3.37A and WT hD$_{2L}$ receptors. *CHO hD$_{2L}$ membrane fragments were incubated with increasing concentrations of [^{3}H]-N-methylspiperone for 15 min at 37 °C. WT assays used hot saturation, whereas the T3.37A used a cold saturation design (see Methods). Data shown are representative of three independent experiments conducted in triplicate.*

Effect of T3.37A on receptor expression and antagonist radioligand binding

The hD_{2L} WT receptor and T3.37A were stably expressed in CHO K1 cells, and saturation radioreceptor assays with [^{3}H]-N-methylspiperone were used to characterize both receptors. The WT receptor was expressed at 4.8 pmol/mg protein with a dissociation constant (K_D) of 0.58 nM (Figure 4.3). The T3.37A (T119A) mutant receptor was expressed at a much higher density (39.5 pmol/mg protein), but with a somewhat decreased affinity for the radioligand (K_D = 4.8 nM). For the T3.37A mutant, a cold saturation assay was used to decrease the amount of [^{3}H]-N-methylspiperone consumed. In such a design, non-specific binding is also dramatically decreased.

Effect of T3.37A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus [^{3}H]-N-methylspiperone in membranes from both WT and mutant receptors. An apparent affinity constant, $K_{0.5}$, was determined from experimental IC50 values that were corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). Competition binding studies were also conducted with several structurally different antagonists to rule out gross structural changes induced by the receptor. Although all of the antagonists had slightly decreased affinity (as found for N-methylspiperone), their relative affinity (i.e., rank order) was unchanged, suggesting that no major changes to overall receptor structure were induced by this mutation.
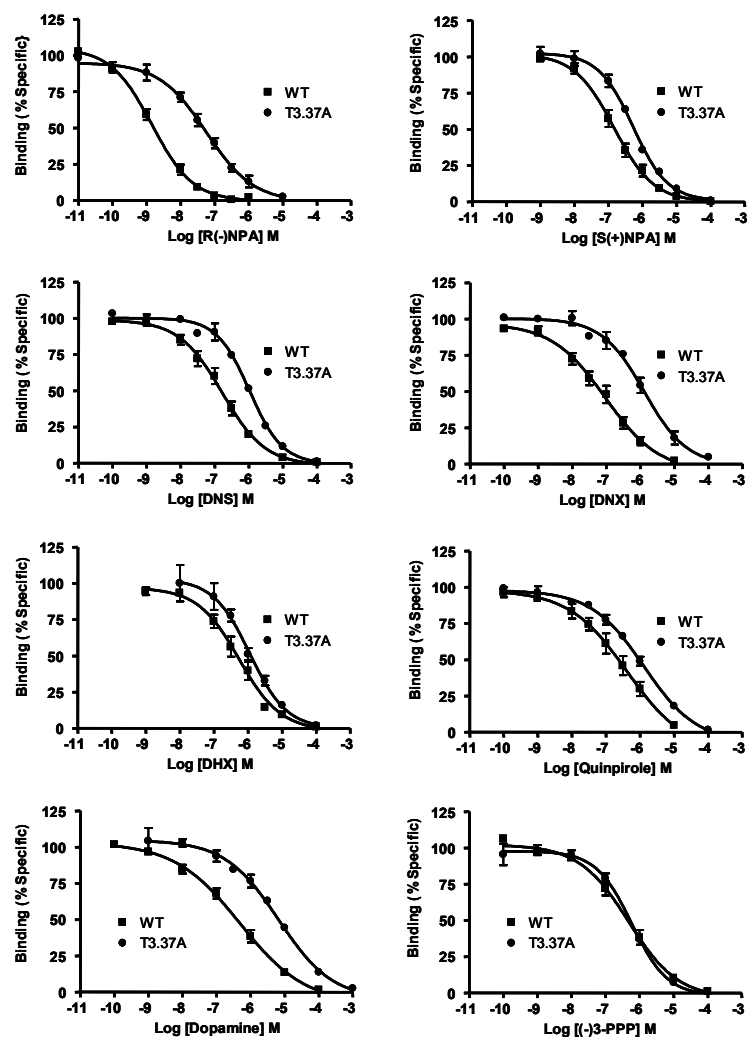
Figure 4.4. Competition binding of test compounds with hD$_{2L}$ WT and T3.37A. *Membrane fragments were incubated with [^{3}H]-N-methylspiperone at K$_D$ for 15 min with varying concentrations of test compounds. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC50s, reported as corrected affinity values (K$_{0.5}$) using Prism 4.0. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 4.1. Competition binding data for mutants with test compounds.

	WT		T3.37A	
Compound	$K_{0.5}$ (nM)	n_H	$K_{0.5}$ (nM)	n_H
R(-)NPA	1.3 ± 0.9	0.74 ± 0.03	38 ± 7	0.71 ± 0.04
S(+)NPA	129 ± 30	0.76 ± 0.04	395 ± 52	0.86 ± 0.02
Quinpirole	365 ± 66	0.59 ± 0.04	$1,180 \pm 290$	0.58 ± 0.06
Dopamine	447 ± 172	0.56 ± 0.02	$5,140 \pm 430$	0.60 ± 0.03
DNS	144 ± 17	0.67 ± 0.03	$1,150 \pm 440$	0.89 ± 0.06
DNX	83 ± 5	0.57 ± 0.03	$1,160 \pm 170$	0.66 ± 0.04
DHX	490 ± 91	0.72 ± 0.04	825 ± 78	0.83 ± 0.06
(-)3-PPP	422 ± 76	0.65 ± 0.02	395 ± 21	0.82 ± 0.02

Values are means $\pm$ SEM (n=3-5)

Effect of mutations on ligand-induced GTPγS binding

An estimate of G protein turnover was conducted using ligand-induced binding of [^{35}S]-GTPγS (see Figure 4.5 and Table 4.2). Of the full agonists for this function in the WT receptor, the T3.37A mutation caused a slight loss of potency for quinpirole, a marked loss of potency for RNPA, but did not have significant effects on the intrinsic activity of either compound. Conversely, this mutation caused a dramatic decrease in both potency and the intrinsic activity of dopamine. Of the partial agonists, there was a complete loss of intrinsic activity of DHX, DNS, DNX, and (-)3-PPP, yet only a decrease in potency for SNPA.
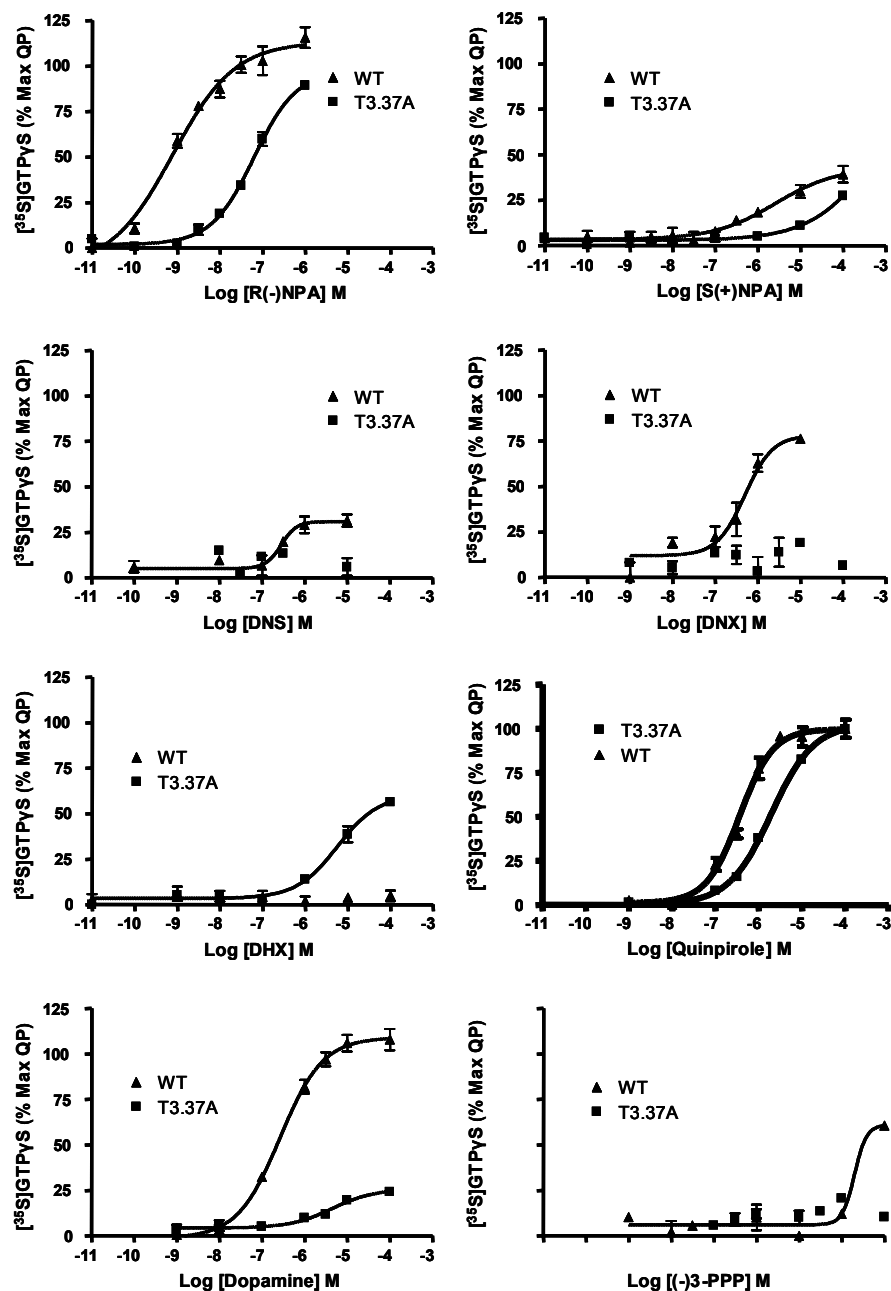
Figure 4.5. GTPγS turnover experiments of test compounds with hD₂ₗ WT and T3.37A. *Membrane fragments were incubated for 15 min with varying concentrations of test compounds until reaching equilibrium. 0.2 nM [³⁵S]-GTPγS was then added for 30 min, and stimulation was measured. Analysis was conducted using non-linear regression and a sigmoidal equation (Prism 4.0) to determine EC50s reported below in Table 4.2. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Representative data for the agonist probe ligands are shown in Figure 4.4 and summarized in Table 4.1. The relative rank order of affinity for the WT receptor [RNPA >> DNX > SNPA = DNS > quinpirole > 3-PPP = dopamine = DHX] is fundamentally altered by

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the T3.37A mutation [RNPA >> 3-PPP = SNPA > DHX > DNS = DNX = quinpirole >>> dopamine]. The four ligands (quinpirole, SNPA, DHX, and 3PPP) that were predicted not to interact with T3.37 (Figure 4.2) were the least affected by the T3.37A. The mutation had no affect on 3-PPP binding, and caused only modest effects (2-3 fold increases in $K_{0.5}$) on the binding of quinpirole, DHX, or SNPA. On the other hand, T3.37A resulted in marked increases in the $K_{0.5}$ of RNPA (30-fold), DNS (8 fold), and DNX (12-fold). There also was a tendency for this mutation to increase the Hill slope of most of the ligands (e.g., see Figure 4.4), although not for RNPA.

Table 4.2. EC50 values for GTPγS binding experiments..

Compound	WT	T3.37A
R(-)NPA	1.0 ± 1.3	94 ± 41
S(+)NPA	1,200 ± 30	3,400 ± 900
Quinpirole	740 ± 120	2,000 ± 100
Dopamine	1,700 ± 1,000	5,700 ± 800
DNS	286 ± 71	--
DNX	550 ± 31	--
DHX	370 ± 180	--
(-)3-PPP	770 ± 10	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments
conducted in triplicate

Effects of T3.37A on ligand-mediated signal transduction

Measurement of ligand inhibition of forskolin-stimulated cAMP accumulation was conducted (Figures 4-6 and 4-7, and Table 4.3). With the WT receptor, quinpirole, dopamine, RNPA, DNS, DNX, and DHX all exhibited full intrinsic activity whereas 3-PPP and SNPA exhibited partial agonist activity. None of these ligands had activity in untransfected cells, and their activity in cells transfected with hD_{2L} was blocked by various D_2 antagonists like domperidone. The T3.37A mutation caused a complete loss of intrinsic activity for all tested ligands except RNPA and quinpirole.
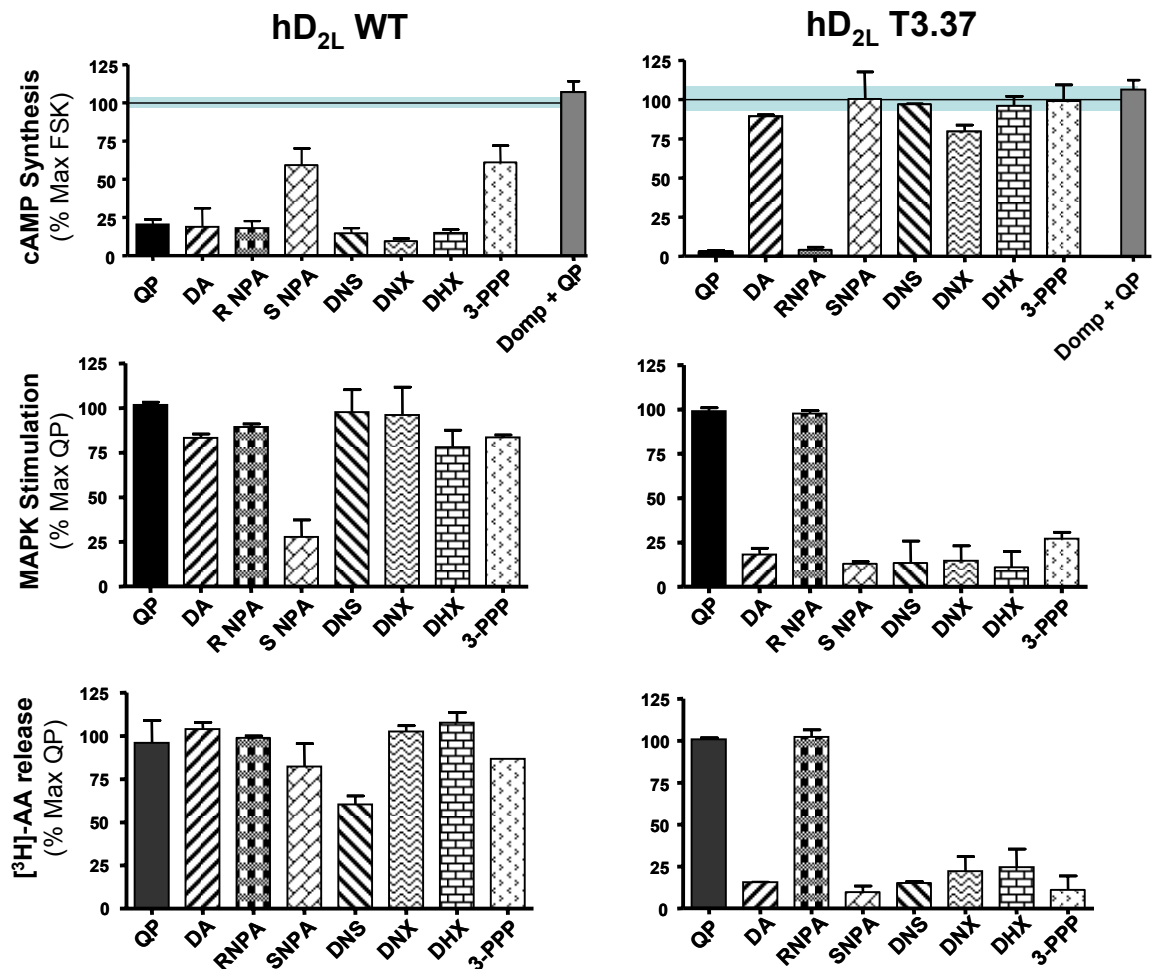
Figure 4.6. Activation of second messenger pathways of hD2L with ligands at T3.37A. Top: *Ligand-mediated inhibition of forskolin-stimulated cAMP accumulation,* ***Middle:*** *Ligand-mediated activation of p44/p42 MAP Kinase,* ***Bottom****: Ligand-mediated potentiation of [^{3}H]-Arachidonic acid release. Note that agonists inhibit AC, but stimulate MAPK and AA release Domperidone alone had no effect on rhese systems. Data are representative of E$_{max}$ values for 3-5 independent experiments conducted in triplicate.*

Measurement of a second pathway, agonist stimulation of the MAPK pathway, gave similar results (Figures 4-6 and 4-7, and Table 4.3). In the WT receptor, quinpirole, dopamine, DNS, DNX, DHX, and RNPA had full intrinsic activity, whereas SNPA and 3-PPP were partial agonists. Similar to inhibition of cAMP accumulation with T3.37A, only RNPA and quinpirole had intrinsic activity, although with modestly decreased potency.
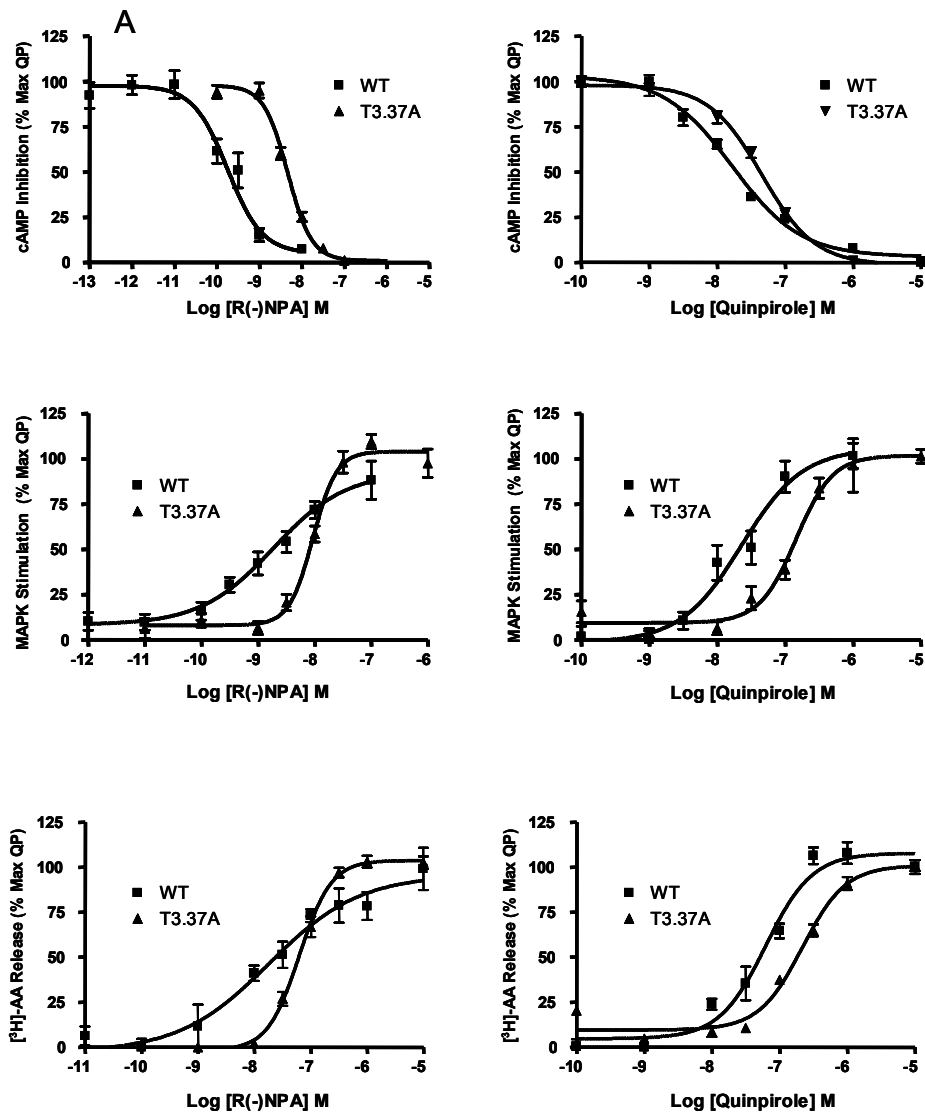
Figure 4.7. Dose response curves of activation of second messenger pathways of hD$_{2L}$ with ligands at T3.37A. Top: Ligand-mediated inhibition of forskolin-stimulated cAMP accumulation, Middle: Ligand-mediated activation of p44/p42 MAP Kinase, Bottom: Ligand-mediated potentiation of [^{3}H]-Arachidonic acid release. Data are representative of 3-5 independent experiments conducted in triplicate.

A third pathway (ligand-stimulated [^{3}H]-arachidonic acid release) also was assessed (Figures 4.6-4.7, and Table 4.3). Quinpirole, dopamine, DNX, DHX, and RNPA were all full agonists, but the classical partial agonists SNPA and 3-PPP also had full intrinsic activity. Conversely, DNS was only a partial agonist with the WT receptor. As above, the T3.37A mutation caused a loss of intrinsic activity for all ligands except RNPA and quinpirole. The overall changes of the T3.37A mutation on intrinsic activity across all three functional pathways are shown in Figure 4.6.

DISCUSSION

These studies are the first to hypothesize a role for T3.37 in the binding interaction of agonists in the D$_{2L}$ receptor or any Class A GPCR. The overall hypothesis underlying this work was that T3.37 has two possible roles for how the D$_{2L}$ receptor interacts with some of its ligands (see Figure 4.2). The docking studies predicted that SNPA, DHX, and 3-PPP do not interact directly with T3.37, whereas RNPA, DNS, and DNX do. Another prediction was that quinpirole, although not interacting directly with T3.37, had its binding stabilized by this residue. This hypothesis was explored by generation of a stable non-conservative mutant receptor (T3.37A), and in depth characterization of the mutant versus wild-type receptor with a series of rationally selected probe ligands.

One of the difficulties in mutagenesis studies such as those performed here is discriminating changes due to global effects from those that are affected by alterations of specific aspects of ligand-receptor interactions. In this regard, there is some evidence that the T3.37A receptor was still reasonably intact. First, both N-methylspiperone and domperidone bound T3.37A with similar affinity as WT receptor. Finally, some of the ligands were able to cause functional activation similar to that seen with the WT receptor.

Table 4.3. EC50 values for activation of second messenger pathways of hD$_{2L}$ with ligands at T3.37A.

Function	AC		MAPK		AA	
Compound	WT	T3.37A	WT	T3.37A	WT	T3.37A
Quinpirole	172 ± 6	49 ± 13	26 ± 5	152 ± 94	63 ± 12	231 ± 37
Dopamine	157 ± 22	--	28 ± 7	--	49 ± 26	--
RNPA	0.2 ± 1.4	4.5 ± 0.3	1.9 ± 0.9	9.5 ± 0.5	18 ± 1.9	77 ± 16
SNPA	118 ± 18	--	26 ± 19	--	1,300 ± 250	--
DNS	193 ± 63	--	72 ± 68	--	345 ± 177	--
DNX	5.6 ± 0.3	--	27 ± 17	--	101 ± 12	--
DHX	93 ± 10	--	213 ± 105	--	395 ± 119	--
3PPP	32 ± 31	--	145 ± 99	--	80 ± 7	--

Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

DHX, DNS, and DNX were originally developed as D$_1$ full agonists ligands (Brewster et al., 1990; Ghosh et al., 1996; Grubbs et al., 2004), and are known to have very similar pharmacophoric elements (Mottola et al., 1996). Thus, although it was not surprising that all three rigid compounds were hypothesized to have somewhat similar binding poses, the predicted differences between them (specifically that only the p-OH of DNS and DNX would form H-bonds with T3.37) were unexpected, at least based on their similar interactions with the D$_1$ receptor (Brewster et al., 1990; Mottola et al., 1996; Ghosh et al., 1996; Grubbs et al., 2004).

The most straightforward prediction from the receptor modeling was that the binding of some ligands (i.e., RNPA, DNS, DNX, quinpirole) would be more affected by the mutation that that of others (SNPA, DHX, and 3PPP). This was, in fact, what was found in our experiments. Of equal interest was the fact that the slope of the competition curves for all of the ligands except RNPA were significantly steeper. Since the expression level in the T3.37A stable cells was much higher than in WT, this could have been explained by a higher proportion of T3.37A receptors that were not precoupled to G proteins. That the shape of quinpirole, dopamine, and RNPA curves was unchanged suggests that these observations are ligand-specific, and not simply due to changes in receptor density. Indeed, antagonists often have competition curves of normal steepness, whereas agonist curves are shallower, and this might suggest that the mutation was going to lead to alterations in functional profile, as was found (vide infra).

GTPγS binding studies were then used to provide a rough estimate on overall G protein turnover. The T3.37A mutation caused a complete loss of acitivty for all of the ligands except quinpirole, RNPA, and SNPA. These effects are consistent with the steepening of the competition curves discussed above. Interestingly, the potency of RNPA was decreased far more than its affinity (90- vs. 30-fold, respectively), whereas the potency and affinity of quinpirole were affected equally (2.6- vs 3.2-fold, respectively. These results provide further support for the hypothesis that the effects of this mutation are ligand specific and not global effects on the mutant receptor. It was therefore of interest to determine if a simlar pattern would be seen in several functional pathways modulated by this receptor.

As was summarized in Figure 4.6, an identical pattern of intrinsic activity was seen in each of the three pathways examined (AC, MAPK, and AA) as was seen with GTPγS

binding. With the T3.37A mutant, there was no intrinsic activity with SNPA, DNS, DNX, DHX, 3-PPP, or dopamine. Conversely, T3.37A had minimal effect on the intrinsic activity of RNPA or quinpirole. What is interesting however, are the effects that the T3.37A mutation had on the potency of quinpirole and RNPA in these three functional assays. Although the mutation decreased the potency of quinpirole about five-fold in the MAPK and AA assays, it increased the potency of quinpirole in the AC assay. On the other hand, this mutation also caused a four-fold decrease in the potency of RNPA for MAPK and AA, yet caused more than a 20-fold decrease in potency in the AC assay. As noted earlier, one of the potential confounding features of our study was the much higher expression level of the T3.37A receptor vs. the WT. One could have hypothesized that the potency differences in the various assays are simply a result of different requirements for receptor reserve in each of these assay systems (Watts et al., 1995). The opposite effects of the mutation on the potency of quinpirole vs. RNPA in the AC assay, yet with similar effects in the other assays, argue against these effects simply being due to differences in receptor reserve.

Why T3.37A minimally affects the function of quinpirole and RNPA while abolishing that of the other tested ligands remains to be addressed. One possible explanation involves the necessity of stabilizing H-bonds between TM3 and TM5 residues. Our molecular model (Figure 4.1) with quinpirole docked into the active site hypothesizes that an intra-molecular H-bond exists between T3.37 and S5.46. This H-bond is an important interaction between two TM regions, and our data suggests that it is critical for ligand-residue interaction/induction of G protein activation. Quinpirole and RNPA interact favorably with both T3.37 and S5.46, and the steric bulk associated with each ligand's interaction in this axis of the active site suggests that these ligands are capable of maintaining

stability between TMs with T3.37A. Dopamine and 3-PPP are, however, flexible and lack the steric bulk that might be associated with maintaining stability between TM3/TM5 with T3.37A. Likewise, SNPA is hypothesized to interact nearer S5.42 and thus the steric bulk of this ligand will be directed away from the S5.46-T3.37 intra-molecular H-bonding, thus SNPA is incapable of stabilizing T3.37A. DNS and DNX however, interact in the binding site similarly to RNPA, yet neither compound elicits functional response with T3.37A compared to RNPA.

There are some clear ways to explore these issues further. The use of double (S5.46A/T3.37A) and reciprocal mutants (S5.46T, T3.37S, and S5.46T/T3.37S) provide an alternative method of studying these critical interactions, possibly deducing the exact critical nature of these particular ligand-residue interactions. Additionally, it is possible that RNPA retained functional activity because of stabilizing effects of its N-n-propyl moiety. In this regard, the N-n-propyl analogs of DNS, DNX, and DHX have all been reported previously (Knoerzer et al., 1995; Sit et al., 2002; Qandil et al., 2003; Grubbs et al., 2004), and study of these compounds with this mutant would provide one test of whether the N-propyl moiety, known to increase D_2 affinity, also plays a key role in functional activation. In either case, these studies of the role of T3.37 provide additional evidence for the unique effects individual ligands may have on signaling. These data are consistent with the hypothesis that ligands unique conformational states that can favor turning on or off specific effector pathways mediated through that receptor, rather than selecting from a pool of preexisting active states.

CHAPTER 5. ANALYSIS OF THE ROLE OF HISTIDINE 6.55 IN MODULATING
FUNCTIONALLY SELECTIVE LIGAND SIGNALING.

To be submitted for publication in:

Molecular Pharmacology

PREFACE

The work presented in Chapters 2-4 provided new information that helps explain the role of certain TM3 and TM5 hydroxyl-containing amino acid residues in the functionally selective ligand activation of effector pathways mediated through the hD_{2L} receptor. This chapter will focus upon a residue that does not directly interact with the agonists tested within this dissertation. Instead, the H6.55 residue will be studied for the role it plays in affecting the global conformational changes associated with receptor activation. Previous studies with several Class A GPCRs suggest the critical nature of this residue and neighboring TM6 residues for receptor activation. This study will address the necessity, or lack thereof, of H6.55 interactions when tested with a subset of our functionally selective ligands, specifically looking at how differences in the rigidity of the test ligands may affect receptor conformations that ultimately mediate downstream signaling pathways.

ABSTRACT

Histidine 6.55 has been suggested to be critical for global conformational changes of catecholamine receptors that lead to activation of receptor signaling pathways. This study examined how H6.55 affected binding to and activation of the dopamine hD_{2L} receptor by a set of typical and functionally selective ligands for which no direct H6.55 interaction was predicted. The H6.55A mutation did not significantly affect the binding of either antagonists or the four agonist ligands (dopamine, quinpirole, dihydrexidine, RNPA) studied. Downstream second messenger pathways (e.g. inhibition of cAMP synthesis, activation of MAPK, and potentiation of AA-release) were measured with each ligand and the mutant receptor. Our findings suggest that H6.55 is necessary for activation of the second messenger pathways linked to cAMP inhibition and AA-release, but not critical for a third pathway, phosphorylation of p44/p42 MAP kinase. These data suggest that H6.55 is not only critical, but that ligand-specific interactions affect receptor conformational changes such that some receptor state populations can be activated (e.g. MAPK) while others cannot (e.g. cAMP and AA-release). Further understanding of the unique nature of H6.55, and how it contributes to activation of only one effector endpoint when mutated, could help elucidate the mechanistic basis for ligand-selective activation of receptor pathways.

INTRODUCTION

Site-directed mutagenesis has been used to characterize the specific interactions that occur between a ligand and receptor. One of the difficulties in such studies is differentiating direct effects of the mutation(s) on ligand-receptor interactions from mutation-induced conformational changes that may cause global, rather, than ligand-specific changes. It has been known for more than a decade that a cluster of TM6 aromatic amino acid residues are critical for agonist-mediated activation of bovine rhodopsin and β_2-adrenergic receptors (Nakayama and Khorana, 1991; Zhang and Weinstein, 1994; Farrens et al., 1996; Gether et al., 1997; Sheikh et al., 1999). Nakayama and Khorana (1991) provided evidence to support a direct interaction between W6.55 and retinal of bovine rhodopsin. The W6.55A mutant had a significant reduction of chromophore regeneration, suggesting the critical nature of this residue for receptor activation. Studies conducted with the galanin receptor GalR1 ($G\alpha_{i/o}$ coupled) showed that H6.55A mutation decreased galanine binding and impaired agonist-mediated inhibition of cAMP accumulation (Berthold et al., 1997). In addition, it has been shown that the β-OH of isoproterenol and N6.55 interact directly in the β_2-adrenergic receptor (Wieland et al., 1996), providing further evidence for critical roles of 6.55 residues in Class A GPCRs. Although SCAM studies (Javitch et al., 1998) support H6.55 accessibility in the hD_{2L} binding pocket, the only data about direct interactions between H6.55 and dopamine ligands is with antagonist binding (Woodward et al., 1994).

Recently, studies have been conducted that show that H6.55 and H6.60 is a Zn^{2+} binding site on the dopamine D_{2L} receptor (Liu et al., 2006). Zinc allosterically modulates antagonist interactions of the D_{2L} receptor (Schetz and Sibley, 1997; Schetz et al., 1999), further suggesting the importance of H6.55. There have been no subsequent studies of H6.55

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that explore their potential role in the action of dopamine agonists. We sought to address the necessity of this receptor residue through mutagenesis studies using a set of ligands with unique functional profiles. Our initial hypothesis from molecular modeling studies was that no direct interactions take place between our test compounds and H6.55, but that mutation of this residue might provide additional information to support what I have termed the energy landscape hypothesis of functional activation. We ascertained the effects of the H6.55A mutation of ligand binding and receptor function. Further understanding of how H6.55 contributes to activation could help elucidate the mechanistic basis for ligand-selective activation of receptor pathways in the D_{2L} and possibly other Class A GPCRs.

RESULTS

Effect of mutation on receptor expression and radioligand binding

The hD_{2L} WT receptor and H6.55A were stably expressed in CHO K1 cells, and saturation radioreceptor assays with $[^3H]$-N-methylspiperone were used to characterize both receptors. The WT receptor was expressed at 4.8 pmol/mg protein with a dissociation constant (K_D) of 0.58 nM (Figure 5.1 and Table 2-1). The H6.55A (H393A) mutant receptor was expressed at a lower density (919 fmol/mg protein) with a K_D of 0.37 nM.
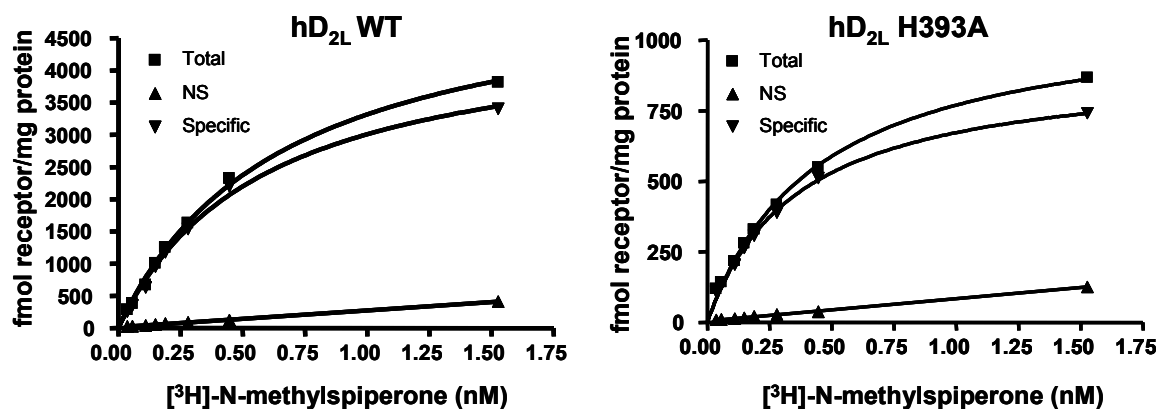
Figure 5.1. Saturation binding assays for stably expressed mutant and WT hD_{2L} receptors. CHO hD_{2L} membrane fragments were incubated with increasing concentrations of $[^3H]$-N-methylspiperone for 15 minutes at 37 °C. Data shown are representative of three independent experiments conducted in triplicate.

Effect of H6.55A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus $[^3H]$-N-methylspiperone in membranes from both WT and mutant receptors. An apparent affinity constant, $K_{0.5}$, was determined from experimental EC50 values that were corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). Competition binding studies also were conducted with several structurally different antagonists to rule out gross structural changes induced by the receptor. Although all of the antagonists had slightly decreased affinity (as found for domperidone), their rank order and relative affinity was unchanged, suggesting that no major changes to overall receptor structure were induced by this mutation.

None of the four compounds tested were hypothesized to interact directly with H6.55. Representative data for the agonist probe ligands are shown in Figure 5.2 and summarized in Table 5.1. Although Table 5.1 shows no significant differences in absolute affinity, a paired t-test of experiments run with mutant and WT receptor on the same day indicated that the H6.55A mutant had significantly lower affinity for dopamine, and that RNPA had both higher affinity and a steeper slope.

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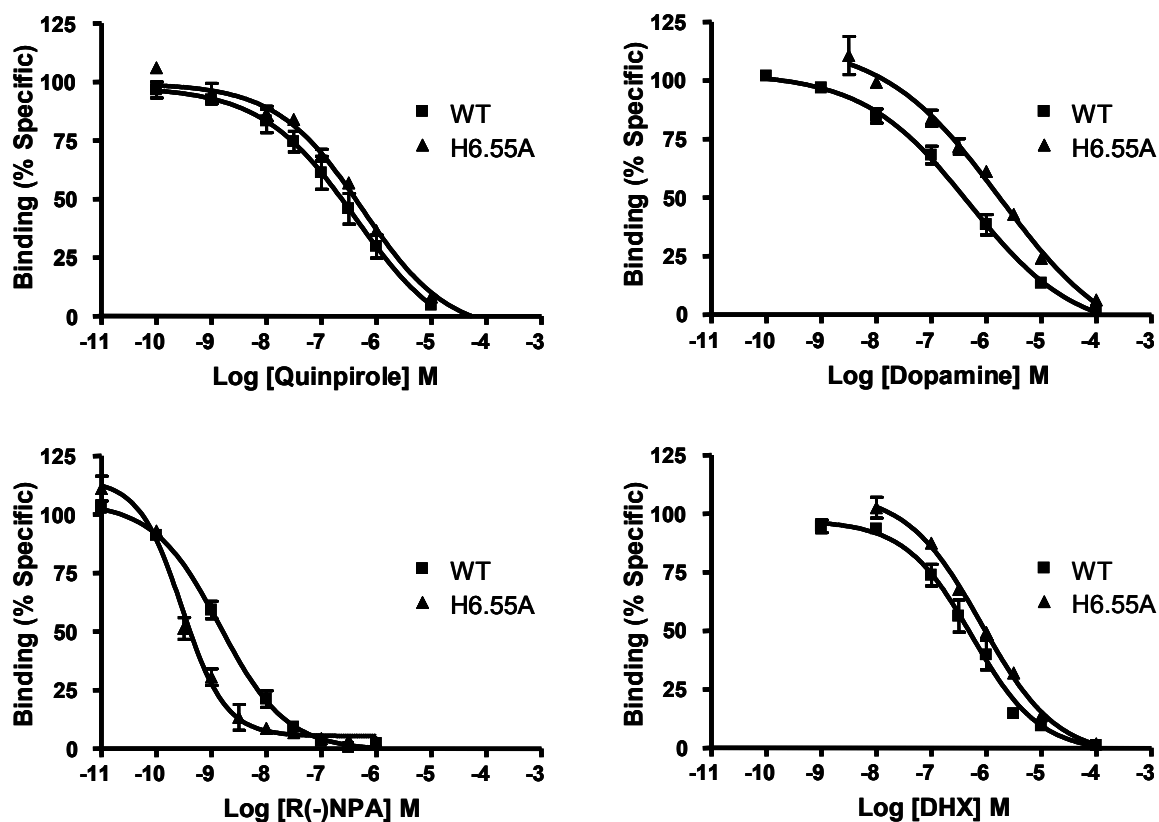
Figure 5.2. Competition binding of test compounds with hD$_{2L}$ WT and H6.55A. *Membrane fragments were incubated with [^{3}H]-N-methylspiperone at K$_D$ for 15 min. with varying concentrations of test compounds. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC50s, reported as corrected affinity values (K$_{0.5}$) using Prism 4.0. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 5.1. Summary of saturation and competition binding data.

Saturation Binding			Competition Binding [K$_{0.5}$ (nM)]			
Mutant	K$_D$ (nM)	Bmax (pmol/mg)	R(-)NPA	quinpirole	dopamine	DHX
WT	0.58	4.8	1.3 ± 0.9	365 ± 66	447 ± 172	490 ± 90
H6.55A	0.37	0.92	$0.22 \pm 0.01*$	443 ± 76	$1440 \pm 590**$	490 ± 170

$*p < 0.103$, $**p < 0.007$.

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Effect of mutations on ligand-induced GTPγS binding

An estimate of G protein turnover was conducted using non-hydrolyzable [^{35}S]-GTPγS (see Figure 5.3 and Table 5.2). All four agonists stimulated [^{35}S]-GTPγS binding with similar intrinsic activity in H6.55A as in WT, with all except DHX being full agonists. Conversely, H6.55A decreased potency for [^{35}S]-GTPγS coupling with all four ligands tested. Specifically, H6.55A exhibited significant decreased potency with dopamine and RNPA compared to WT. H6.55A minimally affected quinpirole and DHX compared to WT. The obvious hypothesis from these data is that the potency, but not intrinsic activity of these ligands (especially dopamine and RNPA), will be decreased at downstream effector pathways in the H6.55A versus WT receptor
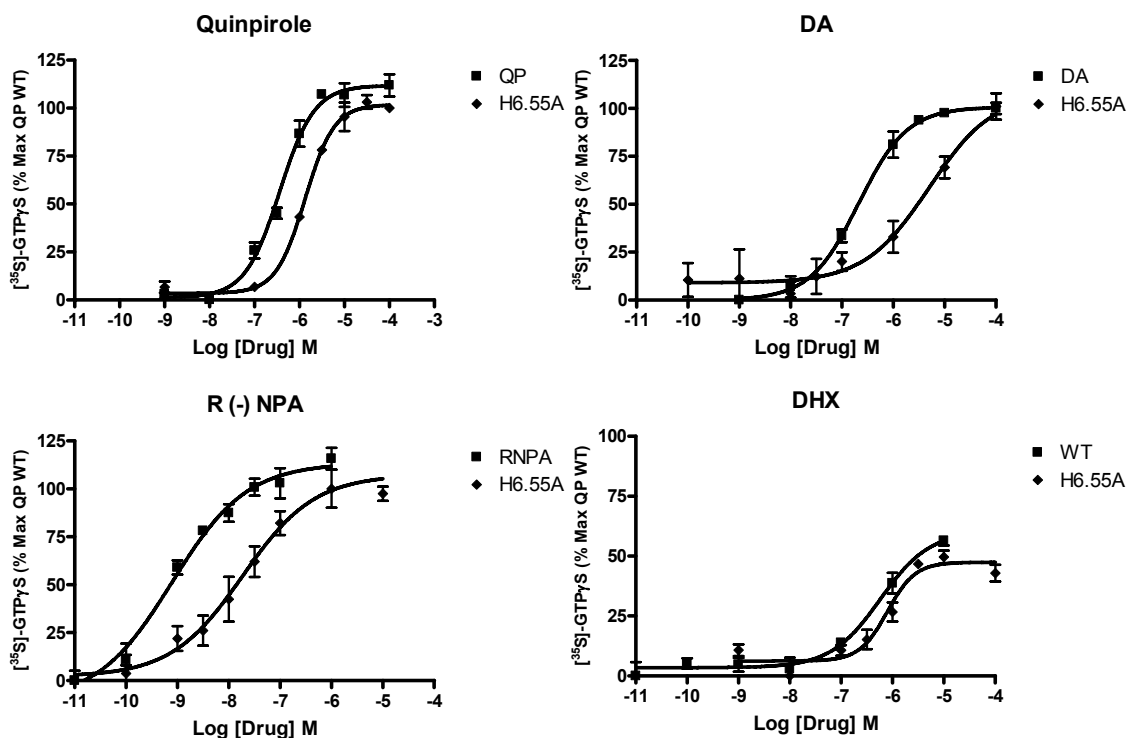
Figure 5.3. GTPγS turnover experiments of test compounds with hD$_{2L}$ WT and H6.55A. *Membrane fragments were incubated for 15 min. with varying concentrations of test compounds until reaching equilibrium. 0.2 nM [^{35}S]-GTPγS was then added for 30 min. and stimulation was measured. Analysis was conducted using non-linear regression and a sigmoidal equation (Prism 4.0) to determine EC50s reported below in Table 2-2. Assays were conducted in triplicate and data represents 3-4 independent experiments.*

Table 5.2. EC50 values for GTPγS turnover experiments. **Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.**

GTPγS (nM)				
Mutant	R(-)NPA	quinpirole	dopamine	DHX
WT	1.0 ± 1.3	735 ± 120	1711 ± 1039	373 ± 184
H6.55A	38 ± 17	1336 ± 205	6497 ± 3049	919 ± 127

T3.37A: Activation of second messenger pathways

The hD$_{2L}$ receptor couples to Gα$_{i/o}$ subunits resulting in inhibition of adenylate cyclase (AC) activity. Measurement of agonist inhibition of FSK-stimulated cAMP accumulation in a whole cell assay system for WT and H6.55A was conducted (see Figure 5.4). All four agonists robustly inhibit FSK-stimulated cAMP accumulation with WT receptor. Conversely, and in contrast to effects on [^{35}S]-GTPγS binding, no inhibition of AC was seen with any ligand with H6.55A.

Measurement of agonist stimulated [^{3}H]-arachidonic acid release then was conducted in a whole cell assay system for WT and H6.55A (see Figure 5.4). All four test compounds robustly stimulated [^{3}H]-arachidonic acid with WT. Conversely, none of these ligands potentiated [^{3}H]-arachidonic acid release with H6.55A, consistent with the loss of agonist effects caused by H6.55A on ligand-induced inhibition of adenylate cyclase.

Finally, assessment of a third pathway, agonist-induced stimulation of p44/p42 MAP kinase phosphorylation was conducted in a whole cell assay system for WT and H6.55A. Similar to cAMP and AA-release assays, all four agonists tested exhibited full agonist activity as expected when evaluated with the WT receptor (see Figures 5.4 and 5.5). Measurement of MAPK activation yielded quite different results to those expected from the

adenylate cyclase and AA-release experiments, with all four test compounds being able to activate MAPK with the H6.55A receptor (see Figure 5.5). The intrinsic activity of all compounds was lower, with the effects being greatest for quinpirole relative to WT. There were only modest effects on the potency of these ligands (see Table 5.3).
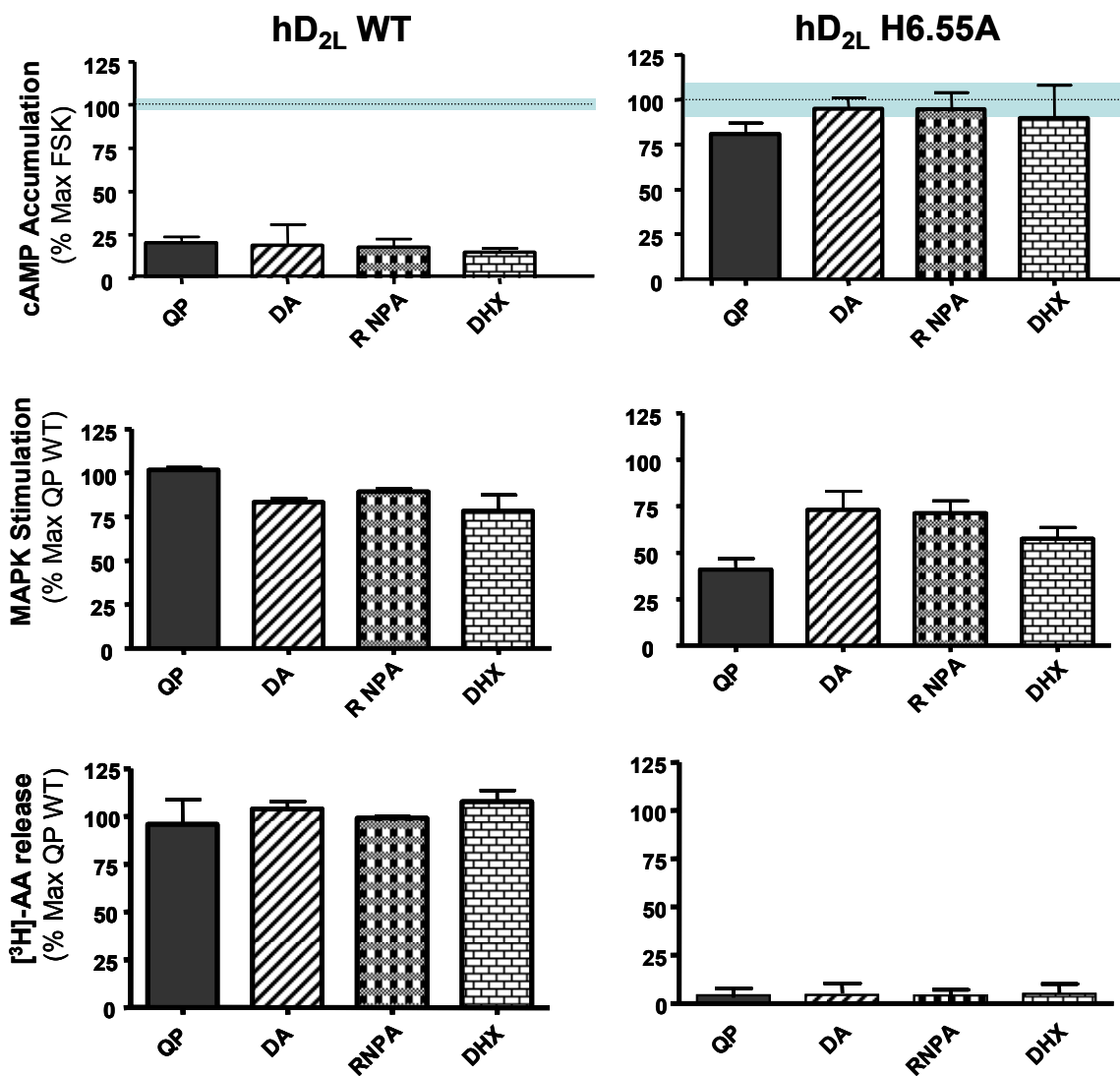
Figure 5.4. Activation of second messenger pathways of hD_{2L} with ligands at WT and H6.55A receptors. Top) agonist mediated inhibition of FSK-stimulated cAMP accumulation, Middle) agonist mediated activation of p44/p42 MAP Kinase, Bottom) agonist mediated potentiation of [³H]-arachidonic acid release. Domperidone was without effect alone in these assays, but blocked the effects of quinpirole (data not shown). Data are representative of Emax values for 3-5 independent experiments conducted in triplicate.

Table 5.3. **Potency estimates (EC50 in nM) for functional response of test ligands with WT and H6.55A hD$_{2L}$.**

Ligand	Receptor	cAMP	MAPK	AA-release
Quinpirole	WT	172 ± 5.5	26 ± 5	63 ± 12
	H6.55A	--	73 ± 31	--
Dopamine	WT	157 ± 22	28 ± 7	49 ± 26
	H6.55A	--	109 ± 25	--
RNPA	WT	0.18 ± 1.4	1.85 ± 0.9	18 ± 1.9
	H6.55A	--	0.43 ± 2.2	--
DHX	WT	93 ± 10	213 ± 105	395 ± 119
	H6.55A	--	740 ± 110	--

Values represent EC50 ± S.E.M. for 3-5 independent experiments conducted in triplicate
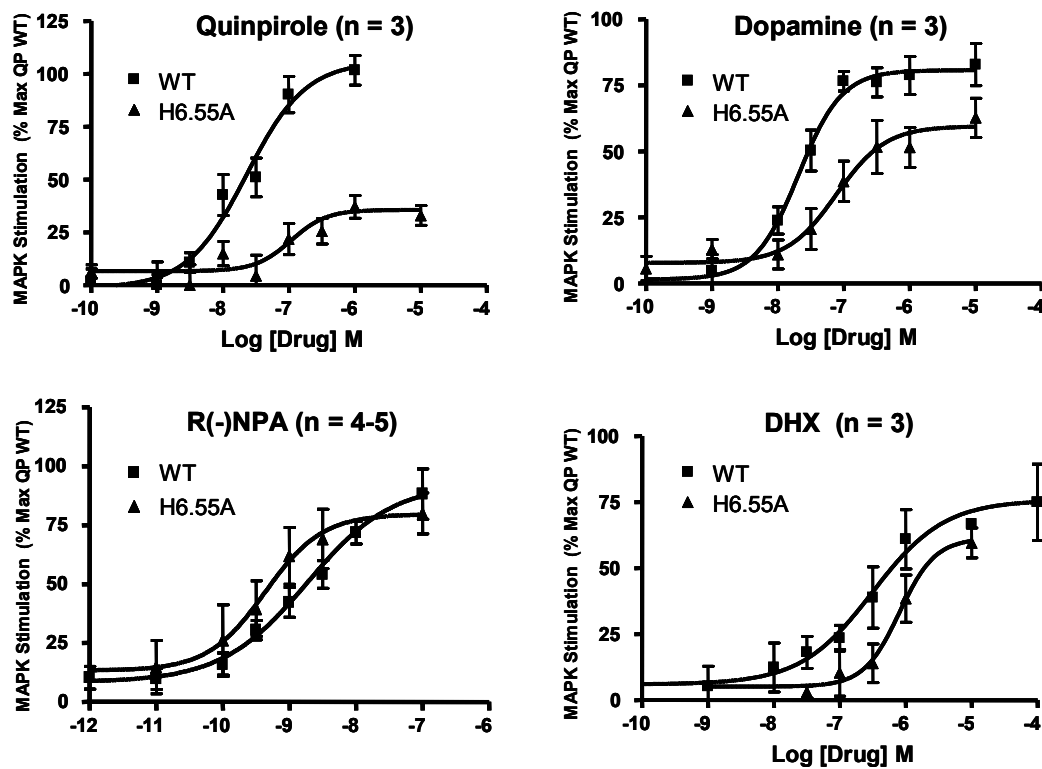
Figure 5.5. ***Dose response curves of activation of second messenger pathways of hD$_{2L}$ with ligands at H6.55A.*** *Agonist mediated activation of p44/p42 MAP Kinase. Data shown is representative of 3-5 independent experiments conducted in triplicate.*

DISCUSSION

This work examined the role of H6.55 mediation of receptor signaling with a set of typical and functionally selective compounds. Previous studies with H6.55 mutants provide support for agonist-receptor residue interactions with β_2-adrenergic, GalR1, and bovine rhodopsin receptors (Nakayama and Khorana, 1991; Zhang and Weinstein, 1994; Wieland et al., 1996; Farrens et al., 1996; Gether et al., 1997; Berthold et al., 1997; Sheikh et al., 1999), but the only data with the D_{2L} receptor suggest an interaction between H6.55 and dopamine antagonists (Woodward et al., 1994). Additionally, recent studies have provided evidence to support H6.55 and H6.60 as the zinc sensitive sites of hD_{2L} (Liu et al., 2006). Our molecular modeling studies suggest that none of the eight compounds in this dissertation directly interact with H6.55. Together, the available information suggested that H6.55 might influence receptor conformational changes associated with second messenger signaling. Because of our hypothesis of ligand-specific changes in patterns of signaling, H6.55A was tested with two functionally selective compounds (RNPA and DHX), with the prototypical agonist quinpirole, and the endogenous ligand dopamine for its effects on receptor binding, ligand-induced coupling to GTPγS, and activation of second messenger pathways mediated through hD_{2L}.

This mutation did not cause marked effects on either the affinity of our test ligands or the ability of these ligands to stimulate GTPγS binding. Based on these results, we hypothesized that agonist-mediation of downstream signaling would show only small differences in potency or intrinsic activity with the H6.55A versus the WT receptor. Thus, it was quite surprising that none of the four test compounds were capable of either inhibiting FSK-stimulated adenylate cyclase activity or AA-release as they were with the WT receptor.

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Equally surprising was that each of these ligands retained a reasonable level of intrinsic activity for activation of a third second messenger pathway, MAPK, although H6.55A reduced the intrinsic activity of quinpirole to a greater extent than it affected RNPA, dopamine, or DHX.

The lack of dramatic effects of the H6.55A mutation on the binding of either agonists or antagonists suggests that this residue does not play a direct role in the D_{2L} binding pocket. A prior mutagenesis study suggested that F6.51 interacts with dopamine through orthogonal pi-stacking interactions (Floresca and Schetz, 2004). H6.55 is located one helical turn above F6.51 and is predicted itself to interact with F6.51 through pi-stacking. Mutagenesis of this residue (H6.55A) could affect the torsional angle of the alpha carbon of F6.51 such that orthogonal pi-stacking with dopamine is abolished, hence causing the modest decreased in the affinity of dopamine. Less likely is the possibility of a direct interaction between dopamine and both F6.51 and H6.55. The basis for the slightly increased affinity and increased slope for RNPA with H6.55A is much less clear. My prior work (see Chapters 2 and 3) hypothesized interactions of RNPA and quinpirole with S5.46 and T3.37 in the floor of the binding site. The fact that H6.55A has no affect on quinpirole binding whereas RNPA slightly increased affinity could be the result of the elimination of steric hindrance for RNPA binding. Because DHX was not so affected, an alternate possibility is that it is the presence of a propyl group that differentiates these compounds. It would be interesting to determine how N-n-propyldihydrexidine compares to RNPA and DHX itself.

The TM6 aromatic amino acid cluster has also been shown to be critical for conformational changes associated with activation of several receptors (Zhang and Weinstein, 1994; Farrens et al., 1996; Gether et al., 1997; Sheikh et al., 1999; Floresca and

Schetz, 2004). This is clearly relevant to the finding of greatest interest here, the differential effects of the H6.55A mutation on functional patterns. The current data collectively suggest that the H6.55A mutation altered the receptor in such a way that GTPγS binding and MAPK activation were only modestly affected, whereas agonist-induced effects on either adenylate cyclase or AA-release were completely lost. Traditional views of ligand-mediated activation of receptor would posit that all second messenger pathways would be similarly affected by H6.55A, in stark contrast to the current results. H6.55A is unique because each ligand tested (regardless of structural differences or hypothesized critical binding interactions with the receptor) induced activation of only a single hD_{2L}-mediated second messenger pathway. The question this raises are the possible mechanisms.

GTPγS binding is known to be only a rough estimate of GPCR function, as it reflects the stoichiometry of which alpha subunits are available and their rate of turnover rather than a specific function (Gazi et al., 2003). Nonetheless, it is quite surprising that the intrinsic activity of GTPγS binding was essentially the same in the H6.55A and WT receptors whereas the mutant showed no agonist stimulation of two of the three measured functions. Indeed, inhibition of adenylate cyclase by the hD_{2L} receptor clearly is associated with activation of $G\alpha_{i/o}$ (Missale et al., 1998; Neve et al., 2004), and these α-subunits are thought to comprise a major part of the GTPγS signal. It would be of interest to determine if there are marked changes in specific labeling of this population of α-subunits that might explain the lack of effect on adenylate cyclase. On the other hand, GPCR mediated effects on MAPK and AA-release pathways have been hypothesized to be mediated in large measure through Gβγ signaling (Neve et al., 2004), although this can also occur indirectly through cAMP-mediated processes. Analysis of MAPK activation via Gβγ signaling in the presence of added

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pertussin could rule out the role of pertussin-sensitive $G\alpha_{i/o}$ proteins. Further analysis of the permutations of $G\beta\gamma$ with fusion proteins could be utilized to determine the specificity of interactions with G protein-H6.55A receptor compared to G protein-WT receptor. Subsequent studies with purified G protein subunits and purified receptor systems could enhance these studies by providing key information to correlate conformational changes of the receptor with agonist-selective activation of specific G protein complexes.

In conclusion, the current data underscore the complexity of signaling and the likely involvement of other partners in signal transduction (Urban et al., 2006a). The interesting anomaly reported in this work not only opens the door for some interesting mechanistic studies, but also impacts on the recent debate over concepts of ligand activation. Classic pharmacological models have been based on the notion of receptor-active states, starting with simple two-stage models that have grown increasingly complex as new data needed to be incorporated (see Chapter 1). Another viewpoint that has been offered is that the number of possible receptor active states is essentially infinite and depends on the population of conformations induced by the binding of a ligand to a receptor (Urban et al., 2006a). The latter is markedly influenced by the environment of the receptor, including a variety of protein partners as well as the lipid environment (Urban et al., 2006a). It seems difficult to reconcile the current data with the classic type of model. In any event, detailed understanding of the involved mechanisms are heuristically interesting, and may enhance drug discovery endeavors which seek to selectively turn on therapeutic pathways while eliminating activation of secondary pathways associated with unwanted side effects.

CHAPTER 6. ANALYSIS OF THE ROLE OF ASPARTATE 2.50 IN ALLOSTERICALLY MODULATING FUNCTIONALLY SELECTIVE LIGAND SIGNALING.

To be submitted for publication in:

Neuropharmacology

PREFACE

Previous chapters addressed specific ligand-residue interactions in the active site of the dopamine receptor. The work presented in this Chapter, however, will address the role of an allosteric site D2.50(80), the sodium and pH sensitive site of hD_{2L}. Previous studies with D2.50A mutant suggest a critical role for cAMP synthesis, but extensive analysis with multiple effector endpoints and functionally selective ligands has yet to be conducted. This chapter will address D2.50 allosterism and its relationship to ligand-induced conformational changes with a set of functionally selective ligands. This chapter will further previous studies by utilizing a set of structurally different compounds (including functionally selective compounds) to characterize the role of D2.50 allosterism at multiple effector endpoints.

ABSTRACT

The most conserved residue of TM2, aspartate D2.50, was studied to understand its role in allosterically modulating signaling of functionally selective ligands with the hD_{2L} receptor. Previous mutagenesis studies of catecholamine receptors suggest that D2.50A and D2.50N can artificially create functionally selective systems. Our studies measured the affect of ligand binding affinity, G protein turnover, and activation of second messenger pathways (e.g. cAMP synthesis, MAPK activation, and AA-release) with the test compounds RNPA, DHX, DA, and QP with D2.50A. All four test compounds had lower affinity for D2.50A than WT, with binding curves shifted from shallow to steep, suggesting a possible loss of high affinity coupling to G protein. The test compounds were neither able to stimulate GTPγS binding with D2.50A, nor activate any of the three receptor-coupled second messenger pathways with this mutant receptor. Unlike prior studies with the cannabinoid CB1 and α_{2A}-adrenergic receptor studies where selective signaling was maintained with D2.50A and D2.50N, hD_{2L} signaling is abolished without this highly conserved aspartate residue.

INTRODUCTION

The focus of drug discovery for decades has been on designing ligands targeting the active sites of receptors or enzymes. Although research on orthosteric sites remains most common, there has been a recent push to explore the potential of allosteric sites. These can be aspects of the target receptor sensitive to interacting proteins, ions, or even other endogenous small molecules, but also any portion of the target receptor where ligand binding can affect function. Allosterism can affect orthosteric ligand binding, induce conformational changes that activate or inactivate receptors, but can also affect the receptor in many other ways. The HIV field has benefited greatly from targeting allosteric sites with non-nucleoside reverse transcriptase inhibitors such as nevirapine (Pommier et al., 2005). These drugs exhibit much greater potency and have significantly longer half lives than the orthosteric nucleoside reverse transcriptase inhibitors such as AZT (Pommier et al., 2005).

A growing literature supports the existence of allosteric sites within G protein coupled receptors (GPCRs) (Birdsall and Lazareno, 2005; Gao et al., 2005; Schetz, 2005; Hoare, 2005; Pin et al., 2005), as well as the potential the clinical utility of such allosteric ligands (Zobel et al., 2000; Petersen and Sullivan, 2001; Li et al., 2002; Li et al., 2003; Grillon et al., 2003; Olesen et al., 2004; Bertini et al., 2004; Poon, 2005; Lindberg et al., 2005; Kellner et al., 2005). Not surprisingly, dopamine receptors have sites of allosteric modulation including Zn^{2+}, Na^+, and amiloride sensitivity (Schetz, 2005). Neve first discovered the sodium and pH sensitivities of the D_2 receptor (Neve, 1991), and subsequent site-directed mutagenesis experiments provided evidence to support aspartate D2.50 as the sodium-sensitive site (Neve et al., 1991). When mutated, the D_{2L} receptor exhibited

decreased affinity for binding and loss of inhibitory action on cAMP accumulation. What is interesting, however, is that the latter study only examined a single functional D_{2L} endpoint.

Recent results have shown that such site-directed mutations can differentially affect signaling pathways. In addition to data presented in earlier Chapters, D2.50N with the cannabinoid receptor CB_1 exhibited full agonist activity for adenylate cyclase inhibition and Ca^{2+} currents similar to WT, but decreased agonist-mediated potentiation of GIRK channels and receptor internalization (Roche et al., 1999). In addition, D2.50N of the α_2-adrenoceptor exhibited agonist-stimulated inhibition of cAMP and Ca^{2+} currents similar to WT, but markedly decreased receptor-mediated Na^{2+} currents (Surprenant et al., 1992).

These data suggest that D2.50 not only allosterically regulates ligand-mediated function, but that it can exhibit a differential role for activation of specific second messenger pathways that signal through a single receptor isoform. We therefore hypothesized that the D2.50A mutation of the hD_{2L} receptor would result in differential effects similar to those seen with the cannabinoid and adrenoceptors. The current data, however, show that D2.50 is critical for the hD_{2L}, and that mutation to alanine causes a complete loss of G protein coupling and subsequent loss of intrinsic activity at several downstream effector pathways.

RESULTS

Effect of mutation on receptor expression and radioligand binding

The hD_{2L} WT receptor and D2.50A were stably expressed in CHO K1 cells, and saturation radioreceptor assays with [^{3}H]-N-methylspiperone were used to characterize both receptors. The WT receptor was expressed at 4.8 pmol/mg protein with a dissociation constant (K_D) of 0.58 nM (Figure 6.1 and Table 6.1). The D2.50A (D80A) mutant receptor

was expressed at a much higher density (34.7 pmol/mg protein) with a slightly decreased

radioantagonist affinity ($K_D = 0.69$ nM).
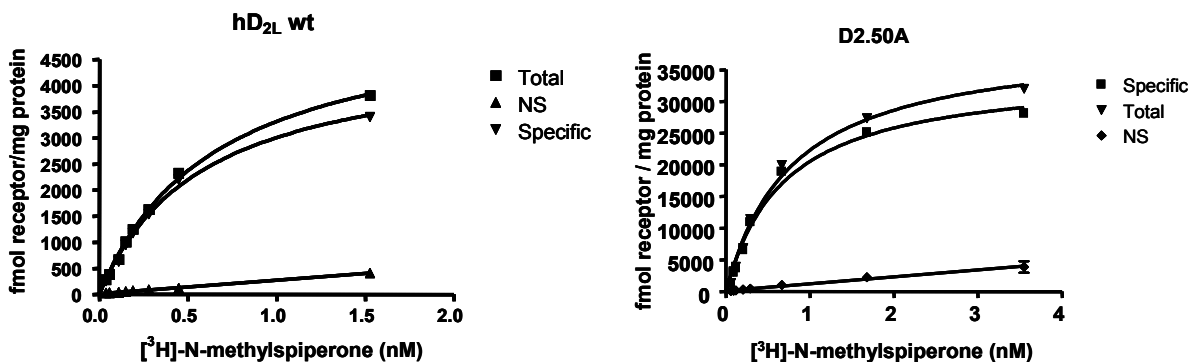
Figure 6.1. Saturation Binding for stably expressing mutant and WT hD$_{2L}$ receptors. *CHO hD$_{2L}$ membrane fragments were incubated with increasing concentrations of [^{3}H]-N-methylspiperone for 15 minutes at 37 °C. Data shown is representative of 3-4 independent experiments conducted in triplicate.*

Effect of D2.50A on affinity of agonist probe ligands

The affinity for each probe ligand was determined using competition radioreceptor assays versus [^{3}H]-N-methylspiperone in membranes from both WT and mutant receptors. An apparent affinity constant, $K_{0.5}$, was determined from experimental IC50 values and were corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973) as reported in Table 6.1. Competition binding studies were also conducted with several structurally different antagonists to rule out gross structural changes induced by the receptor. Although all of the antagonists had slightly decreased affinity (as found for N-methylspiperone), their rank order and relative affinity were unchanged, suggesting that no major changes to overall receptor structure were induced by this mutation.

Representative data for the agonist probe ligands are shown in Figure 6.2 and summarized in Table 6.1. Most notably, the relative rank order of affinity for WT (RNPA >

DHX > DA ≈ QP) is greatly affected by D2.50A (RNPA > DHX > QP >> DA). Specifically, D2.50A caused large affinity shifts with DA and RNPA compared to WT whereas D2.50A had only modest effects on QP and DHX. Interestingly, the Hill coefficients for RNPA, DA, QP, and DHX with WT all shifted to steeper slope with D2.50A, suggesting loss of the high affinity (G protein-coupled) state.
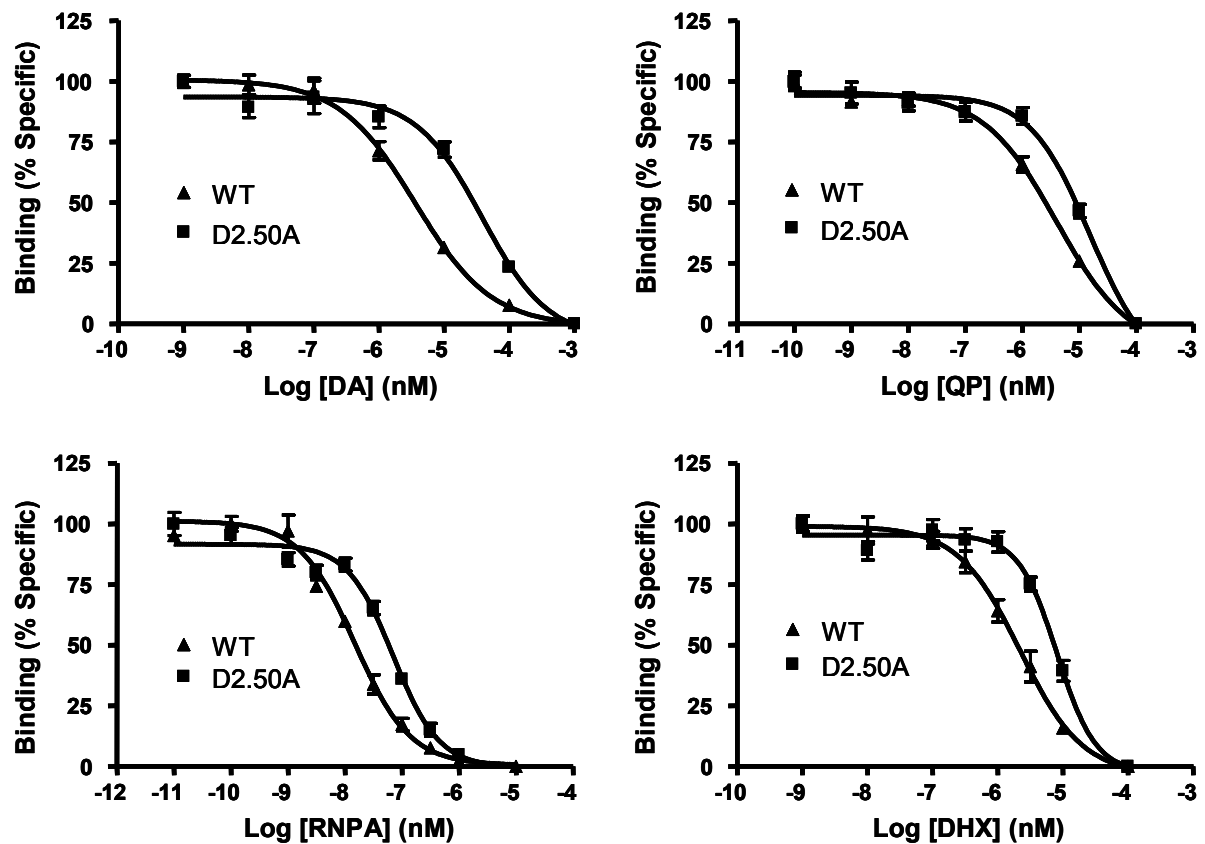
Figure 6.2. Competition binding of test compounds with hD_{2L} WT and D2.50A. *Membrane fragments were incubated with $[^3H]$-N-methylspiperone at K_D for 15 min. with varying concentrations of test compounds. Analysis was conducted using non-linear regression and a sigmoidal equation to determine IC50s, reported as corrected affinity values ($K_{0.5}$) using Prism 4.0. Assays were conducted in triplicate and data represents three independent experiments.*

Table 6.1. Saturation and competition binding data for D2.50A mutant and WT.

	Receptor	
	WT	**D2.50A**
Saturation		
K_D **(nM)**	0.58	0.69
Bmax (pmol)	4.8	34.7
Competition Ligand	$K_{0.5}$ *(nM)*	
R(-)NPA	16 ± 5	63 ± 2
QP	3100 ± 50	9500 ± 80
DA	3050 ± 390	41400 ± 7200
DHX	1990 ± 530	5950 ± 150
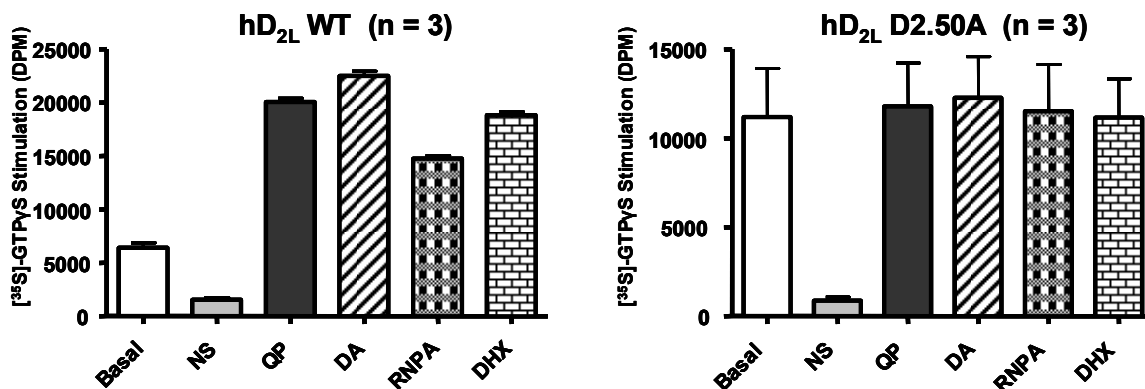
Figure 6.3. GTPγS turnover experiments of test compounds with hD$_{2L}$ WT and D2.50A. *Membrane fragments were incubated for 15 minutes with varying concentrations of test compounds until equilibrium was reached. [^{35}S]-GTPγS (0.2 nM) was then added for 30 minutes and stimulation was measured. Assays were conducted in triplicate and data represents Emax values of 3-4 independent experiments.*

Effect of mutations on ligand-induced GTPγS binding

An estimate of G protein turnover was conducted using non-hydrolyzable [^{35}S]-GTPγS (see Figure 6.3 and Table 6.2). All four test compounds robustly stimulated [^{35}S]-GTPγS turnover with WT receptor. In contrast, D2.50A inhibited all compounds from

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stimulating [^{35}S]-GTPγS turnover. These results suggest that D2.50A affects ligand-induced receptor conformations necessary for G protein coupling. We hypothesized that downstream effector pathways would also be devoid of function.

Table 6.2. EC50 values for GTPγS turnover experiments.

EC50 (nM)				
Mutant	R(-)NPA	QP	DA	DHX
WT	1.0 ± 1.3	740 ± 120	1710 ± 1040	370 ± 180
D2.50A	NA	NA	NA	NA

NA = no activity
Values represent EC50 ± S.E.M. for 3-4 independent experiments conducted in triplicate.

Measurement of agonist inhibition of FSK-stimulated cAMP accumulation in a whole cell assay system for WT and D2.50A was conducted (see Figure 6.4). At the WT receptor, all four test compounds exhibited full dopamine agonist activity, robustly inhibiting FSK-stimulated cAMP accumulation. In contrast, the D2.50A mutation eliminated any stimulatory effect of these four compounds. In addition, in this experiment the effects of domperidone alone, and domperidone plus quinpirole were also assessed, with neither condition causing stimulation or inhibition versus forskolin alone (data not shown).

Measurement of agonist activation of MAPK phosphorylation was conducted in a whole cell assay system for WT and D2.50A (see Figure 6.4). In the WT receptor, all four ligands fully stimulated MAPK phosphorylation. Conversely, the D2.50A mutation eliminated this activity. Measurement of agonist stimulated [^{3}H]-arachidonic acid release was conducted in a whole cell assay system for WT and D2.50A (see Figure 6.4). At the WT

receptor, all four test compounds robustly stimulated [³H]-arachidonic acid release. Conversely, the D2.50A mutation eliminated activity of all four test compounds.
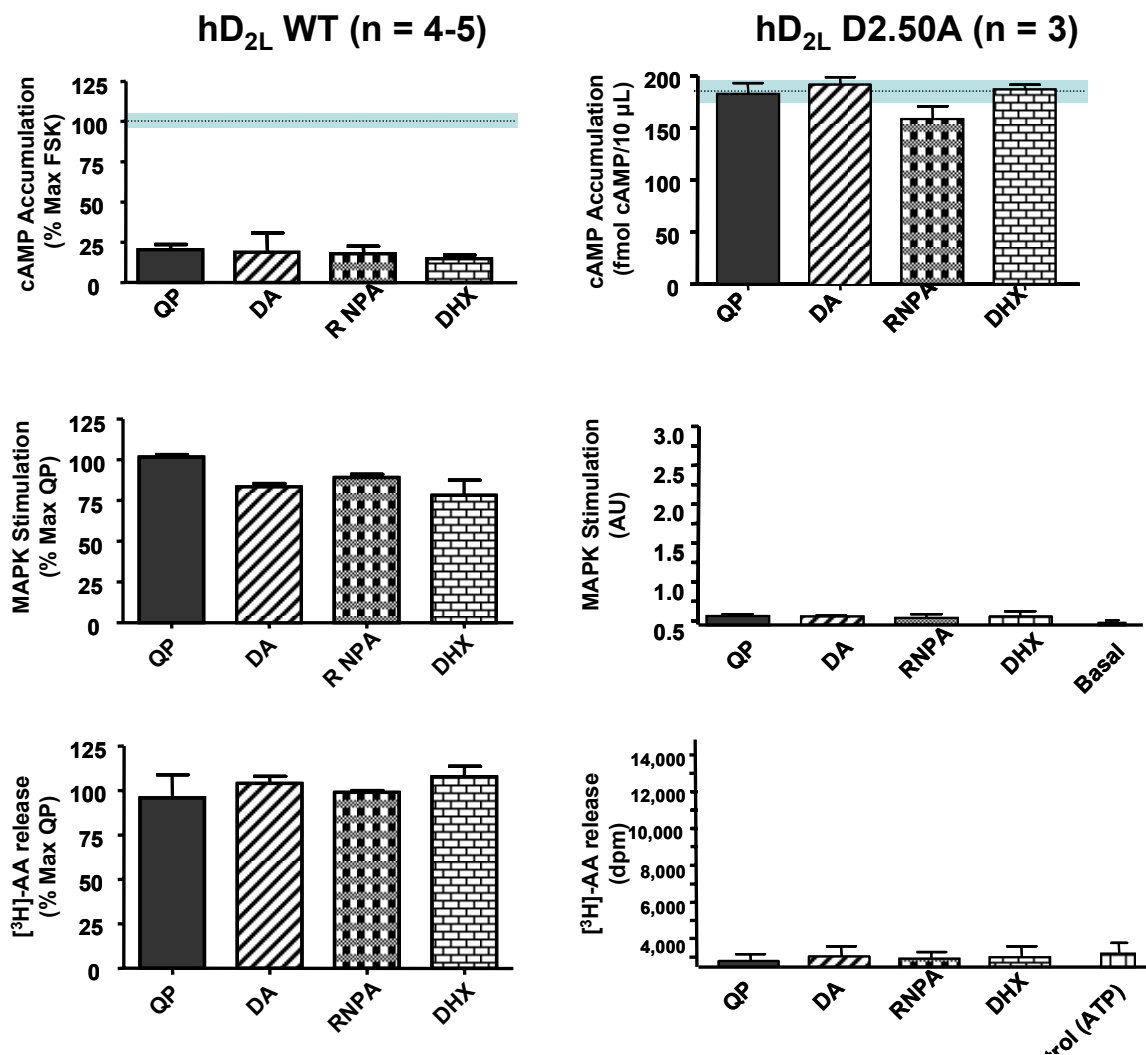
Figure 6.4. Activation of second messenger pathways of hD₂ₗ with ligands at D2.50A. Top) *Agonist mediated inhibition of FSK-stimulated cAMP accumulation, **Middle)** Agonist mediated activation of p44/p42 MAP Kinase, **Bottom)** Agonist mediated potentiation of [³H]-arachidonic acid release. There were no effects of domperidone or quinpirole plus domperidone with any of the assays with the D2.50A mutant. Data are representative of E_max values for 3-5 independent experiments conducted in triplicate.*

DISCUSSION

This dissertation chapter studied the allosteric nature of the TM2 conserved aspartate residue of hD₂ₗ. Previous research has suggested that D2.50 plays a critical role in allosterically regulating receptor conformations based on its Na⁺ and pH sensitivities (Neve

et al., 1991). This study addressed the necessity of D2.50 regulation of signaling when typical or functionally selective ligands were tested at three distinct second messenger endpoints of hD_{2L}. Critical to this study was the evaluation of multiple endpoints, as the previous mutagenesis work (Neve et al., 1991) focused on only receptor binding and one function (i.e., agonist inhibition of adenylate cyclase). D2.50 is located greater than 10 Å from the orthosteric site, and modeling suggests that this residue does not form any direct contact points with ligands used in this study. Our goal, however, was to determine if there was an interaction between changes induced by functionally selective ligands at the active site and allosteric regulation of second messenger pathways mediated by this aspartate.

The data gained from binding studies suggested significantly decreased binding of test ligands to the mutant receptor. The shapes of the binding curves also proved significant. Ligand binding with WT suggested that a mixture of high and low affinity receptor states were present, indicated by a shallow Hill slope (n_H < 1). Conversely, ligand binding to D2.50A exhibited not only a decreased affinity, but also a shift of Hill slope from less than unity (mixed population of receptor states) to unity or greater. These data suggest that ligand binding with D2.50A is to an uncoupled receptor.

Ligand-receptor activation of G protein turnover was evaluated following binding studies. In agreement with the binding data hypotheses, none of the four compounds stimulated coupling to $[^{35}S]$-GTPγS with D2.50A, unlike their robust stimulation of coupling over basal observed with WT. This finding further suggested the critical nature of D2.50 allosteric regulation of hD_{2L} signaling. Second messenger endpoints were subsequently studied, with the hypothesis that D2.50A would inhibit agonist activation of these pathways. As expected, none of the four test compounds activated any of the functional endpoints

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measured with D2.50A whereas each of the four test compounds robustly activated these functional endpoints with hD_{2L} WT. Together, these data provide overwhelming evidence to support the critical role of D2.50 in regulating ligand-induced conformational changes that activate second messenger pathways coupled to D_{2L}.

Complete loss of G protein coupling and subsequent function could be explained by our choice of an alanine point mutant. Replacement of aspartate with alanine removes the polar carboxyl group that interacted with Na^+ to affect binding affinity, hence the decreased affinity observed with D2.50A. Removal of this charge however, could also significantly affect the intra- and interhelical interactions that take place between D2.50 and the surrounding amino acids of hD_{2L}. While receptor binding with antagonist did not reveal any significant changes in affinity, such studies would not provide information about the loss of subtle contact points that could be critical for conformational changes associated with receptor-G protein coupling. Replacement of the –OH moiety with $-NH_2$ (D2.50N) or extension of the carbon chain (D2.50E) might have served as better substitutes than alanine (D2.50A). D2.50E or D2.50N might have provided further information about the H-bonding interactions and steric interactions respectively in addition to information about binding affinity, G protein turnover, and activation of second messenger pathways. The data herein, however, suggest that removal of the aspartic side chain significantly impairs turnover and activation of second messenger pathways regardless of the type of ligand (functionally selective or endogenous) selected for study.

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CHAPTER 7. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

SUMMARY OF STUDIES CONDUCTED

Overview of original goals

A firm understanding of ligand-specific interactions with particular receptor locales and their subsequent effects on second messenger signaling is critical for elucidating mechanisms of functional selectivity. The initial hypotheses for this research came from an existing computational model that led to experiments using lines of CHO stably expressing D_{2L} WT and mutant receptors. A set of structurally diverse and rationally selected probe ligands [including functionally selective ligands (RNPA, SNPA, DNS, DNX, DHX), partial agonists (3-PPP, SNPA), and endogenous and prototypical full agonists (dopamine, quinpirole)] were used in the testing of the specific hypotheses. The resulting data are the first to provide evidence showing how specific regions of a receptor can modulate, in a differential fashion, activation of individual effector pathways mediated through a single receptor isoform. The work of this dissertation provides evidence which lays the groundwork for future studies that include isolation of ligand-specific G protein activation and further design of pathway-selective ligands.

Analysis of TM5 serine residues

The focus of Chapters 2 and 3 addressed the role of each of the three conserved serine residues of TM5. Previous studies conducted with these serines suggest that S5.42 and S5.46 are of significance for dopamine agonist interactions with hD_2 receptors (Mansour et al.,

1992; Cox et al., 1992; Woodward et al., 1996; Wiens et al., 1998). My studies provide results to suggest that these two serines are critical for activation of signaling pathways via effects on direct ligand-residue interactions. Analysis of second messenger pathways with S5.42A mutant provided evidence to support the critical necessity of this receptor-ligand interaction with most all test compounds at AC inhibition (quinpirole and RNPA are unaffected). Activation of MAPK with S5.42A, however, was critical for some ligands (dopamine, DNS, DNX, SNPA, and 3-PPP) but not RNPA, quinpirole, and DHX, whereas S5.42 was critical for activation of AA-release with all ligands but RNPA and quinpirole. RNPA, quinpirole, and 3-PPP exhibited intrinsic activity for AC inhibition similar to WT with S5.46A, whereas all other ligands exhibited loss of function. Activation of MAPK with S5.46A significantly impaired DNS, DNX, DHX, and dopamine, but had minimal affects on RNPA, SNPA, quinpirole and 3-PPP. Conversely, S5.46A significantly impaired agonist potentiation of AA-release for all ligands but RNPA and quinpirole. Binding and coupling data do not correlate in a one-to-one fashion with functional observations, suggesting that each ligand interaction with receptor is unique and this interaction may or may not subtly affect ligand-induced activation of second messenger pathways.

The other serine located in this region of TM5, S5.43, also has been suggested as critical for dopamine and p-tyramine interactions (Cox et al., 1992). Our modeling data and my experiments suggest, however, that S5.43 is not directly involved in interactions with these ligands, but instead plays a role in receptor stabilization and/or activation. The S5.43A mutant significantly affected binding of all ligands, but had minimal affects on GTPγS, AC inhibition, and MAPK. Conversely, S5.43A exhibited differential affects on Arachidonic acid release (loss of function for dopamine, DNS, and DHX, but minimal affects on the other

ligands studied). These findings suggest that this mutation results in a receptor with which most "typical" D_2 agonists that tends to have a high degree of functional selectivity. One implication of these data is that the mutation results in different patterns of induced conformations than WT receptor, suggesting that each ligand-specific receptor state uniquely affects activation of second messenger pathways.

Analysis of TM3 threonine residue

Chapter 4 addressed the role of a novel threonine residue in TM3. Previous literature utilizing SCAM techniques suggested that T3.37 was not water-accessible to antagonist binding (Javitch et al., 1995a). Molecular modeling studies conducted with our agonists suggested otherwise. Results from these studies suggest that not only is T3.37 critical for binding to select ligands (RNPA, quinpirole, dopamine, DNS, DNX), but that function is significantly impaired with the mutant receptor. Only RNPA and quinpirole exhibit intrinsic activity for the second messengers assayed with T3.37A. All other ligands exhibit complete loss of function. Interestingly, SNPA and dopamine markedly increased GTPγS binding, but lose second messenger activity. We do not however understand what this means. It is conceivable that T3.37A impairs SNPA and dopamine coupling to inhibitory G proteins but that T3.37A allows promiscuous coupling to other G proteins. Subsequent studies have yet to be conducted to address these observations but should be considered.

Structural inference from RNPA and quinpirole interactions with T3.37 and S5.46 suggests the hypothesis that T3.37 might not only be critical for binding, but could involve a significant interaction between the helices of TM3 and TM5. The working hypothesis is that T3.37 and S5.46 interact across the receptor pocket, H-bonding in the absence of ligand. Mutagenesis to either residue removes a key interaction between TM3 and TM5, rendering

the receptor inactive. RNPA and quinpirole interact in the pocket differently than our other test ligands, and mutagenesis has minimal affect on ligand interaction between TMs 3 and 5 and subsequent coupling and second messenger function is maintained. This hypothesis needs to be tested further, as understanding of the structural implications of T3.37 may provide insight into mechanisms of receptor activation, and the rational design of functionally selective compounds.

Analysis of TM6 histidine residue

Chapter 5 addressed the role of the TM6 histidine residue. The 6.55 position is critical for Class A GPCRs. Direct agonist interaction at this locus has been shown for the β_2AR, GalR1, and for bovine rhodopsin (Nakayama and Khorana, 1991; Zhang and Weinstein, 1994; Wieland et al., 1996; Farrens et al., 1996; Gether et al., 1997; Berthold et al., 1997; Sheikh et al., 1999). Although no dopamine agonist in this study was hypothesized to interact with H6.55, there are data to support an interaction between H6.55 and dopamine antagonists (Woodward et al., 1994). Additionally, recent studies have provided evidence to support H6.55 and H6.60 as the zinc-sensitive sites of hD_{2L} (Liu et al., 2006). Such data suggest the hypothesis that H6.55 can influence receptor conformational changes associated with second messenger signaling. My studies with H6.55A suggest that RNPA and dopamine receptor binding is affected, whereas that of quinpirole and DHX are not. Agonist-induced coupling to GTPγS is minimally affected at best. Conversely, AC inhibition and AA-release with H6.55A are completely lost, suggesting the critical necessity of H6.55 for receptor activation. Interestingly, H6.55A had no effect on ligand mediated MAPK activation vs. WT, but significantly decreased intrinsic activity for quinpirole. These data, again, seem inconsistent with the notion of limited numbers of discrete active states.

Analysis of TM2 aspartate residue

Chapter 6 addressed the role of the highly-conserved TM2 aspartate residue D2.50. After the sodium and pH sensitivity of D2.50 was discovered (Neve et al., 1991), D2.50 was hypothesized to form direct contacts with dopamine agonists. With the availability of the first GPCR crystal structure (Palczewski et al., 2000), homology analysis made it likely that D2.50 was quite distant (> 10 Å) from the ligand-binding site. Nonetheless, the report of allosteric regulation of binding affinity by sodium (Neve et al., 1991) led to studies with many GPCRs. The latter work suggested that D2.50 was differentially involved in receptor activation, that is, mutagenesis abolished activation of one pathway while maintaining WT-like activation of a second pathway (Surprenant et al., 1992; Roche et al., 1999). My data suggest that D2.50 is critical for receptor mediated activation of all functional pathways. Mutagenesis decreased affinity and shifted receptor binding curves ($n_H < 1$ with WT, $n_H > 1$ with D2.50A). GTPγS binding studies exhibited complete loss of coupling consistent with the binding data (increased Hill slopes), and subsequent activation of second messenger pathways was lost. Together, these data support a critical role for D2.50 regulation of ligand-induced conformational changes that activate second messenger pathways coupled to D_{2L}.

Implications of this work

The work presented in this dissertation provides a wealth of new information that broadens our understanding of how ligand-receptor interactions affect function. Previous work, including extensive SCAM studies, provided a plethora of data to characterize the ligand-receptor interactions of hD_{2L}. Built on this foundation, my research focused on how this affected signaling through multiple second messenger endpoints, an approach that is

vital to an in-depth understanding the mechanisms of activation, and ultimately, the mechanisms of functional selectivity. My research suggests that rather than digital activation of a few active states of a receptor, that each ligand induces a landscape of conformations unique to that ligand-receptor complex. For example, the S5.43A mutant alters ligand-induced coupling of G protein such that heterotrimeric subunits signaling through this mutant do not potentiate AA-release (for dopamine, DNS, and DHX), yet activate AC and MAPK similar to WT (for these same ligands). Signaling at all endpoints with S5.43A was unhindered for the other agonists tested in this dissertation. These data suggest that individual ligands can selectively induce a conformation of the receptor that preferentially activates a single effector pathway while turning off other effector pathways. The potential for drug discovery with functionally selective ligands is very promising. To understand the specific heterotrimeric components activated and their direct links to physiological processes would significantly enhance future therapeutics.

RELATED STUDIES

Structural changes involved in GPCR activation

Many studies have focused on the mechanisms involved in GPCR activation. Receptor mutagenesis can provides evidence to test hypotheses about specific interactions between ligand-receptor complexes, residue-residue contacts, and residue-G protein complexes (Shi and Javitch, 2002). The availability of the GPCR crystal structure of bovine rhopdopsin (Palczewski et al., 2000) and genome analysis of sequence alignments has facilitated molecular structural studies by providing a roadmap of residues for study. This interface has been further implemented, combining mutagenesis via SCAM (see Shi and

Javitch for review) with rational design of homology models of other GPCRs (Ballesteros et al., 2001b). This multicollaborative effort has made possible the rigorously studied and continuously tested molecular model of hD_{2L} that was a foundation of my research. One of the major weaknesses of receptor mutagenesis studies, however, has been the difficulty of separating mutation-induced changes on ligand interaction from mutation-induced changes to receptor structure. In many cases, these alternate (although sometimes related phenomena) and can be resolved by the data, but in other cases this is difficult. I believe my research has shown that the analysis of multiple functional endpoints, although experimentally painful, markedly aids in the discrimination of these two alternate mechanisms.

Further mutagenesis studies have elucidated key receptor residues that participate in conformational changes (upon ligand binding) that activate the receptor. Termed the "ionic lock," D3.49, R3.50, and E6.30, located at the cytoplasmic ends of TM3/TM6 are responsible for activation of the β_2AR (Ballesteros et al., 2001a). Additionally, studies have suggested that C6.47, W6.48, and F6.52 are involved in modulating a TM6 proline kink. These data provide evidence to support rotamer changes with these residues in what has been termed a "rotamer toggle switch" mechanism of receptor activation (Shi et al., 2002).

One way to explore some of the underlying mechanisms in a direct fashion is to utilize fluorescent labeling of receptor residues. Such studies have been conducted with β_2AR whereby a sulfhydryl-reactive fluorescent probe (fluorescein maleimide, FM) has been attached to a cysteine residue (Cys-265) in the carboxy-terminal region of IC3. Agonist activation leads to conformational changes that are manifested as changes in fluorescence characteristics that are proportional to the biological efficacy of the agonist. This technology was used to observe a rotation and/or tilting of TM6 upon agonist activation (Ghanouni et al.,

2001b). Further use of FM provided evidence to support ligand-induction of distinctly different conformations for partial agonist, full agonist, and antagonist ligands (Ghanouni et al., 2001a). One additional step was implemented such that structurally related catechol derivatives could induce ligand-specific structural interactions with the receptor, essentially isolating unique conformational states (Swaminath et al., 2004). Probing the differences in binding and activation with agonists and partial agonists of β_2AR suggested that GPCR activation is a multistep process (Swaminath et al., 2005). In my view, these studies provide strong support for ligand-selective activation of unique receptor states.

Crystallographic studies can, under optimal conditions, provide conclusive evidence for such structural interactions. Unfortunately, the only available data are with a protein (bovine rhodopsin) that is in the same superfamily, but does not have dissociable ligands, and for which only an inactive state structure exists. Fortunately, functional selectivity is not limited to GPCRs. There exists a subset of ligands (e.g. tamoxifen and raloxifene) that act upon estrogen receptors (nuclear receptors) as functionally selective ligands, although that phenomenon has been termed "selective estrogen receptor modulation" (SERM; Dutertre and Smith, 2000). Crystallographic studies with estrogen receptors bound to SERM ligand have provided a wealth of evidence to suggest that ligand-specific receptor conformational states exist (Shiau et al., 1998; Pike et al., 1999). Structural studies conducted with both ERα (2.03 Å) and ERβ (0.93 Å) provide evidence for induction of distinctly different conformational states by ligands of various classes including full agonists, partial agonists, and antagonists. In addition, these studies provide conclusive evidence to support a physiological role for ligand-induced conformational states. What currently is not understood, however, is how

these unique ligand-receptor sub-states affect functional measurements with G protein-coupled receptors.

Functional Selectivity *in vivo*

Data presented in this dissertation provides overwhelming evidence to support functional selectivity *in vitro*. It is imperative, however that functional selectivity be demonstrated *in vivo* if physiological relevance is to be established. Indeed, as was summarized in Chapter 3, my host laboratory entered the arena of functional selectivity kicking and screaming when it was found that dihydrexidine (DHX), a ligand originally designed to be a D_1 dopamine agonist showed functional selectivity in rat brain preparations (Mottola et al., 1991); (Mottola et al., 2002). Studies in preparations not confounded by other dopamine receptors, such as pituitary lactotrophs and MN9D cells confirmed, showed conclusively that DHX, as well as a D_2-selective analog propylDHX, were indeed functionally selective. This provided clear evidence for the unexpected behavioral responses observed with propylDHX in which there were dose-dependent decreases in locomotion as opposed to the increases expected for dopamine agonists (Smith et al., 1997). These data make a compelling case for functional selectivity *in vitro* being able to predict novel effects *in vivo*, although it would be very interesting to determine the relative behavioral effects of DHX, propylDHX, and a typical drug like quinpirole, in D_2-receptor knockout mice.

A particularly controversial area of direct relevance relates to the recently introduced antipsychotic drug aripiprazole (Abilify). Unlike the other antipsychotics available on the market, aripiprazole exhibits no significant extrapyramidal side effects (e.g. akathisia, dystonia, parkinsonism, and tardive dyskinesia), no hyperprolactinemia, and no significant weight gain compared to placebo (Kane et al., 2002). Because aripiprazole is a high affinity

D_2 ligand (Lawler et al., 1999; Shapiro et al., 2003), this is surprising because all other high affinity and highly selective D_2 antagonists cause a characteristic side effect profile that includes akathisia, dystonia, and parkinsonism. Preclinical studies showed that aripiprazole had unique behavioral and neurochemical actions, such that it activated presynaptic D_2 autoreceptors, whereas it antagonized D_2 postsynaptic receptors (Kikuchi et al., 1995). The developers of the drug have postulated that this novel clinical profile is due to the fact that aripiprazole is a D_2 partial agonist. They theorize that aripiprazole would boost dopamine signaling in areas of hypofunction, and decrease dopamine signaling in regions of hyperfunction (Burris et al., 2002). An alternate hypothesis that has been proposed, and for which strong supporting data using a variety of functional endpoints, is that aripiprazole is a functionally selective D_2 ligand (Lawler et al., 1999; Shapiro et al., 2003; Urban et al., 2006b). It seems quite clear that the behavioral and neurochemical data of Kikuchi et al. (1995) can only be explained by functional selectivity, not partial agonism. These data suggest that further understanding of the complex signaling mechanics of aripiprazole could significantly enhance future drug discovery endeavors in this realm of central nervous system therapeutics.

FUTURE STUDIES

The primary goal of this dissertation was to explore mechanisms related to the phenomenon of functional selectivity, knowing that such studies would also impact our understanding of mechanisms of receptor-mediated/signaling. These studies tested the hypothesis that selective interactions between ligand and critical receptor residues would differentially affect signaling through hD_{2L}. There are at least three major conclusions that I have drawn from my experiments. First, that specific interaction between ligand and receptor

significantly affect signaling patterns observed through a single receptor isoform. Second, that mutagenesis of receptor can create an artificial system in which either the endogenous ligand or non-functionally selective drugs become functionally selective. Last, that functional selectivity reflects the dynamic nature of ligand-receptor interactions that might be considered to reflect an energy landscape unique for a given ligand and its signaling complex. These ideas suggest a variety of follow-up directions for future experimentation.

Further mutagenesis studies

The mutations discussed in this dissertation were based upon hypotheses generated from molecular modeling techniques. Except for H6.55 and S5.43, the other target residues were hypothesized to form direct contact with ligands. There are however, other receptor residues in the active site that could indirectly affect binding of our ligands. The phenylalanines F6.51 and F6.52 are solvent accessible (Javitch et al., 1998) residues that comprise part of the TM6 aromatic amino acids that have been hypothesized as critical for receptor activation (Zhang and Weinstein, 1994; Farrens et al., 1996; Gether et al., 1997; Sheikh et al., 1999; Floresca and Schetz, 2004). Previous mutagenesis studies support F6.51 interaction with dopamine through orthogonal pi-stacking (Floresca and Schetz, 2004). F6.51 and F6.52 are good candidates for extensive mutagenesis studies and characterization of receptor signaling. Further understanding of these subtle interactions (whether direct or indirect) will add to our knowledge base of ligand-induced receptor activation.

The results discussed in Chapter 4 of this dissertation support a novel interaction not previously supported in the literature. SCAM studies concluded that T3.37 was not accessible to the binding site crevice (Javitch et al., 1995a), but our molecular modeling did lead to hypothesized interactions with ligands, a hypothesis supported by some of the current

data. What made this residue interesting, however, was that only two ligands (quinpirole and RNPA) exhibited functional activity. These findings and data with S5.46A suggested that some interhelical interplay might take place. Such data lead to the hypothesis that S5.46 and T3.37 may form an H-bond across the pocket of the active site. Altering this interaction through mutagenesis leads to loss of signaling for most all ligands except quinpirole and RNPA. This hypothesis could be tested by a reciprocal double-mutant (i.e., T3.37S and S5.46T), and this is currently being studied, utilizing quinpirole and RNPA as test ligands. Increased understanding of TM3 and TM5 interactions at this location in the active site could alter the approaches used to design dopamine agonists.

Involved signaling partners

As functional selectivity became a focus of our laboratory, mechanistic studies begun. One obvious target was the specific heterotrimeric partners that couple to receptors activated with functionally selective ligands. These studies were conducted by testing ligand-induced activation of GTPγS coupled to an immunoprecipitation pulldown assay with antibodies selective for individual $G\alpha_{i/o}$ subunits. Unfortunately, these studies yielded minimal results due to poor selectivity of currently available antibodies. Such studies are crucial if we are to understand ligand-selective activation of heterotrimeric G proteins.

Another viable approach for determining G protein specificity involves the use of fusion constructs. CHO cells used in this dissertation contain three inhibitory α-subunits (Gerhardt and Neubig, 1991), three β-subunits, and ten γ-subunits (unpublished results) that contribute to the diverse signaling profiles observed with functionally selective ligands. C-terminal peptides are of interest because previous work suggests that the C-termini of α-subunits form direct contacts to receptors (Hamm et al., 1988). C-terminal peptide fusion

constructs of each α-subunit can be generated and tested for differences in binding affinity in the presence of different functionally selective ligands. Less structural information is known for β- and γ-subunits, and thus more work must be conducted to understand the structural interaction with receptor before screening with peptide constructs can be conducted. After isolating the specific receptor-Gα interactions, further work with reconstitution systems, and the total G protein heterotrimer would need to be done. An understanding of ligand-selective activation of specific heterotrimeric components at the molecular level would provide a wealth of data to assist screening techniques for selection/or design of future drugs.

Energy landscape visualization

Individual components of a ligand have been studied with fluorescent probes, isolating the kinetic role of each component in the activation of effector pathways (Swaminath et al., 2004; Swaminath et al., 2005). These studies and earlier work provided evidence to support distinct conformational changes for partial agonists, full agonists, and antagonists (Ghanouni et al., 2001a; Ghanouni et al., 2001b; Cohen et al., 2005). In light of the previously discussed energy landscape hypotheses outlined in this dissertation and the data to support this hypothesis, it would be of interest to study our functionally selective ligands using fluorescent techniques. One has to ask just what type of spectrum a functionally selective ligand would exhibit? How would the endogenous ligand, which can be made functionally selective with receptor mutant, be affected? My hypothesis is that each ligand would exhibit a unique fluorescence profile in a manner similar to my functional data. If that were the case, not only would the differential activation of effector pathways be observed quantitatively (this dissertation), but the actual induction of an energy landscape of conformations unique to each ligand-receptor complex would be observed. Further studies

that seek to parse the subtle kinetic differences between activation of one pathway over another with fluorescent analyses would also provide further visual evidence that distinct conformational states do exist, and that no distinct set of rigid states mandates signaling through GPCRs.

Behavioral profiling

A great deal of *in vitro* research is required before we can fully understand how a single ligand can interact with its target, induce a unique conformational state that selectively couples to a specific heterotrimeric unit, and activate a single effector pathway preferentially over another. The translation of this *in vitro* data to *in vivo* studies is a giant leap that we are not ready to tackle, however, the data within this dissertation offers some interesting places to start. Current technology provides one clear direction by which my research might be translated into physiologically-relevant systems. One would be to test some of the most important hypotheses using transgenic mice. Assume for example, that a specific mutation caused a marked change in the signaling profile of one ligand. Using an example I cited in the Discussion of Chapter 3, the S5.43 mutation completely abolishes AA signaling for dopamine, SNPA, DNS, and DHX, but not for RNPA, quinpirole, and DNX. The signaling of these compounds at other pathways (e.g., AC and MAPK) was largely unaffected. Thus, one could prepare a transgenic mouse expressing S5.43 rather than the WT D_2 receptor. One could then use a variety of levels of analysis ranging from overall behavioral responses, to cellular response (e.g., isolated lactotrophs) to single unit recordings (e.g., in the substantia nigra) to ascertain whether the change in signaling *in vitro* translated into effects *in situ*. Of course, it would be equally of interest (if unexpected) if the development of the mice was

affected markedly. This would suggest that those pathways no longer activated by dopamine (AA in the S5.43A example) have a critical development role.

Ramifications of this work

The work detailed in this dissertation provides an abundance of new information to suggest that functional selectivity not only exists, but that there are distinctly different mechanisms involved with such signaling patterns than that currently accepted by the general scientific community. If we are to make progress in this ever increasing world of pharmacology, the interface between rational drug design, receptor screening, and detailed pharmacological analyses must be reevaluated. At present, ligand-based and structure-based drug design can suggest new compounds that then are tested for functionality through a variety of screening processes. Missing from current approaches is consideration for the complexity if signaling through a single receptor. Generally, new compound screening involves only binding affinity, often with studies of a single effector pathway (e.g. for hD_{2L} receptors AC inhibition is the gold standard). There are two inherent weaknesses in this strategy that can be illustrated using the D_{2L} receptor as an example. If one is looking for an agonist, and if a "hit" in a binding assay fails to inhibit AC, one would assume the compound is completely inactive. Indeed, some such "rejected" compounds may actually have useful activity at another D_{2L}-$G\alpha_{i/o}$ regulated pathway. The potential of such functionally selective drugs thus will never be known. It seems to me that in the future, doing basic research to elucidate the signaling pathways important to the physiological processes modulated by a given receptor would provide the necessary foundation for truly rational drug design.

Another implication of my work relates to a common strategy of developing high-throughput screens (HTS) by creating receptor chimeras that are amenable to devices like

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FLIPPR. One common approach is to convert a $G\alpha_{i/o}$ receptor into a $G\alpha_q$ receptor that will then couple to calcium mobilization that can be assessed by fluorescence. Although very cost effective, my research would suggest that such chimeric receptor systems are very artificial, and may markedly alter the signaling profile of a given ligand. Although this may be of lesser importance when looking for neutral antagonists, it may be crucial if agonist-type ligands are being sought. On the other hand, if one is seeking new functionally selective ligands, the use of a selective mutational strategy with key target residues (such as employed in this research) may be of potential value as one may be able to detect such functional differences that might not be found using only the WT receptor. In its entirety, my work suggests that there is both richness and subtlety in how ligands may activate multiple signaling pathways that is scientifically interesting, and may have profound impact on drug discovery and the approaches of medicinal chemists.

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