STRUCTURAL BASIS FOR LIGAND-BINDING AND ACTIVATION OF D_1-LIKE DOPAMINE RECEPTORS

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ABSTRACT

JUSTIN T. BROWN: STRUCTURAL BASIS FOR LIGAND-BINDING AND ACTIVATION OF D_1-LIKE DOPAMINE RECEPTORS

(Under the direction of Richard B. Mailman, Ph.D.)

The D_1-like dopamine receptors have been implicated in the etiology of several neurological and psychiatric disorders. Recent advances in neurobiology have demonstrated the potential utility of D_1-like dopamine receptor agonists as therapeutic compounds. Despite immense promise, there are no D_1 centrally available agonists currently available as therapeutic compounds. Moreover, there are no selective ligands that can distinguish between the two D_1-like receptors (D_1 and D_5). One of the major obstacles to the discovery of such agents is limited information about the structural basis for ligand-binding and activation of the D_1-like receptors. There are few such studies that have been done with the D_1 receptor, and virtually none with the D_5 receptor. This dissertation was aimed at gaining a greater understanding of the structural mechanisms necessary for ligand-binding, receptor activation, and receptor internalization. Rationally-selected point mutations of the D_1-like receptors were made, and detailed analysis of binding and function made for a series of structurally and functionally diverse test compounds. Work in this dissertation provides the first experimental evidence that T3.37 plays an important role in binding and activation of D_1-like receptors. Studies of a TM6 phenylalanine at residue at position 6.51 revealed that this residue plays a key role in coupling ligand binding to receptor activation. Studies of another aromatic residue

located in TM6, W6.48, provided evidence that this amino acid serves as an important switch residue for creating an active receptor conformational state. Furthermore, this work revealed what may be the largest structural differences ever seen between the D_1 and D_5 receptor subtypes. Results from this dissertation provide important insight into the structural mechanisms that govern ligand-binding and receptor activation, and may aid in the design of clinically relevant D_1 agonists.

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 summary chapter, and an appendix with a listing of common 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 *Molecular Pharmacology*.

Current publications related to this work

Ryman-Rasmussen JP, Griffith A, Oloff S, Vaidehi N, Brown JT, Goddard WA 3rd, Mailman RB. Functional selectivity of dopamine D_1 receptor agonists in regulating the fate of internalized receptors. Neuropharmacol. 52: 562-575, 2007

Brown JT, Kant AC, Mailman RB. Rapid, semi-automated, and inexpensive radioimmunoassay of cAMP: Application in GPCR-mediated adenylate cyclase assays. J Neurosci Methods 2008 (in press).

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I want to thank my family and friends who have provided support, encouragement, and relaxation throughout these years.

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4-Me-DHX	4-methyl-dihydrexidine, (4-methyl-trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[*a*]phenanthridine)
5-HT	5-hydroxytryptamine (serotonin)
A68930	1-aminomethyl-5,6-dihydroxy-3-phenylisochroman
AC	adenylate cyclase
cAMP	cyclic AMP; adenosine 3',5'-cyclic monophosphate
COMT	catechol-O-methyl-transferase
CP	caudate putamen
DA	Dopamine
DHX	dihydrexidine; (trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexa-hydrobenzo[*a*]phenanthridine)
DOPAC	Dihydroxyphenylacetic acid
EC50	effective concentration at 50% receptor occupation
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
HVA	homovanillic acid
IBMX	Isobutylmethylxanthine
$K_{0.5}$	apparent affinity constant ($n_H < 1.0$)
KCl	potassium chloride

MAO	monoamine oxidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	nucleus accumbens
NE	Norepinephrine
n_H	Hill coefficient
OT	olfactory tubercule
SCH23390	7-chloro-8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SKF38393	7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SN	substantia nigra
V_{max}	maximum enzyme activity
VTA	ventral tegmental area

CHAPTER 1. INTRODUCTION

DOPAMINE RECEPTORS: BIOLOGY AND PHARMACOLOGY

History of dopamine systems

Until the late 1950's dopamine was considered to function solely as an intermediate in the synthesis of epinephrine and norepinephrine. Arvid Carlsson discovered an important role for dopamine in 1957 (Carlsson *et al.*, 1957), and subsequent work that demonstrated localization of dopamine in the basal ganglia led Carlsson to suggest that dopamine depletion was responsible for parkinsonism (Carlsson *et al.*, 1958). Biochemical studies of dopamine receptors began in the 1970's (Iversen, 1975), with a direct biochemical mechanism linking dopamine to the stimulation of cAMP production first being demonstrated in the laboratory of Paul Greengard in 1971 (Kebabian and Greengard, 1971). Greengard's laboratory showed that dopamine could dose-dependently stimulate the accumulation of cAMP, and an ensuing study demonstrated that this response could be inhibited by antipsychotic drugs (Clement-Cormier *et al.*, 1974). In the mid-to-late 1970's, experiments utilizing new antipsychotic agents (i.e. butyrophenones and substituted benzamides) resulted in discrepancies between the experimental data that was obtained and the hypotheses held by most investigators (Kebabian, 1977). These new antipsychotic drugs bound receptors with low affinity and exhibited little potency in inhibiting dopamine stimulated adenylate cyclase (Garau *et al.*, 1978; Trabucchi *et al.*, 1975). These observations, along with parallel studies characterizing dopamine receptor localization, led to the notion of the existence of

two distinct subtypes of dopamine receptors, D_1 and D_2. (Garau *et al.*, 1978; Kebabian and Calne, 1979). The D_1 receptor, the original receptor reported by Greengard's group, linked to the stimulation of adenylate cyclase and bound thioxanthines and phenothiazine antipsychotics with high affinity, but had lower affinity for the butyrophenone and benzamide classes (Garau *et al.*, 1978). The D_2 receptor exhibited high affinity for the butyrophenones and benzamides, but was not linked to the stimulation of adenylate cyclase.

Dopamine neurotransmission

Dopamine is the predominant neurotransmitter in the mammalian brain, where it contributes to the regulation of motor and limbic processes. Dopamine systems in the brain originate from cells bodies in three brain regions: the substantia nigra, the ventral tegmentum, and the hypothalamus.

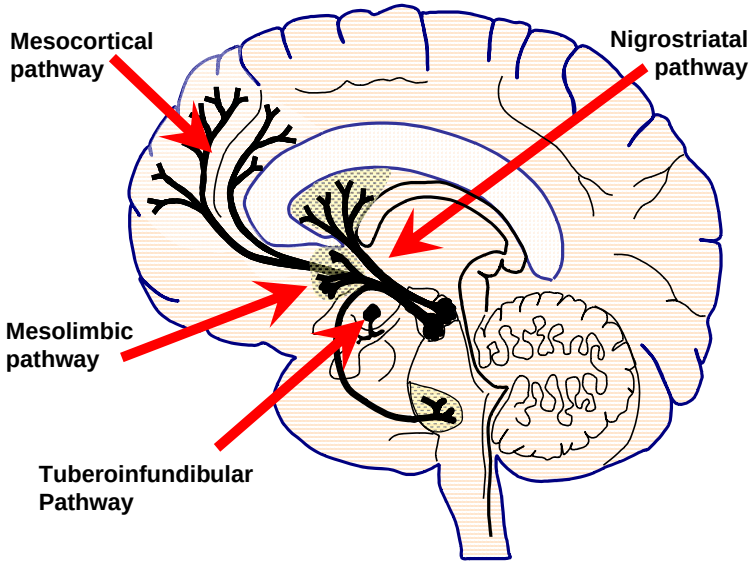


Figure 1.1. Schematic of brain dopamine pathways.

There are four major dopaminergic systems in the brain (Figure 1.1): (i) The nigrostriatal pathway projects from the substantia nigra to the dorsal striatum and is the

pathway that degenerates in Parkinson's disease. (ii) The mesolimbic and (iii) mesocortical pathways include those neurons that project from the ventral tegmental area (VTA) to regions of the limbic system (e.g. nucleus accumbens and amygdala) and prefrontal cortex. The mesocortical system is thought to be involved in motivation and emotional response, while the mesolimbic system is associated with feelings of reward and desire. Finally, the (iv) tuberoinfundibular pathway comprises those neurons that extend from the hypothalamus to the median eminence and is responsible for controlling prolactin levels (Cooper *et al.*, 1996).

Dopamine biosynthesis and metabolism

Like all catecholamines in the nervous system, dopamine synthesis originates from the amino acid precursor tyrosine. L-tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase, representing the rate-limiting step in dopamine biosynthesis. Subsequent removal of the carboxyl group by L-aromatic amino acid decarboxylase converts L-DOPA to dopamine. Tyrosine hydroxylase is susceptible to endogenous mechanisms of- as well as pharmacological-regulation. Dopamine is converted to dihydroxyphenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO) following reuptake by the nerve terminal. Dopamine that is not taken up by the dopamine transporter is degraded by the extracellular enzyme catechol-*O*-methyltransferase to homovanillic acid (HVA), the major brain metabolite in primates and humans.

Classification of dopamine receptors

G protein-coupled receptors (GPCRs) are commonly divided into five or six distinct classes based on sequence homology: Class A (rhodopsin-like), Class B (secretin

family), Class C (metabotropic glutamate/pheromone), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors), and Class F (frizzled/smoothened). Dopamine receptors, members of the rhodopsin-like subfamily of GPCRs, are comprised of five distinct dopamine receptor genes divided into two classes, D_1-like and D_2-like dopamine receptors. D_2-like receptors, consisting of D_2, D_3, and D_4, couple to inhibitory G-proteins leading to the inhibition of adenylate cyclase activation. Members of the D_1-like dopamine receptor family, referred to as D_1 and D_5, preferentially couple to stimulatory G-proteins resulting in an increase in cAMP accumulation.

Molecular biology of D_1-like dopamine receptors

The D_1 receptor was cloned in 1990 (Dearry *et al.*, 1990; Sunahara *et al.*, 1990; Zhou *et al.*, 1990) and the D_5 subtype the following year (Sunahara *et al.*, 1991; Tiberi *et al.*, 1991). The D_1 receptor is localized on chromosome 5 and D_5 is on chromosome 4. In humans the D_1 and D_5 receptors consist of 446 and 477 amino acids, respectively. The receptors share approximately 60% acid amino identity overall, and 82% identity in the putative transmembrane-spanning regions (Jarvie and Caron, 1993). The D_5 receptor displays higher constitutive activity than the D_1 receptor, which may account for its higher affinity for most agonists (Tiberi and Caron, 1994). Currently, no selective ligands are available that can distinguish between the D_1 and D_5 receptor subtypes.

The human D_1 receptor has two potential sites for N-linked glycosylation and several potential sites for phosphorylation by PKA, including Thr-136 and Thr-268. The human D_5 receptor has a potential N-linked glycosylation site at Asn-7 and a second potential site in the third extracellular domain (Asn-194). Potential sites for phosphorylation by PKA exist at Thr-153 and Ser-260.

Anatomical localization of D_1-like dopamine receptors

Three methods have been used to determine the distribution of dopamine receptors in the body: i) receptor binding, ii) immunological methods, and iii) mRNA localization experiments. Studies utilizing quantitative receptor autoradiography to map the distribution of D_1-like receptor binding sites within the brain revealed the highest levels of binding to be in the forebrain areas such as the caudate-putamen, olfactory tubercle, and nucleus accumbens (Boyson *et al.*, 1986; Savasta *et al.*, 1986; Wamsley *et al.*, 1991). Binding was also observed in the basal ganglia pathways, as well in several limbic areas including the dentate gyrus of the hippocampus and amygdaloid nucleus. The lack of a subtype selective ligand precludes the use of autoradiography to determine the distribution of each D_1 subtype. A study using D_1 receptor null mutant mice, however, showed putative D_5 binding sites in the hippocampus (Montague *et al.*, 2001).

Cloning of the D_1-like receptors enabled examination of D_1 and D_5 subtypes distribution using immunological and mRNA localization studies. D_1 receptor mRNA was found to be most abundant in the neostriatum, whereas the presence of D_5 receptor mRNA in the striatum is less than one-tenth the level of the D_1 receptor. High levels of D_5 receptor mRNA is present within other brain regions such as the hippocampus, hypothalamus, and mid-brain. The differential expression of D_1 vs. D_5 receptors in specific brain regions suggests that there may be a novel physiological role for the D_5 receptor.

D_1-like dopamine receptor signaling

D_1-like receptors and G proteins

The signaling pathway most commonly associated with the D_1-like receptors is increased synthesis of adenylate cyclase. This action occurs via activation of specific G proteins. The inactive forms of G proteins are heterotrimers composed of α, β, and γ subunits. At least 21 Gα subunits, encoded by 16 genes, have been identified in the human genome (Hurowitz *et al.*, 2000). The Gα units can be grouped into 4 main classes: $G\alpha_s$, $G\alpha_i$, G_q, G_{12}. The D_1-like receptors signal primarily through the activation of stimulatory G proteins, $G\alpha_s$ and $G\alpha_{olf}$, which activate adenylate cyclase leading to an increase in intracellular cAMP (Corvol *et al.*, 2001; Sidhu *et al.*, 1991). Studies of neostriatum, a region with high D_1 expression and low expression of $G\alpha_s$ and an abundance of $G\alpha_{olf}$, have demonstrated that D_1 receptors couple to $G\alpha_{olf}$ in this region of the brain (Le *et al.*, 1991; Watson *et al.*, 1994). $G\alpha_{olf}$ null mutant mice have a minimal response to dose-dependent adenylate cyclase activation and also lack behavioral responses attributed to D_1 receptor stimulation, thereby suggesting that $G\alpha_{olf}$ is the primary G protein coupled to D_1 in the basal ganglia nuclei (Corvol *et al.*, 2001; Zhuang *et al.*, 2000). Studies have shown that the D_5 receptor signals via $G\alpha_s$ (Kimura *et al.*, 1995), but there is no evidence to indicate that D_5 couples to $G\alpha_{olf}$ (Sidhu *et al.*, 1998; Sidhu, 1998). Recent *in vitro* studies have suggested that the D_1 receptor can couple to other G proteins, such as $G\alpha_z$, $G\alpha_o$, $G\alpha_{i1}$ and $G\alpha_{i2}$ (Sidhu and Niznik, 2000). The relevance of the ability of D_1-like receptors to couple to G proteins other than $G\alpha_s$ and $G\alpha_{olf}$ has yet to be determined.

To date, 6 Gβ and 12 Gγ subunit genes have been identified (Oldham and Hamm, 2008). Gγ subunits exhibit considerable structural diversity, while the Gβ subunits are

structurally similar. Although little is known about the influence of Gβ and Gγ subunits on receptor-G protein interactions, studies using reconstitution systems (Corvol *et al.*, 2001; Figler *et al.*, 1996; Kisselev *et al.*, 1994; Butkerait *et al.*, 1995) and reverse genetic approaches (Kleuss *et al.*, 1993; Wang *et al.*, 1997) indicate that the nature (i.e. subunit type and/or post-translational isoprenylation) of the Gγ subunit of the Gβγ dimer plays a role in receptor-G protein interaction. Using ribozyme-mediated suppression of Gβγ subunits, a recent study demonstrated that the depletion of $G\gamma_7$ in HEK293 cells reduces D_1 receptor stimulation of adenylate cyclase but not D_5 stimulation (Wang *et al.*, 2001). The role of other Gγ subunits in diverse cell systems has yet to be elucidated but it is clear that Gγ, and perhaps Gβ, subunits can be important for D_1 receptor signaling.

Regulation of adenylate cyclase

Regulation of adenylate cyclase underlies several CNS functions such as learning, synaptic regulation, and signal transduction. Nine adenylate cyclase (AC) isoforms have been identified to date, and each isoform contains a binding site for $G\alpha_s$. Cyclase activity can be modulated by $G\alpha_s$, $G\alpha_i$, and Gβγ subunits (Simonds, 1999). D_1-like receptors activate adenylate cyclase in multiple cell lines (Cumbay and Watts, 2004) by coupling to $G\alpha_s$ and $G\alpha_{olf}$. A study using neostriatal tissue demonstrated that type 5 adenylate cyclase mediates dopamine receptor signaling in the striatum (Glatt and Snyder, 1993). This observation was supported by studies of adenylate cyclase type 5 null mice that showed greatly diminished D_1 receptor stimulated cyclase activation (Lee *et al.*, 2002; Iwamoto *et al.*, 2003).

Stimulation of phospholipase C & D

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) producing 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 then binds to the IP_3 receptor, stimulating the release of Ca^{2+} from intracellular stores within the endoplasmic reticulum. DAG recruits protein kinase C (PKC) to the membrane leading to downstream NF-κB activation and actin reorganization.

Several studies have demonstrated D_1 receptor regulation of the phospholipase C/inositol triphosphate pathway (PLC/IP_3), but the mechanism(s) by which the D_1 receptor couples to the stimulation of phospholipase C is unclear. Two distinct potential mechanisms for D_1 linked PLC activation have been proposed. Investigators have postulated the existence of a novel D_1-like receptor, distinct from the G_s-coupled D_1 receptor, linked to PLC via $G_{\alpha q}$ (Pacheco and Jope, 1997; Undie and Friedman, 1990). However, the high concentration of agonist (100 μM) used to elicit the response in these studies, one at which the agonist may bind other receptors, confounds interpretation. Recent studies, demonstrating the ability of D_1 and D_2 dopamine receptors to form functional hetero-oligomeric units that rapidly generate PLC-mediated calcium release via $G_{\alpha q/11}$ (Lee *et al.*, 2004), provide evidence for a novel mechanism of PLC signaling through D_1 receptor oligomerization with the D_2 receptor (Lee *et al.*, 2004; Rashid *et al.*, 2007). Given the important role of calcium in neuronal function, this theory warrants further investigation.

Bergson and colleagues (Lezcano *et al.*, 2000) demonstrated intracellular calcium release when calcyon, a D_1 receptor interacting membrane protein, is co-expressed with D_1 and D_5 receptors. The authors suggested that calcyon functions as a molecular switch

enabling signaling through either adenylate cyclase or PLC at a single D_1 receptor, however the paper describing this observation was recently retracted (Lezcano *et al.*, 2006). Further evidence for a cAMP/PKA-independent signaling pathway was exhibited in studies with adenylate cyclase V deficient mice (Iwamoto *et al.*, 2003; Lee *et al.*, 2002). While 85-90% of cyclase activity is abrogated, locomotion is enhanced. It is not clear from this study whether the behavioral effects are due to a non-cyclase dependent PLC pathway.

A recent finding suggests that the D_5 dopamine receptor can regulate the activity and expression of phospholipase D_2 (PLD2) (Yang *et al.*, 2006). PLD2 and the D_5 receptor are membrane bound proteins localized in the brush-border membrane of renal proximal tubules (Exton, 2002), indicating a role for the D_5 receptor in the pathogenesis of hypertension.

Regulation of ion channels

D_1-like receptors modulate numerous voltage- and ligand-gated ion channels via phosphorylase kinase A (PKA) phosphorylation and stimulation of DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa) (Neve *et al.*, 2004). D_1-like receptor stimulation of cAMP production can lead to the activation of PKA, which can subsequently modulate ion channels by combinations of direct PKA-phosphorylation and DARRP-32 mediated inhibition of protein phosphatase 1 (PP1). DARRP-32, a neostriatum enriched signaling protein, is activated via PKA-stimulated protein phosphatase-2A (PP2A) and modulates ion channel function by inhibiting PP1 (Greengard *et al.*, 1999; Hemmings, Jr. *et al.*, 1984). The exact mechanism(s) by which D_1-like receptors regulate various ion channels is complex and ill-defined.

Regulation of other signaling pathways

Several reports indicate that D_1-like receptors activate mitogen activated protein kinases (MAPK) including ERK1/2 (Chen *et al.*, 2004), p38 MAPK, and c-jun amino-terminal kinase (Zhen *et al.*, 1998). Regulation of MAP kinase pathways appear to be PKA-dependent, but the prevalence of D_1 receptor-mediated MAP kinase activation remains unclear and is likely system-dependent *in vitro*.

Evidence suggests that D_1 receptors may modulate arachidonic acid (AA) release but data is ambiguous at best and requires further study. Piomelli *et al.* (1991) demonstrated that expression of D_1 receptors alone in CHO cells does not elicit AA release, but co-activation of D_1 and D_2 receptors in CHO cells resulted a synergistic response (Piomelli *et al.*, 1991). Release of arachidonic acid via D_1 receptors is likely dependent on co-activation of one or more additional receptors.

D_1-like receptors are capable of forming functional interactions with several other membrane bound receptors. Studies have shown that D_1 receptors can physically interact with NMDA receptors, as well as adenosine A_1 receptors (Gines *et al.*, 2000; Kreipke and Walker, 2004). D_5 receptors can form a functional complex with $GABA_A$ receptors, thereby enabling D_5 receptors to modulate synaptic strength independent of G proteins (Liu *et al.*, 2000). These data raise the possibility that D_1 compounds may not only have utility in the treatment of disorders linked to dopamine dysregulation but those diseases attributed to receptors with which they form hetero-oligomers as well.

D_1 receptor internalization

Internalization plays an important role in regulating D_1 receptor responsiveness. Internalization *in vivo* has been observed in striatal neurons under hyperdopaminergic

conditions in both rats (Dumartin *et al.*, 1998) and humans (Muriel *et al.*, 1999). The process has been well studied for dopamine in various cell lines, and is mediated by the GRK/arrestin pathway (Tiberi *et al.*, 1996; Zhang *et al.*, 1999). Phosphorylation of the D_1 receptor occurs within minutes of dopamine binding, allowing arrestin to bind to the third intracellular loop of the receptor thus leading to D_1 receptor internalization. Characteristic of "Class A" GPCRs, arrestin is not trafficked into the cell with the D_1 receptor (Oakley *et al.*, 2001). The D_1 receptor is recycled rapidly back to the cell surface following internalization caused by the endogenous ligand (Vargas and von Zastrow, 2004; Vickery and von Zastrow, 1999). However, recent studies in our lab have demonstrated differential ligand induced internalization and long-term trafficking with structurally diverse agonists. The experiments in this Dissertation explore the structural mechanisms responsible for this phenomenon.

Disorders of dopamine neurotransmission and the role of D_1-like receptors

Dopaminergic systems have been the subject of extensive research over the past 40 years as disruption of dopaminergic transmission has been implicated in the etiology of a variety of neurological and psychiatric disorders such as Parkinson's disease, Huntington's disease, dysfunction of learning and memory, schizophrenia, and attention deficit hyperactivity disorder (ADHD). While early research efforts focused on the role of D_2 dopamine receptors in disorders of dopaminergic transmission, the development of high affinity, full D_1 agonists has revealed an important role for D_1 receptors in neurological disease.

Parkinson's disease

Parkinson's disease is caused by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta. Ideally, Parkinson's disease would be ameliorated by prevention of disease progression or replacement of lost neurons; unfortunately such treatments are not available. The standard treatment available to Parkinson's disease patients is the use of pharmacotherapy to treat disease symptoms. The current gold standard, levodopa, is extremely effective in the early stages of the disease but loses efficacy and develops side effects after years of treatment. Observations that parkinsonism could be induced by typical antipsychotics, D_2 receptor antagonists, lead to the hypothesis that the beneficial effects of levodopa were due to actions at the D_2 dopamine receptor. As a result, research efforts have been focused on the development of D_2 receptor agonists which have failed to dramatically ameliorate symptoms of Parkinson's disease.

Further elucidation of the role of D_1 receptors in motor control led to the hypothesis that D_1 receptors may in fact have utility as a PD therapeutic. The design and synthesis of the first D_1 full agonist, dihydrexidine (Brewster *et al.*, 1990; Lovenberg *et al.*, 1989), permitted testing of the hypothesis that D_1 agonists will be an effective PD therapeutic. In 1991, Taylor *et al.* (1991) demonstrated that the beneficial effects of levodopa in the treatment of Parkinson's disease was in fact due to the activation of D_1 receptors, not D_2 receptors, resulting in the redirection of efforts towards the development of D_1 receptor agonists to treat Parkinson's disease (Mailman *et al.*, 2001). Subsequent studies in MPTP-treated non-human primates, as well as two human clinical studies (Rascol *et al.*, 1999; Rascol *et al.*, 2001), have confirmed the effectiveness of full D_1

agonists as a symptomatic treatment for PD (Kebabian *et al.*, 1992a; Shiosaki *et al.*, 1996).

Despite the tremendous promise of full D_1 agonists as a treatment for PD, there are no clinically available D_1 receptor-selective drugs. Numerous issues such as development of tolerance, lack of oral bioavailability, and a requirement for full agonism have hindered the development of D_1 receptor drugs. Several of these issues are explored by the work conducted for this Dissertation.

Memory and cognition

Stimulation of the prefrontal cortex potentiates neuronal signaling that is essential to the working memory process (Sawaguchi and Goldman-Rakic, 1991). D_1 receptors are the predominant dopamine receptor subtype (20 times the density of D_2 receptors) expressed in the prefrontal cortex in non-human primates (Goldman-Rakic *et al.*, 1990; Lidow *et al.*, 1991). In 1994, Sawaguchi and Goldman-Rakic demonstrated that local injection of a D_1 antagonist into the prefrontal cortex produced working memory deficits in rhesus monkeys, subsequent studies showed that D_1 agonists can improve cognitive function in rats (Hersi *et al.*, 1995) and non-human primates (Arnsten *et al.*, 1994; Cai and Arnsten, 1997). Dihydrexidine -- a compound used in the experiments in this Dissertation -- improved working memory in both aged monkeys and catecholamine depleted young monkeys (Arnsten *et al.*, 1994), and also caused improved performance in MPTP-lesioned non-human primates (Schneider *et al.*, 1994). Importantly, the effects of D_1 stimulation in cognition and memory are dose-dependent with higher doses impairing working memory in aged monkeys (Castner *et al.*, 2000). The exact mechanism(s) by which D_1 receptors affect cognition and memory is not fully

understood, but it is clear that the D_1 dopamine receptors have a prominent role in modulating cognitive performance.

Schizophrenia

The symptoms of schizophrenia can be grouped into positive (type I) symptoms, such as hallucinations, thought disorder, and delusions, and negative (type II) symptoms, which include poverty of speech, loss of drive and flattening of affect (Crow, 1980; Goldberg and Mattsson, 1967). Dopamine receptor antagonists, that primarily target D_2 receptors, have been the most widely used therapeutic agents in the treatment of schizophrenia over the last four decades. These antipsychotic drugs ameliorate the positive symptoms of schizophrenia but have little effect on the primary negative symptoms. These negative symptoms have been identified as the primary reasons patients experience such difficulty reintegrating into society (Holden, 2003).

Evidence that the negative symptoms manifested in schizophrenia patients are linked to dysfunction of the prefrontal cortex, or hypofrontality (Castner *et al.*, 2000), suggests that D_1 agonists may have utility in treating schizophrenia. Indeed, in 2003 the National Institute of Mental Health-sponsored MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) program chose D_1 agonists as the most promising therapeutic target for treating working memory disabilities in schizophrenics. Recent studies using the D_1 full agonist dihydrexidine not only demonstrated that the compound was tolerated and safe in humans, but that D_1 receptor agonists can increase prefrontal perfusion in patients with schizophrenia (George *et al.*, 2007; Mu *et al.*, 2007). Unfortunately the therapeutic utility of DHX is severely limited due to its short pharmacokinetic half-life thus eliminating it as a drug candidate.

Substance abuse

In general, reward phenomena are mediated by dopaminergic pathways (Hiroi and White, 1991; Nakajima *et al.*, 1993). The discovery that cocaine is a potent inhibitor of dopamine uptake indicated that dopaminergic actions are responsible for the pleasurable and reinforcing effects of this drug (Fibiger, 1978; Ranaldi and Beninger, 1994). Additionally, evidence has lead to the hypothesis that most drugs produce dependence by increasing dopaminergic transmission in the brain. Several reports have indicated that D_1 agonists might decrease likelihood of relapse in the treatment of cocaine users (Self *et al.*, 1996), including a study by Haney *et al.* (1999) that demonstrated ABT-431 (a full D_1 agonist) dose-dependently decreased the effects of cocaine and also reduced cocaine craving at the highest dose tested (4 mg/kg) in humans. Although the effects of selective dopamine agonists are not always clear (Caine *et al.*, 2007; Caine *et al.*, 2000b; Caine *et al.*, 2000a), these preliminary data have made D_1 agonists a high priority target for the treatment of cocaine abuse.

D_1-like receptor selective compounds: Drug design and clinical applications

History

The first selective D_1-like dopamine receptor agonist, SKF38393 (a member of the 1-phenyl-tetrahydrobenzazepine family), was developed by scientists at SmithKlineFrench laboratories in 1978 (Pendleton *et al.*, 1978; Setler *et al.*, 1978). The distinguishing feature of SKF38393 is a pendant phenyl ring that confers D_1 vs. D_2 selectivity by interacting with a "chirally defined accessory site" (Kaiser *et al.*, 1982). In 1983, the selective phenylbenzazepine compound SCH23390 was reported as the first D_1 receptor antagonist (Cross *et al.*, 1983; Iorio *et al.*, 1983). This compound proved to be an

immensely important tool for the characterization of D_1-like receptors and remains the primary antagonist used in the characterization of D_1-like receptors. SKF38393 and SCH23390 were breakthroughs in the characterization of D_1 receptor function, and were equally as important in understanding the structural determinants for ligand recognition at the D_1 receptor. The most important structural feature of these two phenylbenzazepines is the appended pendant phenyl ring that confers D_1 receptor selectivity, thus demonstrating the importance of exploiting this region for the development of D_1 selective compounds.

As discussed previously, the parkinsonian effects elicited by antipsychotic agents (i.e. D_2 receptor antagonists) lead to the belief that D_2 receptors were responsible for the beneficial actions of levodopa in Parkinson's disease. Development of the selective D_1 agonist SKF38393 allowed examination of a possible role for D_1 receptors in PD however SKF38393 failed to produce anti-parkinsonian effects in both the MPTP primate model and humans (Boyce *et al.*, 1990; Close *et al.*, 1985; Falardeau *et al.*, 1988), and even decreased the efficacy of levodopa (Nomoto *et al.*, 1988). These results supported the notion that D_2 receptors were of primary importance in PD, however the pharmacological ramifications of the low efficacy of SKF38393 at the D_1 receptor were not appreciated at that time. The observation that apomorphine, a mixed dopamine agonist with full efficacy at D_2 receptors and partial efficacy at D_1 receptors, is efficacious in severe PD patients (Poewe *et al.*, 1988) led some investigators to theorize that the effectiveness of this drug was due to D_1, not D_2, receptor agonism.

As the role of D_1 receptor function in Parkinson's disease and other neurological disorders was further elucidated, it became apparent that there was a lack of D_1 receptor agonists for receptor characterization and clinical applications. The only available D_1

selective agonists were of the phenylbenzazepine class, whose utility is restricted by pharmacological limitations such as low efficacy (Andersen *et al.*, 1987). For this reason, our lab began to investigate the molecular interactions governing ligand binding and activation at the D_1 receptor. Knowledge of the structural interactions involved in the binding and activation of the phenylbenzazepines, combined with computer assisted drug design, led to hypotheses about the structural features that might yield a high affinity D_1 full agonist (Nichols 1983). The ensuing D_1 receptor model incorporated a few structural features thought to be necessary for high affinity and full intrinsic activity: the ethylamine fragment must be in a *trans*, extended beta conformation, and a hydrophobic accessory ring system near co-planar to the catechol ring (Charifson *et al.*, 1988; Charifson *et al.*, 1989). This knowledge resulted in the synthesis of the first high affinity full D_1 agonist, dihydrexidine (DHX) (Lovenberg *et al.*, 1989), a hexahydrobenzo-[*a*]phenanthridine. Around the same time a second class of full D_1 agonists was developed, the aminotetralins. The design and synthesis of DHX permitted testing of the hypothesis that full D_1 agonists would cause significant antiparkinson effects, and indeed, DHX produced profound antiparkinson effects in MPTP-treated monkeys. This result was later confirmed in non-human primates using another full D_1 agonist, ABT-431 (Abbott Laboratories) (Shiosaki *et al.*, 1996; Taylor *et al.*, 1991).

Our group then used computer-aided conformational analysis, with DHX as a structural template, to refine the agonist pharmacophore for D_1 recognition and activation (Mottola *et al.*, 1996). The essential features of the D_1 full agonist pharmacophore include two hydroxyl groups, an equatorially oriented electron lone pair on the basic nitrogen atom, and near co-planarity of the accessory ring system with the catechol ring

(Ghosh *et al.*, 1996; Mottola *et al.*, 1996) (see Figure 1.2). The orientation of the accessory ring system, and thus the nature of its interaction with the hydrophobic accessory region, is thought to be an important determinant of agonist efficacy. The D_1 receptor full agonist dinapsoline (naphth[1,2,3-*de*]isoquinoline; DNS) was developed using this pharmacophore model, thereby demonstrating the utility of this approach. The rigid nature of DHX, DNS, and their congeners makes them invaluable tools for probing the molecular interactions governing binding and activation of D_1 dopamine receptors. Several structurally and pharmacologically diverse D_1-like receptor ligands, including DHX and DNS, were utilized for the work in this Dissertation (see Figure 1.3).

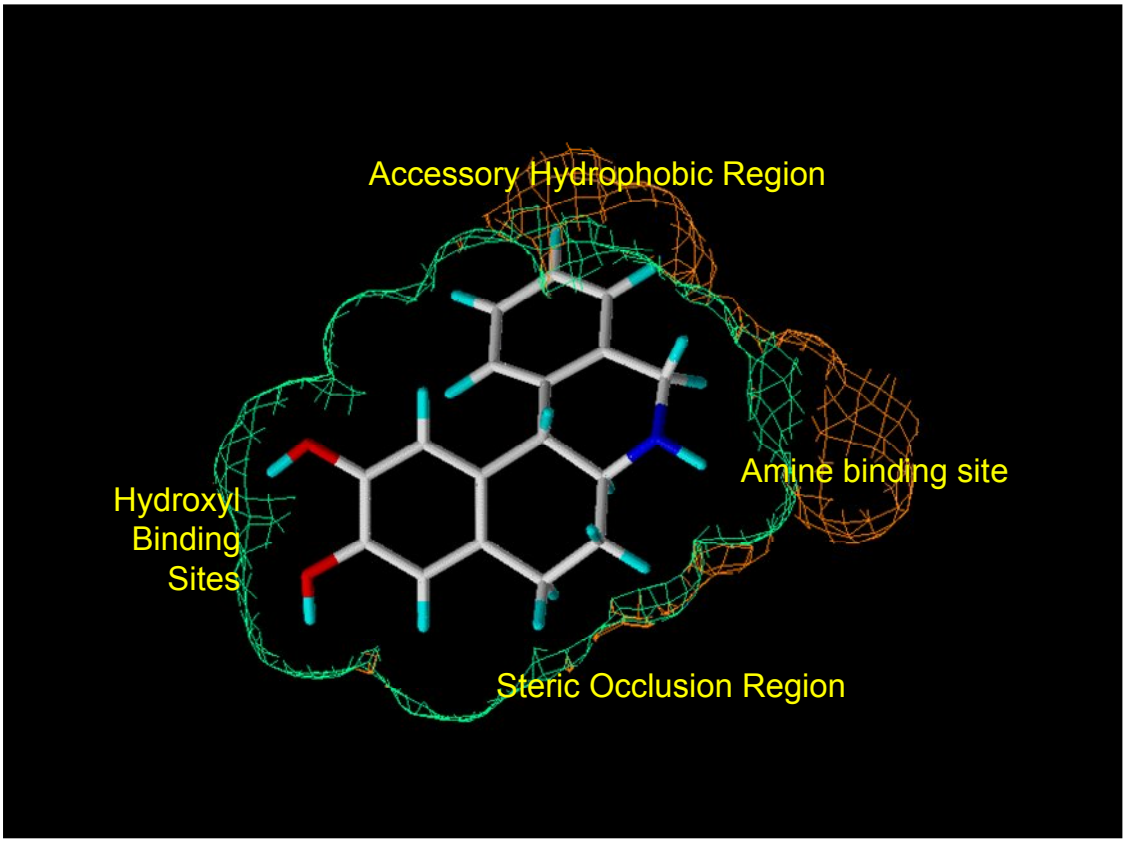
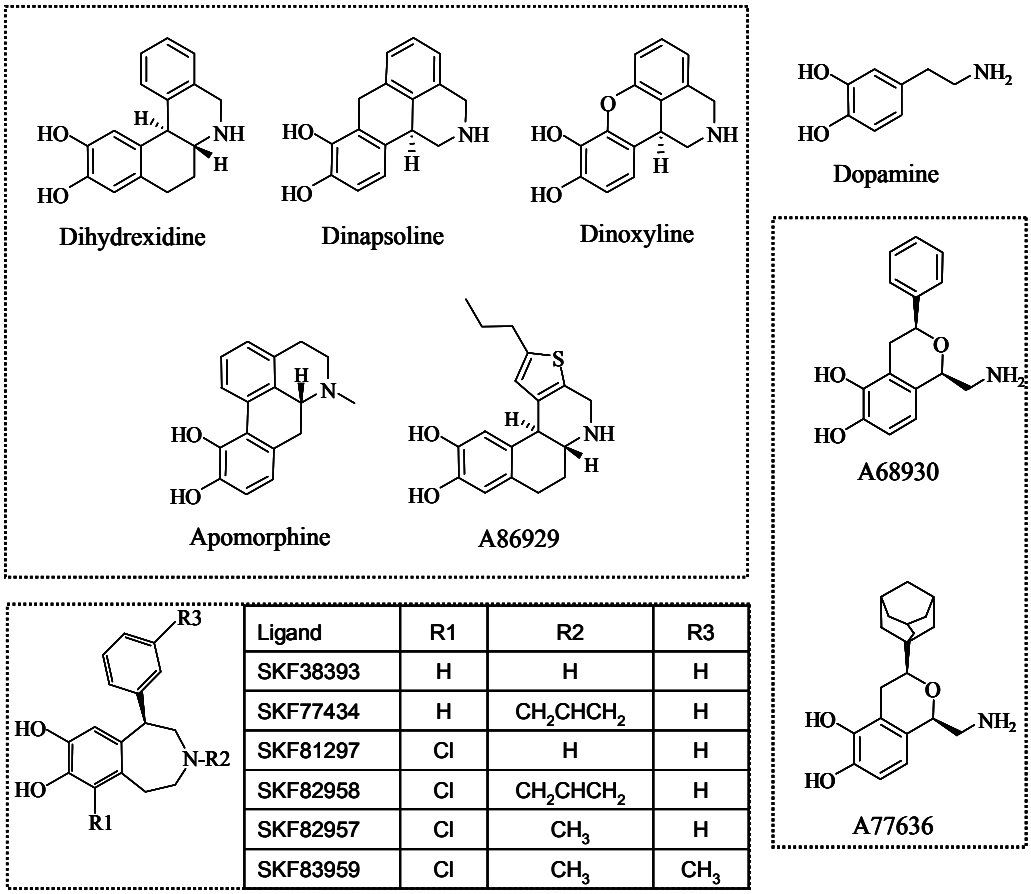


Figure 1.2. Pharmacophore of the D_1 dopamine receptor (adapted from Mottola et al., 1996)



Ligand	R1	R2	R3
SKF38393	H	H	H
SKF77434	H	CH_2CHCH_2	H
SKF81297	Cl	H	H
SKF82958	Cl	CH_2CHCH_2	H
SKF82957	Cl	CH_3	H
SKF83959	Cl	CH_3	CH_3

Figure 1.3. Ligands used in this dissertation.

Issues in the development of clinically useful D_1 receptor agonists

Despite tremendous therapeutic promise for D_1 dopamine receptor agonists, no D_1 selective drugs are currently available for clinical use. Several factors have severely hindered the viability D_1 receptor agonists as therapeutics. The first D_1 full agonists, DHX, ABT-431 and the phenylbenzazepines, exhibit poor oral bioavailability and a short duration of action. Isochromans (e.g. A77636 & A68930), the second class of true full D_1 agonists developed (Kebabian *et al.*, 1992b), appeared to overcome the poor bioavailability plaguing DHX and ABT-431, but further development was precluded due to the rapid and profound behavioral tolerance A77636 produced when administered to animals or humans (Asin and Wirtshafter, 1993; Blanchet *et al.*, 1996; Britton *et al.*,

1991). DNS demonstrated improved oral bioavailability and good pharmacokinetic half-life, but development of this compound was terminated for reasons thought to be related to toxicity.

The design and development of clinically useful D_1 receptor agonists is contingent on understanding the structural features responsible for both desirable (i.e. efficacy, bioavailability) and unwanted (i.e. tolerance, toxicity) properties. The degree of intrinsic activity produced by a D_1 receptor agonist appears to be of particular therapeutic importance. For example, effective amelioration of PD symptoms requires full D_1 agonism while evidence suggests that partial D_1 agonists may be more effective in treating cognitive dysfunction. It is therefore important, for the design of effective D_1 receptor drugs, to determine the molecular interactions responsible for conferring such properties. The goal of the work in this Dissertation was to provide insight into the design of D_1 ligands that have specific functional characteristics (e.g. full agonist), and/or are selective for the D_1/D_5 subtype.

STRUCTURE AND FUNCTION OF CLASS-A GPCRS

The G-protein-coupled receptor (GPCR) superfamily is one of the largest and most diverse protein families in mammals with almost 1,000 members (Takeda *et al.*, 2002; Takeda *et al.*, 2002). GPCRs are comprised of seven stretches of membrane-spanning α-helices, an intracellular C terminus and three interhelical loops. Understanding GPCR function is of great interest as ~30% of the pharmaceuticals currently on the market target these proteins (Hopkins and Groom, 2002). The rhodopsin-like subfamily of receptors is the largest and most studied family of GPCRs, and provided some of the early insight into structural features of this receptor superfamily.

Rhodopsin crystal structure

Initial insight concerning the structure of GPCRs was derived from homology modeling using bacteriorhodopsin as a template. The low sequence similarity to mammalian GPCRs and the fact that bacteriorhodopsin is not coupled to G-proteins limits the utility of this protein as a template for other GPCRs. In 2000, the x-ray structure of bovine rhodopsin was solved (Palczewski *et al.*, 2000), and until very recently (*vide infra*) served as the primary source of insight into GPCR structure and function. The structure of rhodopsin has provided a useful structural framework that has been used widely in the creation of homology models for other GPCRs.

Helical structure of rhodopsin

The structure of rhodopsin is stabilized by seven intrahelical hydrogen bonds. In its ground state rhodopsin, the endogenous ligand 11-*cis*-retinal is bound within the binding site crevice. Several kinks, bends, and varying degrees of tilting within each helix have functionally important consequences in the activation of rhodopsin. Helix 1 is bent by 12^{o} around a proline (Pro^{53}) residue, and forms multiple hydrogen bonds with TM2, 7, and 8. TM2 is tilted ~25 perpendicular to the membrane surface and is bent by 30^{o} due to a Gly-Gly motif (Gly^{89} and Gly^{90}) (Okada *et al.*, 2002; Palczewski *et al.*, 2000). This helix forms intrahelical hydrogen bonding with TM1, TM3, and TM4. TM3, extremely important in rhodopsin, is the longest (48 A) and most tilted (33º) helix. This helix contains two bends, Gly^{120}-Gly^{121} (12º) and Ser^{127} (11º), with the largest tilt facilitating multiple inter-helical interactions (TMs 2, 4, 5, 6, and 7). In the C-terminal of TM3 there is a E(D)RY motif (conserved among all class A GPCRs) that is critical for the regulation of the receptor-G protein interaction. Salt bridges between Glu^{134}, Glu^{247}, and Arg^{135} are

thought to play a critical role in restraining rhodopsin in an inactive conformation. TM4 is the shortest and least tilted transmembrane helix. It has a bend due to the presence of two neighboring proline residues, Pro^{170} and Pro^{171}. TM5 is highly tilted (~26º) and contains two small bends at Phe^{203} (25º) and His211 (15º) (Okada *et al.*, 2002; Palczewski *et al.*, 2000). Residues (Met^{207}, His^{211}, and Phe^{212}) in this TM provide the binding site for the b-ionone ring of the chromophore. TM6 is the most bent and second longest helix. The 30º bend is caused by the highly conserved residue Pro^{267}. Ionic/hydrogen bond interactions between TM6 with TM3 (in the DRY region) and TM7 (Cys^{264}-Thr^{297}) constitute restraints important for an inactive conformation. An aromatic cluster (Tyr^{268}, Phe^{261}, and Trp^{265}) surround the β-ionone ring, with indole ring Trp^{265} positioned in close proximity to the C_{13}-methyl group of the chromophore. Tm7 contains a critically important residue (Lys^{296}) that forms a link with the chromophore within the transmembrane region. Significant distortion around this portion of the helix accommodates the conformational changes needed during isomerization. Large bends around Pro^{291} (24º) and Pro^{303} (21º) permit accommodation of the linkage with the chromophore. The highly conserved NPXXY motif is located in the C-terminal of TM7. In addition to the transmembrane helices, a short helix (TM8) exists in the cytoplasm surface and forms the fourth cytoplasmic loop. This helix is connected to TM1 and TM7 through ionic/hydrogen bonds and thought to be the site of non-covalent binding of all-trans-retinal to opsin (Sachs *et al.*, 2000).

Activation of rhodopsin

Rhodopsin is unique among GPCRs in that the chromophore, 11-*cis*-retinal, is covalently bound within a binding crevice formed by the transmembrane helices, thus

restricting the receptor in an inactive conformation (Sakmar, 1998). The retinal binding cavity is largely surrounded by hydrophobic residues and 11-*cis*-retinal is covalently linked to Lys^{296} on TM7 by a protonated Schiff Base. There are several polar atoms nearby, such as Glu^{113}, which is believed to serve as the counter-ion to the Schiff base. The β-ionone ring makes contact with a group of aromatic residues such as Trp^{265}, Phe^{212}, and Phe^{261}. The inactive state of rhodopsin is stabilized by numerous interhelical interactions, some of which are mediated by water molecules (Okada *et al.*, 2002). When exposed to light, 11-*cis*-retinal absorbs a photon and isomerizes to the all-*trans*-retinal conformation which triggers formation an active receptor state, metarhodopsin II (Sakmar, 1998). Meta II is the active form of the protein capable of interacting with the G protein transducin (G_t).

While the structure of rhodopsin in its ground state provides important insight into receptor structure, the lack of an x-ray structure of the active receptor state makes it difficult to elucidate the conformational changes that occur during activation. Recently, biophysical studies have been used to provide greater insight towards the structural rearrangements that occur during receptor activation. The use of techniques such as UV absorption spectroscopy (Lin and Sakmar, 1996) and site-directed spin labeling (Altenbach *et al.*, 1996; Altenbach *et al.*, 1999) have provided evidence that rhodopsin photoactivation involves rigid-body motion of TMs 3 and 6. Studies by Farrens *et al.* suggest that TM6 moves counterclockwise when viewed from the extracellular side, resulting in an outward movement of the cytoplasmic end of TM6 away from TM3 (Dunham and Farrens, 1999; Farrens *et al.*, 1996). Further site-directed spin labeling

studies conducted by Altenbach *et al.* (2001b; 2001a) indicate that the cytoplasmic portion of TM7 moves away from TM1.

β_2-adrenergic receptor crystal structure

While bovine rhodopsin has served as a useful template for understanding GPCR structure, there are limitations to extrapolating its structure to other GPCRs. The most serious limitation is that, unlike other rhodopsin-like GPCRs, in its ground state rhodopsin is covalently bound to the inverse agonist 11-*cis*-retinal. Recently, a high-resolution crystal structure of the β_2-adrenergic receptor was obtained by engineering the receptor to include lysozyme or a monoclonal antibody to provide conformational stability (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007).

Like rhodopsin, the β_2-adrenergic receptor consists of 7TM helices, as well as a short segment that forms an eighth helix. The ligand-binding site of the β_2-adrenergic receptor is located in a position similar to that of the covalently bound ligand of rhodopsin, G_t. Unexpectedly, the second extracellular loop (ECL2) of the β_2-adrenergic receptor contains an extra short helical segment not present in rhodopsin. The apparent solvent accessibility of this segment lead the authors to hypothesize that it may help stabilize the receptor core and prevent ECL2 from hindering ligand access to the binding pocket (Cherezov *et al.*, 2007). Several shifts in the structural alignment of the β_2-adrenergic receptor transmembrane helices were observed relative to rhodopsin, including TMs 1, 3, and 4, but the largest structural difference founds was in TM1 which lacks the proline-induced kink found in rhodopsin. The ramification of this difference in TM1 is not yet clear. Transmembrane alignment likely varies across different GPCR classes serving to accommodate binding of an assortment of structurally diverse ligands.

The crystal structure of the β_2-adrenergic receptor is an important breakthrough towards understanding the structural features important in mammalian GPCRs. The structure alone, however, does not permit an understanding of the conformational changes that occur from ligand binding to receptor activation. Indeed, the structural engineering that was required for crystallization clearly causes perturbations from the "native" receptor that cannot be elucidated using current technology. Moreover, myriad conformational receptor states adopted upon ligand binding must be elucidated before a complete picture of the structural changes involved in the transduction of ligand-binding to receptor activation can be assembled. Furthermore, in order to gain complete understanding of GPCR function it may be necessary to obtain crystal structures of agonist bound not only to the GPCR, but to the GPCR-G-protein signaling complex.

Mutagenesis of catecholamine receptors

Nomenclature

Throughout this dissertation, I shall use the universal indexing system initially described by Ballesteros and Weinstein (1995) to describe amino acid positions. This system facilitates the comparison of analogous residues in different GPCRs. The single letter code for the amino acid is provided first followed by the residue position with respect to the most conserved amino acid in each transmembrane helix (designated X.50, where X is the transmembrane domain 1-7). The most conserved residue in each transmembrane is designated 0.50. The decimals are indexed positively (towards the carboxy-terminus) or negatively (towards the amino-terminus). Table 1.1 shows the most conserved residue in each TM helix in the D_1 and D_5 receptors, as well as the residues targeted in this research.

Table 1.1. Most conserved residue in each TM helix in the D_1 and D_5 receptors and residues targeted in this research.

Universal Index	D_1	D_5	Chapter where studied
N1.50	N41	N58	-
D_2.50	D70	D87	-
R3.50	R121	R138	-
W4.50	W148	W165	-
P5.50	P206	P237	-
P6.50	P287	P311	-
P7.50	P328	P356	-
T3.37A	T108A	T125A	Chapter 3
S5.46A	S202A	S233A	Chapter 3
F6.51W/L/I/Y	F288	F312	Chapter 4
W6.48A	W285A	W309A	Chapter 5
N6.55A	N292A	N316A	Chapter 5
V4.60A	V159A	V176A	Chapter 6
W4.64A	W163A	W180	Chapter 6
L6.54A	L291A	L315	Chapter 6
X6.58A	L295A	V319	Chapter 6

Structure-function analysis of D_1-like dopamine receptors

Few efforts have been made towards understanding the interactions governing ligand recognition and activation of the D_1-like receptors, especially the D_5 dopamine receptor. The following summarizes efforts that have been made to date using methods such as site-directed mutagenesis, molecular modeling, and receptor chimeras.

The role of TM3 & TM5 in D_1-like ligand-receptor interaction

Strader *et al.* (1988) was the first group to demonstrate that an aspartate residue at position 3.32 serves as the counter-ion to the amine of catecholamine ligands. As expected, studies have shown that D3.32 is critical for ligand interaction at both the D_1 and D_5 dopamine receptors (Pollock *et al.*, 1992) (unpublished observations for D_5). Abrogation of this interaction results in a complete loss of ligand binding as well as functional activity at D_1-like receptors.

The role of TM5 serines in ligand interaction with catecholamine receptors is well established (Strader *et al.*, 1989) though specific interactions vary by receptor type and ligand structure. Pollack *et al.* demonstrated a role for S5.42 and S5.43 in D_1 receptor recognition of the phenylbenzazepines, and for S5.46 in the interaction with dopamine. The knowledge that could be gleaned from this study, however, was limited by a lack of structurally diverse D_1 ligands available to use as receptor probes. The synthesis of structurally and pharmacologically diverse classes of D_1 compounds (i.e. DHX, DNS, and the isochromans) allowed additional insight to be gained concerning the molecular interactions involved in binding and activation at both D_1 and D_5 dopamine receptors. Studies in our lab, focused on the role of the three TM5 serines in the recognition of an array of test compounds, established that S5.42 (and possibly S5.43) interacts with the meta-OH and that S5.46 interacts with the para-OH of catechol-containing compounds at the D_1 and D_5 dopamine receptors (unpublished observations). Our findings also indicate that S5.43 may be promiscuous and interact with the *meta*-OH and *para*-OH of the catechol through a bifurcated bond. Importantly, these findings demonstrated that compounds of different chemical classes (e.g. A77636 and SKF82958) have distinct modes of interaction with the TM5 serines of the D_1-like receptors

The work conducted for this Dissertation further examines the molecular interactions that contribute to binding of the catechols of D_1-like compounds.

The role of TM6 & TM7 in D_1-like ligand-receptor interaction

Several studies have demonstrated a critical role for TM6 residues in ligand recognition and subsequent receptor activation of catecholamine receptors, particularly those residues positioned in the rotamer toggle switch region. In a previous study we

examined the role of two phenylalanine (F6.51 & F6.52) residues in D_1 and D_5 receptor binding and activation. We constructed non-conservative point mutations (i.e. to alanine) in both residues and examined the effects on ligand-binding and receptor activation. The changes observed in the F6.52A mutant receptor were non-specific and appear to be the result of global changes in receptor structure, indicating that F6.52 does not play a prominent role in ligand binding or receptor activation of the D_1-like receptors. The results obtained for the D_1- and D_5-F6.51A mutant receptors, however, indicate a critical role for this residue in ligand-binding and receptor activation. The F6.51A mutant receptor exhibited more dramatic effects in efficacy and potency for dopamine, A77636 and the rigid ligands (i.e. DHX & DNS) than for the phenylbenzazepine compounds (i.e. SKF38393, SKF82526, & SKF82958), thus providing evidence for agonist-specific conformational states.

Hydrophobic residues in TM7 have been predicted to interact with the accessory ring system of dopaminergic compounds, and to possibly be involved in the distinction between receptor subtypes. To examine this hypothesis, experiments in our lab targeted three hydrophobic residues in TM7 (F7.35, F7.38, and W7.43) of the D_1 and D_5 dopamine receptors by mutating each residue to alanine (unpublished observations). The results indicate that F7.38 and F7.35 do not directly interact with any of the test compounds, however a reduction of basal cAMP accumulation suggests that F7.35 may be important for efficient coupling of the agonist-induced receptor to G-protein turnover. Mutation of W7.43 to alanine resulted in a dramatic loss in receptor expression suggesting that this residue is necessary for correct protein folding and/or processing of D_1-like receptors. The work in this dissertation further investigates the role of F6.51 in

binding and receptor activation, and also examines the role of other TM6 residues positioned in and around the putative toggle switch region of D_1-like receptors.

Mechanisms of ligand-dependent receptor activation

Activation of Class A GPCRs occurs when an agonist diffuses into an unliganded receptor and induces-or stabilizes- a structural rearrangement of the receptor resulting in activation of intracellular G-proteins. GPCRs are restrained in an inactive conformation by a network of non-covalent intramolecular interactions between the TM helices. Evidence suggests that all Class A GPCRs share, in general, a common activation mechanism.

Many unliganded receptors have a basal level of G protein activation. The degree of inactivation can vary by receptor type, and even among subtypes of the same family as with the D_1 and D_5 dopamine receptors. There are numerous examples of discrete mutations in any receptor domain that can dramatically increase the constitutive activity of a receptor (Allen *et al.*, 1991; Lefkowitz *et al.*, 1993; Samama *et al.*, 1993; Scheer *et al.*, 1996). Until recently GPCR activation was viewed as a bimodal process, consisting of inactive or active receptor states. Recent evidence suggests that receptor activation is a multistep process consisting of several discrete receptor conformational states.

When an orthosteric agonist diffuses into the GPCR binding pocket, the restraining intramolecular interactions are broken and new interactions are formed that stabilize the receptor in an active conformational state. Recent studies indicate that GPCR activation occurs by the disruption and creation of specific highly conserved structural motifs that serve as molecular switches in GPCR activation (Kobilka and Deupi, 2007). The exact conformation of the receptor is dependent on the structure of the ligand, thus

its specific interaction with these molecular switches, and such distinct states may produce differential activation of signaling pathways (see section on Functional selectivity). Three of the more prominent examples of these molecular switches include: (i) Protonation of the highly conserved D/ERY motif at the cytoplasmic side of TM3. Mutation of Arg3.50 results in reduction or abolishment of ligand binding and receptor activation in most Class A receptors. In the 5HT-$_{2A}$ and β_2-adrenergic receptors, ionic interactions between Arg3.50 and Glu3.49 and Glu6.30 stabilize the inactive state. These interactions are eliminated on receptor activation (Ballesteros *et al.*, 2001a). (ii) The NPxxY motif, conserved in TM7 of rhodopsin-like receptors, serves to connect TM7 with the cytoplasmic helix 8. This connection is thought to be critical for regulation of C-terminal interaction with intracellular signaling partners (Fritze *et al.*, 2003). Additionally, mutations of the NPxxY motif have been shown to affect receptor expression, G-protein coupling, and ligand affinity (Barak *et al.*, 1995; Mitchell *et al.*, 1998; Wess, 1993). (iii) A cluster of aromatic residues in TM6, surrounding the conserved proline, that has been coined the rotamer toggle switch. The rotameric conformations of a group of conserved aromatic residues (Phe6.51, Phe6.52, Phe6.44, Trp6.48) surrounding a proline (Pro6.50) in TM6 are postulated to be interrelated (Shi and Javitch, 2002). Agonist interaction with one or more of these aromatic residues in TM6 induces rearrangement of a Trp (6.48) residue that is proposed to cause a drastic reduction in the proline kink in TM6 (Huang *et al.*, 2002), resulting in the movement of the cytoplasmic ends of TM6 away from TM3 (Ebersole *et al.*, 2003). This action may serve to transduce agonist-induced conformational changes in the extracellular receptor region to changes in the cytoplasmic portion of the receptor that is involved in receptor-

G- protein coupling (Chen *et al.*, 2002b; Chen *et al.*, 2002a). The ligand binding site is tightly coupled to the G-protein nucleotide binding site but the exact mechanism by which agonist binding is translated into structural changes that result in G-protein activation remains unclear. Until recently, ligand binding and receptor activation was considered a bimodal process. Recent studies have demonstrated that the receptor conformation is agonist specific and can exist in numerous conformations that may have diverse functional implications (vide infra). Ligands of different chemical structure can stabilize distinct receptor conformations and may thus engage the aforementioned molecular switches differentially, yielding differential functional responses.

Molecular modeling of GPCRs

Although many investigators have used bacteriorhodopsin as a template for modeling the TMs of GPCRs (Palczewski *et al.*, 2000), bacteriorhodopsin is not a GPCR, even though it is a seven transmembrane-spanning protein. By contrast, a deduced GPCR template based on a comprehensive analysis of hundreds of GPCR sequences (Baldwin, 1993) actually was found to agree reasonably well with the 2.8 Å crystal structure of rhodopsin (Palczewski *et al.*, 2000). For the amine-like subfamily, there is general agreement about the broad generalities of mechanisms of ligand recognition and receptor activation. Small ligands bind primarily within the core of the seven transmembrane-spanning helices (TMs), and subsequently induce conformational changes in the receptor that alters the relative positions of the seven TMs. The movements of the TMs produce changes in the three intracellular loops (ICLs) leading to efficient coupling to a heterotrimeric G protein(s) and/or activation of precoupled G proteins. It is the details about specific receptors and their ligands that remain unclear. The major issue in such

modeling is the reliability and predictability of a model, something often difficult to assess.

Weinstein and colleagues (Zhou *et al.*, 1994; Ballesteros *et al.*, 1998) have used models of the GnRH receptor to note that the highly conserved pattern of N55 in TM1, D83 in TM2, and N302 in TM7 probably stabilizes the helical bundle, an idea confirmed by experiments using site-specific mutations of these residues and functional analyses. In the RH 3D crystal structure (Palczewski *et al.*, 2000; Palczewski *et al.*, 2000), the conserved N55 in TM1 interacts with the peptide backbone carbonyl of G51 and A299, whereas D83 in TM2 weakly interacts with N55 and with a structural water molecule that bridges between D83 and the backbone carbonyl of G120 in TM3. N302 in TM7 interacts with the backbone carbonyl of S298 in TM7, possibly with the OH of Y301, and also with the water molecule that bridges D83 with the backbone carbonyl of G120. Weinstein's group also predicted that TM7 is not an ideal α-helix but contains a kink caused by the Asn-Pro/Asp-Pro motif (Konvicka *et al.*, 1998). The RH structure, however, has the Asn-Pro-Xaa-Xaa-Tyr sequence and has a regular helical structure, with a kink further along the helix, as well as distortions in other parts of TM7 (e.g., around the Lys that covalently attaches to retinal).

The DRY motif (Asp/Glu-Arg-Tyr) at the intracellular end of TM3 often is considered important for receptor activation. It was initially shown that the charged pair of Glu-Arg was needed for rhodopsin activation because double mutants of these residues failed to activate transducin (Franke *et al.*, 1992). Scheer *et al.* (2000; 1996) and others have reported theoretical and experimental manipulations that have led to the hypothesis that inactive receptor is restrained by interactions between Arg of DRY and the

hydrophilic residues formed by TM1, 2, and 7. Possibly, when the Asp in DRY is protonated, it causes the Arg of the DRY to move out of the TM bundle, thereby changing the orientation of residues in IL2 and 3, in turn affecting G protein-coupling. A somewhat different hypothesis has been offered for the GnRH receptor in which Arg of the Asp-Arg-Ser sequence at the end of TM3 interacts with the adjacent Asp in the inactive state of the GnRH receptor. Activation involves release of the Arg from interacting with Asp by Asp protonation and promotes movement of the Arg into a hydrophilic pocket in the TM bundle. An Ile one helical turn above the Arg in TM3 sterically directs the Arg into the TM hydrophilic pocket (Ballesteros *et al.*, 1998). In any event, Shapiro *et al.* (2002) hypothesized that E6.30 formed a strong ionic interaction with R173(3.50) of the D(E)RY motif in the 5-HT_{2A} receptor. When they made the E318(6.30)R mutant, it had high constitutive activity and enhanced affinity for agonist. They concluded that the disruption of a strong ionic interaction between transmembrane helices 3 and 6 of 5-HT_{2A} receptor is essential for agonist-induced receptor activation, and that this may represent a general mechanism of activation for many GPCRs.

Whatever the difference, current models predict that movement of the Arg in the highly conserved D(E)RY sequence is a "switch" that converts a GPCR from an inactive to an active state in response to agonist-stimulated receptor protonation. More recent data suggest that the switch can be activated by more general mechanisms other than D-protonation (Ghanouni *et al.*, 2000). Transmembrane helix movement is also known to be important in GPCR activation, and is a mechanism we hypothesize is affected differentially by the rigid ligands on which we focus. In the case of RH, TM3 and 6 have relative motion upon light activation (Farrens *et al.*, 1996) that is also predicted from the

crystal structure (Palczewski *et al.*, 2000). Similar effects have been reported with the ß$_2$-adrenergic receptor (Gether *et al.*, 1997b), and TRH-receptor (Colson *et al.*, 1998), among others. On the other hand, some data suggest that these mechanisms are not universal (Angelova *et al.*, 2000), with other possibilities including release of intrahelical interactions (Fanelli, 2000), movement of TM3, 4, 5, and 6 (Fanelli *et al.*, 1999), and movement of TM5 and 6 (Zhang and Weinstein, 1993). It is not surprising that the activation of each specific receptor, although following a similar general pattern, has critically important inter-individual differences.

As these examples indicate, although there is a solid foundation for homology modeling of the D_1 receptor, it is often difficult to explain the subtleties that are the focus of my goals. As will be detailed in the experimental methods, I have collaborated with the laboratory of William Goddard on some exciting new ways to approach these issues

RECEPTOR THEORY & RECEPTOR PHARMACOLOGY

The concept of a "receptor" is generally attributed to Paul Erhlich (1854-1915) whose studies lead him to the notion of selectivity and "receptive side chains". Erhlich's contemporary, John Langley, coined the term receptor in 1878 while studying the effects of nicotine and curare analogs on muscle contraction.

Efficacy

Efficacy was first used by R.P. Stephenson to describe the property of a drug that caused receptor activation and produced a pharmacological response (Stephenson, 1956). His concept of efficacy allowed for separation of receptor activation from the tissue event of physiologic activity. Stephenson's observations occurred in the context of tissue

response, however we now know that tissue response does not represent the entire repertoire of possible receptor responses (e.g., receptor internalization).

Efficacy is defined as the property of a drug that causes its target receptor to change its functional actions, either stimulatory or inhibitory. Intrinsic efficacy was thought to be a property intrinsic to the ligand, independent of the system or receptor-effector coupling, but data have since indicated that this concept is incorrect. We now know that a single ligand interacting with a single receptor type can elicit responses that cannot be accommodated by the concept of intrinsic efficacy. Thus, I will use the term intrinsic activity to describe the relative maximal functional response produced by the receptors characterized in response to test compounds.

Drugs that elicit a response equivalent to that of the endogenous ligand are referred to as full agonists. Compounds that produce a response less than the tissue maximum are called partial agonists. A partial agonist can have high, medium, or low intrinsic activity and can antagonize the response of a full agonist at appropriate concentrations. Compounds that do not elicit activity are termed antagonists, and those antagonists that decrease the functional response below basal levels are referred to as inverse agonists. The functional characteristics of a ligand can be tissue-dependent, receptor-dependent, and function-dependent at a given receptor in a given system.

Models of receptor activation

Over the years several activation models have been developed to describe the relationship between ligand, receptor, and effector. Our understanding of how drugs bind and subsequently activate receptors has evolved and resulted in the revision and

expansion of activation models. The following provides an overview of the evolution and current state of receptor models.

Equilibrium models

Although numerous models have been developed, each is rooted in the theories described by Clark (1937) and the laws of mass action. In 1983, Black and Leff described a two-state model of receptor activation that proposed a receptor exists in either and active [R*] or inactive state [R]. In this model, the proportion of receptors existing in either state is in equilibrium and agonist binding to the receptor shifts the equilibrium to favor the active state (Black and Leff, 1983).

The original two-state model was developed as a mechanistic model for ion channels and did not account for receptor-G-protein interaction (del Castillo and Katz, 1957). To integrate the G-protein component, a ternary complex model was described that considered the agonist/receptor/G-protein complex (De Lean *et al.*, 1980). Observations, such as the discovery of constitutive receptor activity (Costa and Herz, 1989), revealed behavior that could not be adequately described by the original ternary complex model. The extended ternary complex (ETC) model was subsequently devised by Samama and colleagues (Samama *et al.*, 1993) to allow for the formation of an active receptor/G-protein complex in the absence of agonist. The discovery of inverse agonism demonstrated the need to describe a receptor state in which the inactive receptor can couple with G-proteins. This led to the development of complex but more thermodynamically complete model, the cubic ternary complex (CTC) model.

Recent findings, such as the ability of agonists to selectively activate specific signaling pathways and not others, cannot be accommodated by traditional receptor

theory (i.e. two state and ternary complex). Leff and colleagues attempted to explain such findings by expanding two-state theory to a "three-state receptor model" of agonist action (Leff *et al.*, 1997). To account for the apparent ability of an agonist-bound receptor to activate specific signaling pathways, they proposed that a receptor can exist in two distinct receptor active states, [R*] and [R**], interacting with different G proteins. This model proposes that agonists can selectively interact with one active state over the other. More recent evidence that indicates receptors can couple to numerous G-proteins to elicit more than two functional responses clearly limits the utility of the three state model of receptor activation.

Agonist-specific receptor states: functional selectivity

Traditional receptor theory states that the relative degree of activation produced by an agonist binding to a receptor is independent of the effector pathway activated (28). This theory proposes that receptors can exist in two states: an active or inactive state. Activated receptors are postulated to exist in the same state regardless of the ligand bound. The intrinsic efficacy elicited by the ligand-receptor interaction is assumed to be a property of the ligand, independent of the pathway in which efficacy is measured. Traditional theory assumes that ligands differ only in strength of signal. Accordingly, ligands are classified based on their intrinsic efficacy as full agonists, partial agonists, or antagonists.

Over the past decade, multiple lines of evidence (in numerous receptor systems) have revealed that certain agonists selectively couple a single receptor subtype to specific functional pathways and not others (Berg *et al.*, 1998; Kilts *et al.*, 2002; Kenakin, 1995). This phenomenon has been termed "agonist-directed trafficking" (Kenakin, 1995),

"biased agonism" (Jarpe *et al.*, 1998), "functional dissociation" (Whistler *et al.*, 1999), "differential engagement" (Manning, 2002), and "functional selectivity" (Lawler *et al.*, 1994; Kilts *et al.*, 2002). I shall use the term functional selectivity throughout this Dissertation. Functional selectivity postulates that the ability of a receptor to couple to an effector pathway is dependent on the conformation of the ligand-receptor complex (Kenakin, 1995). Therefore, an agonist is capable of selectively (or differentially) activating effector pathways at a single receptor subtype (see Figure 1.4). A study by Berg *et al.* (1998) demonstrated that agonist trafficking is not due to a difference in stimulus strength by showing a reversal in relative agonist potency for two effector pathways. This finding supports the notion that agonist efficacy is dependent on the pathway in which a response measured.

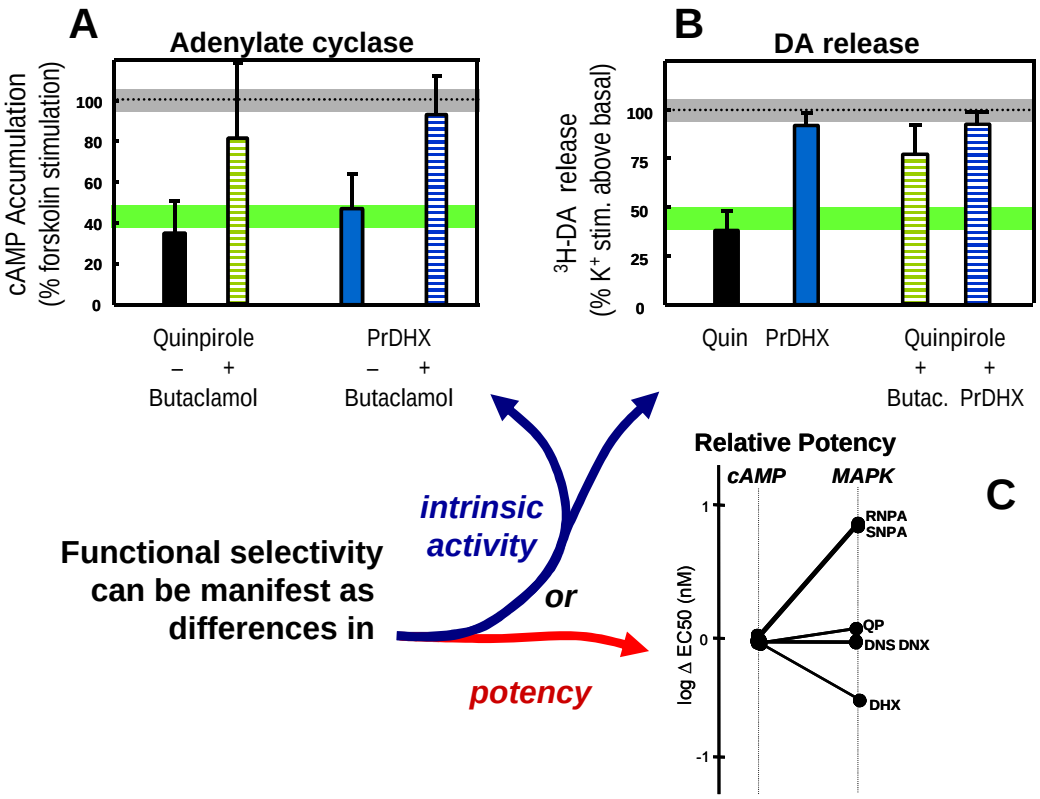


Figure 1.4. Example of functional selectivity.

Recent studies of the β_2-adrenergic receptor have demonstrated that agonist binding occurs through a series of conformational intermediates and that structurally diverse agonists cause distinct conformational states (Ghanouni *et al.*, 2001a; Swaminath *et al.*, 2005). For example, the full agonist isoproteronol produced a conformational state distinct from that of the partial agonist salbutamol (Ghanouni *et al.*, 2001a). It is not yet clear whether specific conformational states are responsible for differential activation of signaling cascades. These studies indicate that structurally diverse agonists engage with the receptor in a distinct manner, thus disrupting different combinations of intramolecular interactions resulting in differential activation of effector pathways. Such findings also demonstrate the need to build more complex ligand-receptor models that incorporate multiple receptor states. New models, such as steady-state and dynamic models, of GPCR activation may better accommodate functional selectivity (Christopoulos and Kenakin, 2002; Lew *et al.*, 2001).

Functional selectivity has important implications in the design and discovery of novel therapeutics, including D_1 receptor agonists. Potentially effective compounds may be disregarded in the drug discovery process on the basis that intrinsic activity is not observed at a specific functional endpoint. However, this may simply imply that the assay is not designed to detect activity at the appropriate functional endpoint. It may be possible to design functionally selective drugs that preferentially activate a specific signaling pathway to enhance therapeutic benefits while ameliorating unwanted side effects.

Evidence for functional selectivity at D_1-like receptors

Functional selectivity has been difficult to demonstrate for D_1-like dopamine receptors due to the lack of clear effectors coupled to the receptor. The clearest evidence of D_1 functional selectivity was shown in two recent studies comparing the functional endpoints of adenylate cyclase activation and receptor internalization. The first study explored the relationship between agonist structure, receptor affinity, and efficacy of adenylate cyclase activation and receptor internalization in response to thirteen agonists from three different structural classes (Ryman-Rasmussen *et al.*, 2005). This study identified several D_1 agonists that activate adenylate cyclase with great efficacy but fail to cause receptor internalization. Interestingly, internalization efficacy was found to be independent of agonist structural class and agonist affinity. This study revealed interesting disparities in the ability of synthetic D_1 agonists to regulate receptor trafficking, and suggested that, at least for the D_1 receptor, functional selectivity is not predictable by simple structural examination.

A subsequent study compared the ability of two structurally dissimilar agonists, A77636 (isochroman) and dinapsoline (DNS, isoquinoline), to regulate receptor internalization and trafficking with that of dopamine (Ryman-Rasmussen *et al.*, 2007). These compounds are full agonists at activating adenylate cyclase, and reach steady-state internalization by 30 minutes. DNS exhibited efficacy similar to dopamine in causing internalization 1 hour after agonist treatment while A77636 caused significantly greater internalization. Investigation of post-endocytic agonist effects on receptor trafficking revealed significant differences in agonist regulation of receptor trafficking. Dopamine caused the D_1 receptor to recycle back to the cell surface within 1h whereas the D_1 receptor persisted intracellularly up to 48 h after removal of A77636. Surprisingly, DNS

caused the receptor to recycle back to the membrane after 48 h. Pulse chase experiments, and use of actinomycin D to inhibit new protein biosynthesis, demonstrated that cell surface recovery was not due to synthesis of new proteins. Together, these data indicate that these agonists target the D_1 receptor to different intracellular trafficking pathways. Experiments revealed a slow dissociation rate of A77636 from the D_1 receptor, suggesting that ligand-receptor interactions distal to the binding pocket may dictate the ability of an agonist to cause receptor internalization and regulate long-term receptor trafficking. While the mechanisms of tolerance are unknown, it is interesting to note that A77636 elicits profound *in vivo* tolerance within 24 h (Lin *et al.*, 1996) whereas DNS does not induce such tolerance in a rat model of Parkinson's disease (Gulwadi *et al.*, 2001).

Receptor reserve and functional effects of spare receptors

The intrinsic activity of a ligand is dependent on the efficiency of receptor-effector coupling and therefore can be influenced by receptor concentration. Receptor reserve is defined as a system in which the stoichiometry of the receptor is of greater molar excess compared to the G protein subunits. According to theory, in such a system a ligand may produce a maximal response (full intrinsic activity) without occupying all receptors available. In some cases, a partial agonist may exhibit full intrinsic activity in a system with a high degree of spare receptors. Thus, a system with high receptor reserve may confound the ability to distinguish the true intrinsic activity of a ligand. Receptor reserve can be examined by titrating the number of available receptors, for example using either non-selective alkylating agents such as EEDQ or molecular approaches. The issue of spare receptors has been an important issue in understanding the action of D_1

dopamine agonists (Pifl *et al.*, 1991; Watts *et al.*, 1993; Gilmore *et al.*, 1995; Watts *et al.*, 1995). One of the important lessons taught by this literature is that one can use SKF38393 as a monitor of whether one has receptor reserve to a degree that is "non-physiological." SKF38393 is a moderate intrinsic activity partial agonist in almost all *in situ* systems (Watts *et al.*, 1995), and thus an excellent control for studies in D_1 heterologous systems.

GOALS OF THIS DISSERTATION AND SPECIFIC AIMS

The overall goal of this research was to elucidate the molecular interactions governing ligand-binding and subsequent activation of the D_1-like dopamine receptors. This work was based on a well-defined D_1 agonist pharmacophore as well as structural information derived from studies of other catecholamine receptors. Target residues were identified rationally, based on a D_1 receptor molecular model, and mutant receptors were characterized using an array of structurally diverse probe ligands. D_1 receptor agonists have tremendous therapeutic potential in the treatment of numerous neurological disorders and it is therefore of great importance to understand the structural features conferring desirable therapeutic properties. The desired outcome of this work was to gain a greater understanding of the structural mechanisms responsible for binding and activation of the D_1-like receptors and to use this information for the following: i) to generate novel findings that can be extended to the understanding of other catecholamine receptors; ii) to understand the structural basis of D_1 receptor properties important for the design of clinically useful full D_1 agonists (e.g. full efficacy, low tolerance, bioavailability, functionally selective properties); iii) to identify structural features of the

D_1-like receptors that may aid in the design of a D_1 vs. D_5 receptor selective agonist. I pursued these objectives through the following aims:

Aim 1: Determine a more effective method for the quantification of GPCR-mediated adenylate cyclase activation.

This aim sought to modify the standard laboratory technique of quantifying cAMP accumulation to expedite the time required to conduct the assay and to reduce costs. The work in this aim yielded a cAMP assay with improved costs and speed while maintaining sensitivity.

Aim 2: Determine the role of a TM3 threonine residue (3.37) in the binding and subsequent activation of the D_1-like dopamine receptors.

Previous studies have elucidated the manner by which TM5 serine residues interact with the catechols of D_1 receptor agonists. Our D_1 molecular model predicts that a threonine residue in TM3 (3.37) is positioned to influence ligand-binding by interacting with the *para*-OH of D_1 compounds. I sought to test this hypothesis by using a group of structurally and pharmacologically diverse D_1 test compounds.

Aim 3: Determine more specifically the role of phenylalanine 6.51 in the interaction of D_1-like dopamine receptors with structurally diverse D_1 agonists.

A previous study in our lab demonstrated an important role for F6.51 in D_1-like receptor activation. The experiments in this aim sought to gain a detailed understanding of the role of F6.51 in receptor activation by creating several conservative (i.e. Ile and Leu) and non-conservative (i.e. Trp and Tyr) point mutations of the phenylalanine residue in both the D_1 and D_5 dopamine receptors.

Aim 4: Determine the role of two TM6 residues (W6.48 & N6.55) hypothesized to play an important role in the transduction of ligand-binding to receptor activation of the D_1-like receptors.

Several studies have demonstrated that residues residing in the rotamer toggle switch region play an important role in receptor activation. The experiments in this aim sought to explore the role of two TM6 residues, one predicted to form part of the toggle switch region and the other to be positioned one turn above this region, involved in binding and activation of the D_1-like dopamine receptors.

Aim 5: Determine the effects of the mutation of four residues distal to the binding site of the D_1 dopamine receptor on ligand binding, receptor activation, and receptor internalization.

In a recent study, we demonstrated differences in long-term trafficking of dopamine, dinapsoline, and A77636 at the D_1 dopamine receptor. We hypothesize that differences in D_1 receptor internalization and long-term trafficking are due to ligand-receptor interactions distal to the binding pocket. This aim sought to explore this hypothesis by constructing non-conservative mutations of four residues thought to contribute to ligand stabilization in the D_1 receptor.

CHAPTER 2:
RAPID, SEMI-AUTOMATED, AND INEXPENSIVE RADIOIMMUNOASSAY OF CAMP: APPLICATION IN GPCR-MEDIATED ADENYLATE CYCLASE ASSAYS

PREFACE

The work presented in this chapter comprises the alterations made to improve the laboratory's method of assessing cAMP accumulation. Major modifications of the original cAMP assay include coupling the primary antibody directly to magnetic beads (opposed to the secondary antibody) and separating the antibody-bound magnetic beads from unbound marker using filtration on microplates. This work greatly improved speed and costs associated with the assay, while retaining the high levels of sensitivity associated with the original method.

ABSTRACT

Cyclic AMP (cAMP) is an important signal transduction second messenger that is commonly used as a functional mirror on the actions of G protein-coupled receptors that can activate or inhibit adenylate cyclases. A radioimmunoassay for cAMP with femtomole sensitivity was first reported by Steiner more than 30 years ago, and there have been several subsequent modifications that have improved this assay in various ways. Here we describe additional improvement to existing methods that markedly improve speed and reduce cost without sacrificing sensitivity, and is also adaptable to analysis of cGMP. The primary antibody is coupled directly to magnetic beads that are then separated from unbound marker using filtration on microplates. This eliminates the need for a secondary antibody, and markedly increases throughput. In addition, we report a simple, reproducible, and inexpensive method to make the radiomarker used for this assay. Although still requiring the use of radioactivity, the resulting method retains a high degree of accuracy and precision, and is suitable for low-cost high-throughput screening. Use of aspects of this method can also improve throughput in other radioimmunoassays.

[Citation: Brown JT, Kant AC, Mailman RB. Rapid, semi-automated, and inexpensive radioimmunoassay of cAMP: Application in GPCR-mediated adenylate cyclase assays. *J Neurosci Meth* (2008) doi: 10.1016 jneumeth.2008.10.016 (epub)]

INTRODUCTION

Cyclic AMP (3',5'-cyclic adenosine monophosphate; cAMP) is a key second messenger involved in numerous intracellular signaling pathways (Antoni, 2000; McPhee *et al.*, 2005). Production of cAMP is controlled by the membrane-bound family of adenylate cyclases (ACs) that convert adenosine triphosphate to cAMP. The activity of most of the ACs is regulated by heterotrimeric GTP-binding proteins (e.g., $G\alpha_{s/olf}$, $G\alpha_{i/o}$) that directly interact with the intracellular region of GPCRs and can both increase or decrease enzyme activity (Hanoune and Defer, 2001). In addition, phosphodiesterases can catalyze the degradation of cAMP (Weishaar, 1986).

The measurement of adenylate cyclase activity can be accomplished using radiometric assays that follow the incorporation of a radioactive precursor into cAMP (Salomon, 1979; Schulz and Blum, 1985). More commonly, however, a variety of methods that quantify cAMP have been used both for assessment of adenylate cyclase activity, as well as for measuring tissue content of cAMP or breakdown of this second messenger. A major advance for the field was the development by Steiner *et al.* (1972) of a radioimmunoassay (RIA) for cAMP that offered a high degree of sensitivity and specificity that was soon improved by Harper and Brooker (1975). Attempts at automating this assay actually led to a commercial instrument (Brooker *et al.*, 1976), but this proved unwieldy.

More recently, other methods for quantifying cAMP have used different radiometric or reporter gene strategies (Williams, 2004). Recently developed radiometric assays such as Flashplate technology (NEN/Perkin Elmer) and scintillation proximity assays (SPA, Amersham Biosciences) are based on the competition of [^{125}I]-labeled

cAMP and analyte cAMP, resulting in the production of light when the labeled compound is in close proximity to a solid scintillant surface. These assays are convenient and reproducible, but are often more expensive than traditional radiometric methods and generally speaking less sensitive. Reporter-gene assays utilize cell lines expressing reporter enzymes such as luciferase, green fluorescent protein (GFP), and β-lactamase. Levels of intracellular cAMP are detected via the expression level of a reporter gene that is modulated by transcription factor binding to upstream cAMP response elements (CRE). Reporter-gene assay are generally less expensive than the radiometric assays discussed above, however, they are often plagued by high false-positive hit rates. Several novel, non-radiometric methods to quantify cAMP also have recently become available. These assays involve the use of luminescent proximity (ALPHAScreen®) (Ullman *et al.*, 1994), enzyme complementation technology (DiscoveRx, HitHunter™ EFC), or electrochemiluminescence (Meso Scale Discovery) to detect receptor-mediated changes in intracellular cAMP. Each method is readily compatible with automated high throughput screening (HTS), and often demonstrates a high level of sensitivity, but requires a high degree of instrumentation to maximize throughput putting it beyond the reach of most academic labs.

For this reason, the RIA (or to a lesser extent, protein binding assays using PKA-enriched tissue) remains the most widely used technique. There has been a recent report detailing an improved procedure for this RIA (Post *et al.*, 2000). Indeed, there are commercial kits available (e.g., Amersham Biosciences) that utilize secondary antibody bound to magnetizable polymer beads, and are separated by magnetic separation or centrifugation. Using the dopamine D_1 receptor as a model system, we now describe

improvements to this procedure that decrease the number of experimental steps, the assay time, and the assay cost, without sacrificing accuracy or precision. In addition, we describe a rapid method for the routine production of the [^{125}I]-labeled cAMP derivative that is used as the radiomarker in this RIA.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and reagents

Dihydrexidine was synthesized according to procedures previously published (Brewster *et al.*, 1990). Acetic anhydride, dopamine, IBMX, pargyline, propranolol, SKF38393, and triethyleneamine, and 2'-O-monosuccinyladenosine 3':5'-monophosphate tyrosyl methyl ester (ScAMP-TME) were purchased from Sigma-Aldrich (St. Louis, MO). HEPES was obtained from Research Organics, Inc. (Cleveland, OH). Dulbecco's modified Eagle's media (DMEM), penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen. UniFilter-96 GF/B RIA filter plates, Microscint™ 20, and Na^{125}I were purchased from Perkin-Elmer (Waltham, MA, USA). Donkey anti-goat antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Amine terminated BioMag® beads were purchased from Polysciences, Inc. (Warrington, PA, USA), and pre-conjugated Biomagnetic Particles (BMP) to donkey anti-goat secondary was obtained from Rockland, Inc. (Gilbertsville, PA, USA).

Sample generation and storage

cAMP is a relatively heat and acid stable compound that does not require special storage. The following procedure illustrates a common way that samples are generated from a GPCR-based cellular system, but the assay that follows can be used for almost any matrix.

Cell culture: Human epithelial kidney (HEK-hD_1) cells transiently transfected with human D_1 dopamine receptor using pcDNA3.1 vector (Invitrogen) cells were maintained using Dulbecco's Modified Eagles' Medium with 50 U/mL of penicillin, 50 μg/mL of streptomycin (Gibco), and supplemented with 10% fetal bovine serum at 37°C, 5% CO_2. Saturation binding experiments with the D_1-selective antagonist [^{3}H]SCH23390 using membrane homogenates provided a B_{max} of approximately 4.5 pmol/mg protein.

Cell membrane adenylate cyclase assay: Assay buffer was prepared containing 100 mM HEPES, 4 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl 10 μM pargyline, 500 μM IBMX, 0.1% ascorbic acid, pH 7.4. Drug dilutions were prepared at a range of 10^{-4} to 10^{-10} M with three replicates per drug treatment. Diluted drugs, ATP (2 mM), GTP (5 μM), phosphocreatine (20 mM), creatine phosphokinase (185 U/tube) and propranolol (100 μM to block endogenous β_1-adrenergic receptors) were added in a total volume of 100 μL in each well of a 48-well plate. The reaction was initiated by addition of HEK-hD1 cell membranes. Plates then were vortexed briefly, and incubated at 30°C for 15 min. The reaction was terminated with 500 μL 0.1 M HCl, and stored at 4°C. Prior to transferring samples for the RIA, plates are centrifuged for 5 min at 2,500 g using a RC-3B centrifuge from Sorvall Instruments (H2000B rotor) to pellet cellular debris. Plates will keep indefinitely at 4°C following the assay.

cAMP Radioimmunoassay

Iodination reaction

The radiomarker 2′-O-[4-monosuccinyladenosine 3′:5′-cyclic monophosphate-3-[^{125}I]iodotyrosyl methyl ester (hereafter termed [^{125}I]cAMP-ScTME) was first reported by Steiner *et al.* (1972) can be purchased commercially. For laboratories that will run a

reasonable number of such assays, it is technically simple and inexpensive to synthesize this in the laboratory as outlined below. The overall reaction scheme as outlined by Steiner and coworkers (Steiner *et al.*, 1972) is shown in Figure 2.1.

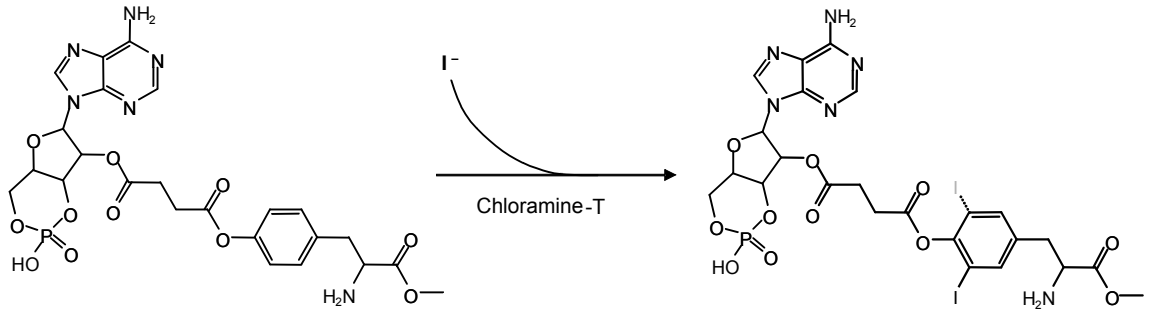


Figure 2.1: Reaction scheme for synthesis of 2'-O-[4-monosuccinyladenosine 3':5'-cyclic monophosphate-3-iodotyrosyl methyl ester. Conditions described in Section 2 (molar excess of precursor) favor the formation of the monoiodinated product (see Figure 2.2).

The following reagents and buffers are required:

- 0.5 M phosphate buffer, pH 7.6. We usually make this by titrating 15 mL of 0.5 M K_2HPO_4 with ca. 1.5 mL of NaH_2PO_4 to pH 7.6.
- 0.05 M phosphate buffer (pH 7.6). This is prepared by adding 10 mL of the 0.5 M phosphate to 90 mL H_2O.
- Carrier-free $Na^{125}I$. We usually use 2 or 5 mCi. If more than 5 mCi is used, the amount of precursor should be increased proportionally.
- Precursor ScAMP-TME [2'-O-monosuccinyladenosine 3':5'-monophosphate tyrosyl methyl ester; Sigma M2257]. From the 1 mg commercial size, we make 1-1.5 mL of a stock solution containing 0.1 mg/mL of distilled water. Aliquots (50 μL) are added to microfuge tubes, labeled, and frozen at -20° C. A single aliquot is used for each radioiodination. The frozen precursor appears stable for several years.
- Chloramine-T: (20 mg/10 mL 0.05 M PO_4).
- Sodium metabisulfite: (24 mg/10 mL 0.05 M PO_4).

The reaction procedure is as follows. Briefly, 80 μL of the 0.5 M phosphate buffer pH 7.6 is added directly to the container in which carrier-free $Na^{125}I$ (Perkin Elmer)

arrives. We usually iodinate with 5 mCi, but this can be varied. Then, the whole content of one of the thawed aliquots of ScAMP-TME (5 μg/50 μL H_2O) is added, the cap screwed back on, and the vial mixed on a vortexer for 15 sec. Following this, Chloramine T (100 μL of 2 mg/mL solution) is added, and timing begun as the mixture is vortexed. After ~45 sec, the reaction is terminated by addition of sodium metabisulfite (200 μL of 2.4 mg/mL solution). [Safety note: Unreacted ^{125}I is potentially volatile, and a potential health hazard. The use of concentrated (0.5 M) phosphate buffer insures that the reaction solution does not become acidic, a condition favoring the liberation of molecular iodine. In addition, this reaction is done in a chemical hood.]

Purification of iodinated product

It is necessary to separate the monoiodinated cAMP-ScTME from free iodine, diiodinated cAMP-ScTME and other minor by-products. Although this can be done using batch chromatography with reverse phase Sep-Pak cartridges (Oehlenschlager *et al.*, 1990), we have dedicated an archaic isochratic HPLC system and fraction collector for this purpose. The total reaction volume (~500 μL) is injected using a Rheodyne 7125 Injector (500 μL loop). The isocratic separation (0.8 mL/min) uses a C18 reverse phase column (Inertsil ODS 2-5 μm, Metachem Technologies). A typical chromatogram is shown in Figure 2.2. The column effluent is collected by a fraction collector (0.5 min samples). As noted above, unreacted ^{125}I is a potential health hazard, and for the separation, 100 μL of 0.1 M sodium hydroxide is added to the first 10 tubes on the fraction collector to insure that all unreacted iodine remains in the form of soluble sodium iodide rather than molecular iodine.

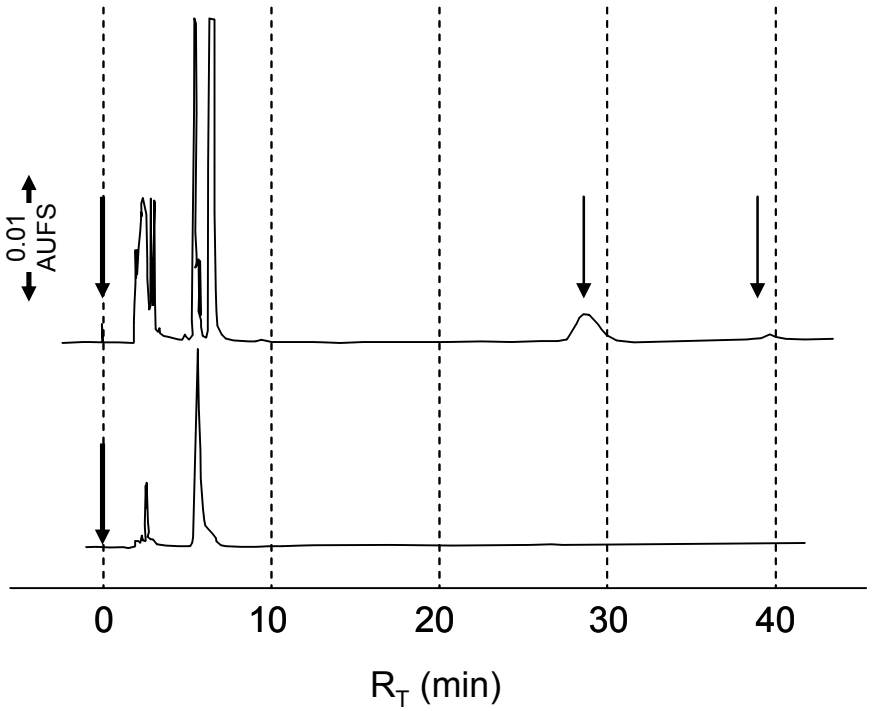


Figure 2.2: Chromatogram of radioiodination. [Bottom tracing] shows injection of cAMP-Sc-TME precursor alone using conditions as described in Methods using 254 nm UV detection. The solvent front emerges at ~ 2 min, and the precursor elutes at ~ 6min. The signal in the solvent front and a detectable shoulder on the major peak is consistent with the 95% purity estimated by the supplier. [Top tracing] Actual results from a radioiodination. The monoiodinated product that is immunologically recognized elutes at ~28 min, and is the fraction to be collected and used for the RIA. This fraction contains from 60 to70% of the radioactivity in a typical reaction. The fraction eluting at ~40 min also contains significant radioactivity (10-20%), and is presumably the diiodinated form. These two peaks account for ~80% of the total radioactivity injected, with the remainder of the radioactivity largely eluting in the solvent front (representing unreacted iodine or highly polar reaction by-products).

The radioactivity is estimated using a hand-held radioactivity detector (or one can count 1 μL aliquots), and the tubes with the highest radioactivity (usually 3-4 tubes) are pooled together, diluted with 1.5 volumes of methanol, and then divided into two or more aliquots for storage at -20°C. Under these conditions, the marker is usable for a minimum of four months, although there is a significant loss of material due to decay.

Preparation of primary antibody conjugation to amine-terminated beads

The primary α-3'-5'-cyclic monophosphate antibody was conjugated to BioMag® amine-terminated beads (50 mg/mL) as directed by the provided protocol (Polysciences,

Inc.). Lyophilized antibody was reconstituted in distilled water to a final concentration of 0.5 mg/mL, and dialyzed in coupling buffer (0.01 M pyridine in distilled water, pH 6.0), changing the buffer three-times over a 9 h period. The beads then were prepared by washing with coupling buffer, and magnetically separating three times. Glutaraldehyde solution (5% glutaraldehyde in coupling buffer) was mixed with the BioMag® beads, and reacted for 3 h with rotation. The beads were washed four times with coupling buffer, and antibody was added to the beads with rotation for 16-24 hrs. Glycine quenching solution (1 M glycine, pH 8.0) was combined with beads and rotated for 30 min. Primary α-cAMP-beads were mixed a volume of 20 mL of storage buffer (0.01 M Tris, 0.1% NaN_3, 0.1% (w/v) BSA, 0.15 M NaCl, 1 mM EDTA, pH 7.4), and stored at 4°C. The antibody-bead conjugate was used for up to 3 months with no appreciable sign of degradation. Fidelity of the conjugate was assessed by determining the ratio of binding between two sets of tubes, one containing radiolabeled cAMP bound to primary antibody and the other containing only radiolabeled cAMP. A ratio of 0.2-0.3 was found to be ideal while less than 0.2 led to inconsistent replicates.

Radioimmunoassay

cAMP standards (2 nM-500 nM) and sample aliquots (5 μL) were transferred from the 48-well microplate in which the cAMP formation was performed to 96-well Skatron plates containing Macrowell tube strips. Sodium acetate buffer (50 mM, pH 6.75) was added to the sample wells to bring the total volume up to 50 μL. Samples that contain cAMP outside of the range of the standard curve can be diluted with additional sodium acetate. An acetylating mixture of TEA/AA (2:1 ratio) was added (5 μL) to the wells and vortexed. Acetylation increases assay sensitivity presumably by creating a

structure that more closely resembles the original hapten. ^{125}I-cAMP was then added within 30 minutes of acetylation. Optimal ranges for radioactivity were determined to be between 280 cpm/μL and 320 cpm/μL for iodinated ^{125}I-cAMP-scTME. An aliquot (20 μL) of conjugated-primary antibody then was added to bind labeled and unlabeled cAMP (in 50 mM sodium acetate, 0.1% BSA, pH 4.75). Plates were incubated overnight at 4°C. Radioimmunoassay reactions were terminated by filtration with UniFilter-96 plates (Perkin-Elmer) with dH_2O. Plates were washed three times and then dried at 50°C for 1 hour. Microscint™ 20 fluid (50 μL) was added to the wells and counted on a TopCount NXT (Perkin-Elmer) for 2 min or $2\sigma = 5\%$.

Data analysis

Standard data were fit to a one-site binding competition model using Prism 4 (GraphPad Inc., San Diego, CA, USA). Sample data were fit by interpolation using standard data to obtain fmol cAMP values. A sigmoidal regression model was used to fit the data to obtain EC50 and maximal efficacy values over the complete dose range (10^{-4} to 10^{-10} M).

DISCUSSION

It should be underscored that the radiosynthesis of the marker does not require a UV detector or radioactivity detector to perform this separation, as a lab radioactivity monitor can easily distinguish the tubes that contain the desired material. Moreover, although we use a dedicated HPLC system for this work, the separation could be optimized for a SepPak, although the disadvantage is that it is difficult to verify the separation. This would not save significant time, but does not require a dedicated “hot” HPLC.

Elimination of secondary antibody allows direct detection

All prior procedures have used secondary antibodies to separate free and antibody-bound ^{125}I-cAMP-ScTME after the incubation of the analytical samples with the primary antibody. Techniques have included ammonium sulfate precipitation (Steiner *et al.*, 1972), charcoal-albumin (Harper and Brooker, 1975), and more recently, polyethylene glycol-assisted secondary separation of bound and unbound ^{125}I-cAMP (Amersham Biosciences) in which samples are pelleted by centrifugation, excess fluid in each tube decanted or aspirated, and bound radioactivity quantified. Subsequent modifications of this method have used secondary antibody conjugated to magnetic beads for detection of cAMP. All of these procedures are relatively laborious and we therefore examined whether both cost and time savings might result from elimination of the use of secondary antibody. We hypothesized that the primary antibody could be conjugated directly to Biomag® amine-terminated beads (see Section 2), and then used in a one-step assay. We therefore used the beads prepared as described above.

To expedite the radioimmunoassay (RIA), we attempted to eliminate the use of secondary antibody by conjugating anti-succinyl-cAMP antiserum to Biomag® amine-terminated beads (see Section 2). Following the conjugation of antiserum to Biomag® beads, we compared the ability of cAMP antiserum to bind cAMP standards. After incubation, the free radiomarker and that bound to the primary antibody-conjugated BioMag® beads were separated using a 96-well harvester and UniFilter-96 GF/B plates (1 μm pore size, PerkinElmer), thus enabling detection of bound radioactivity using a high throughput plate counter (Perkin-Elmer TopCount NXT). Samples (10 μL) were transferred to Macrowell tube strips (using a 12-channel electronic pipette) and necessary reagents were added as described in the Section 2. Following overnight incubation with

30 µL of primary antibody (1:40 dilution), samples were harvested using Filtermate Harvester (Packard) and plates were dried for ~1 h. Scintillation fluid (50 µL) was added to each well, and plates were counted on a TopCount NXT. Cross-well variation was corrected for following the manufacturer's protocol. Not only does this result in useful standard curves, but application to a well-characterized system (the dopamine D_1 receptor) results in EC50 values consistent with earlier literature. Our results demonstrate that cAMP antiserum conjugated to beads can be used to separate bound and free ^{125}I-cAMP with the method of separation utilized in this study.

Optimization of cAMP antiserum conditions

To determine optimal conditions for cAMP antiserum binding, we assessed the ability of the antibody to bind cAMP under variable assay conditions. cAMP standards were incubated with antiserum volumes of 50 µL and 10 µL (1:40 dilution in 50 mM sodium acetate, pH 6.75) for 2 h at room temperature, and overnight at 4ºC. All assay conditions yielded viable standard curves (Figure 2.3). As anticipated, the total amount of cAMP bound was greater for samples incubated with a 50 µL volume of cAMP antiserum than samples incubated with 10 µL. Incubation overnight resulted in increased levels of binding for both dilutions compared to the samples incubated for 2 h at room temperature. These results indicate that antiserum conditions (i.e. dilution, volume, and time of incubation) can be altered according to individual preference and assay requirements. For future experiments we chose to use an overnight incubation using 30 µL (per well) of cAMP antiserum at a 1:40 dilution.

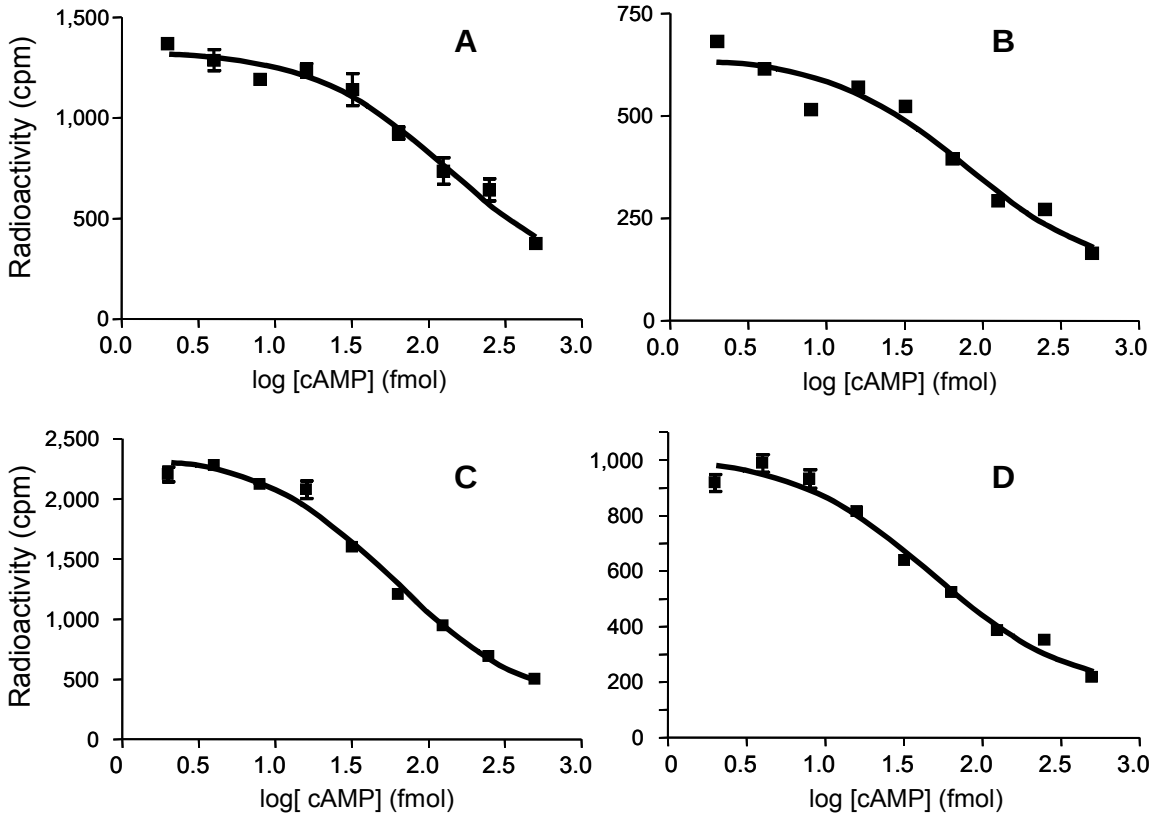


Figure 2.3. cAMP standard curves generated under varying assay conditions. Standards were incubated for 2 hrs. at room temperature with 50 μL (A) and 10 μL (B) primary antibody and overnight at 4° C [50 μL (C), 10 μL (D)]. Each assay condition yielded a viable standard curve, indicating that the conditions can be tailored according to the user's needs.

Assay precision and accuracy

To assess the feasibility of our new cAMP method, we performed an RIA using assay conditions as described by Amersham and our new method. The adenylate cyclase portion of the assay was conducted as described in Section 2. Samples were drawn from the same adenylate cyclase plate and cAMP concentrations were measured using both RIA methods. We determined that the intrinsic activity (Figure 2.4) and potency of dopamine and SKF38393 were the same for the old method and our new method (Figure 2.4). In light of the fact that some assays are limited by their ability to distinguish full and partial agonists (Williams, 2004), it is noteworthy that our method easily detects compounds with partial agonist activity (e.g., SKF38393).

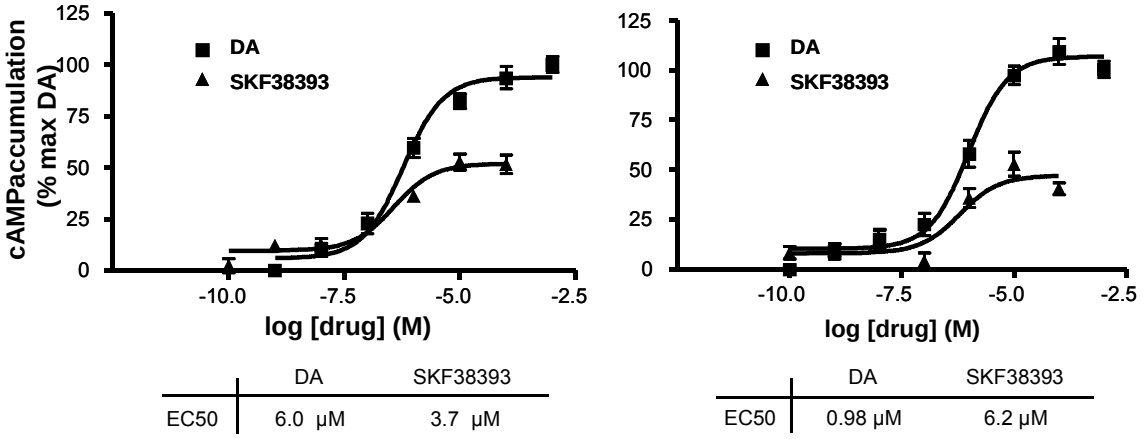


Figure 2.4. Measurement of D_1 dopamine receptor-mediated cAMP accumulation utilizing [left panel] secondary antibody-PEG assisted RIA method, and [right panel] our new RIA method (primary antibody conjugated to beads). cAMP production was measured using HEK293 cell membranes transiently expressing human D_1 dopamine receptors. Data are expressed as % maximal cAMP stimulation caused by dopamine. The curves shown represent mean $\pm$ S.E.M. for quadruplicate determinations of cAMP accumulation from four separate experiments.

To assess the between-assay reproducibility for our method we pooled the standard deviation of duplicate samples for 20 assays (Figure 2.5). The Coefficient of Variation (CV) ranged from 7 to 13%, with the CV being 10% or less over a dynamic range of more than two orders of magnitude. This is an acceptable value for an assay based on protein binding that uses radioactivity as its endpoint. It should be noted that a significant portion of the experimental variance is due to counting error (Mailman and Boyer, 1997; Motulsky, 2007), a factor that can be decreased by longer counting times if desired. It is known that this assay employs very good precision, and these data also show that it has good accuracy, both of which could be improved by longer counting time at the tradeoff of throughput.

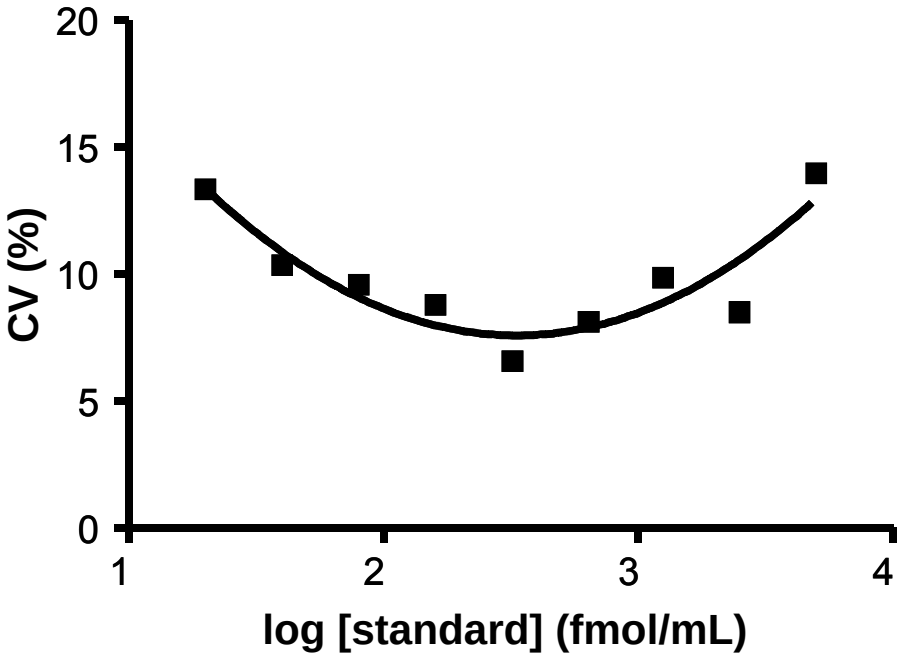


Figure 2.5. Precision profile demonstrates the Coefficient of Variation as a function of the concentration of cAMP standards.

Cost issues and alternative technology

In this study we have demonstrated an improved method of cAMP detection that allows for the quick, accurate measurement of femtomole levels of cAMP. A flowchart of this method is shown in Figure 2.6. We have eliminated the need for secondary antibody and time-consuming separation techniques. By altering the mode of detection and assay format, we have increased throughput and excluded laborious steps inherent to the previous method. Although our research focus is on whole-cell and membrane assays of $G\alpha_{s/OLF}$, $G\alpha_{i/o}$ and $G\alpha_{q/11}$ coupled GPCRs, the method is applicable to any measurement of cAMP and can be easily adapted for cGMP.

The method summarized in Figure 2.6 significantly reduces the costs required to perform the assay. Modification of the assay format and method of detection has yielded a substantial reduction of the time, labor, and costs, as well as a decrease in reagents used for the previous method. At the time of submission of this manuscript, [^{125}I]cAMP-

ScTME cost $1,517 for 50 μCi (Perkin Elmer; NEX130050). Reagents suitable for dozens of radioiodinations cost less than $200, and 5 mCi of $Na^{125}I$ can be purchased from Perkin Elmer for $155 yielding a total cost of finished product for a single iodination of < $100/mCi, several-hundred-fold less than the commercial cost.

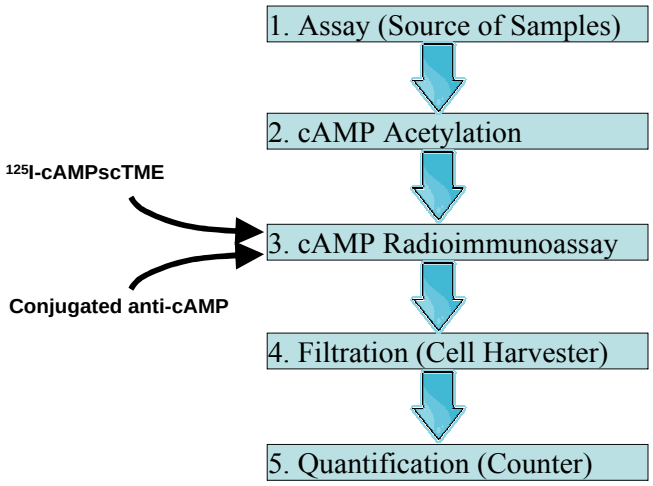


Figure 2.6. Schematic flowchart of the described method.

For the overall assay system we estimate our cost to be ca. $0.50/sample, several-fold less expensive than competing commercial systems. For example, one commercial ELISA assay costs $310 for a single 96 well assay plate, and also has a sensitivity of at least an order of magnitude less than the method we describe. Protein binding assays have also been used in the assay of cAMP for decades ((Brown *et al.*, 1972; Ekins and Brown, 1972). Such protein binding assays are fast and suitable for high throughput, but are of much lower sensitivity, and also require preparation of the cAMP binding protein preparation and the use of a long-lived relatively expensive radioactive marker (i.e., ^{3}H-cAMP). Finally, it should be obvious that this method could be easily adapted to the radioimmunoassay of cGMP. Indeed, the general approach used can also improve the throughput of any radioimmunoassay.

CHAPTER 3: INVESTIGATION OF THE ROLE OF THREONINE 3.37 IN LIGAND-BINDING AND RECEPTOR ACTIVATION.

PREFACE:

Previous studies have demonstrated the importance of TM5 serine residues for interaction with catechol hydroxyls. These serines form a network of hydrogen bonds that serve to anchor the ligand in the binding pocket. We hypothesize that T3.37 is positioned to contribute to the network of hydrogen bonds, specifically by influencing the *para*-OH of D_1 agonists. This chapter focused on exploring the role of T3.37 in ligand binding and activation of D_1-like receptors.

ABSTRACT

Molecular modeling of the D_1-like receptors led to the hypothesis that T3.37 is positioned to interact with the para-OH group of D_1 receptor ligands. To test this hypothesis, we constructed a non-conservative mutation (to alanine) of T3.37 and determined its effects on ligand binding and receptor activation. Rationally-selected, structurally dissimilar probe ligands [e.g., SCH23390, dopamine, dihydrexidine (DHX), A77636, SKF38393] were used to characterize mutant receptors. The T3.37A mutation had marked effects on affinity, potency, and intrinsic activity of rigid D_1-like ligands, but only minimal changes in affinity were observed for the more conformationally flexible phenylbenzazepines. The changes in both affinity and efficacy of the test ligands in these experiments exhibit a strikingly similar trend to the decreases in ligand binding observed at the S5.46A mutant receptor, suggesting that similar ligand-receptor contact points were affected by these mutations. We constructed a double mutation of T3.37A(108) /S5.46A(202) to explore further the molecular interactions involved in catechol binding in this region. The D_1-like T3.37A(108) /S5.46A(202) mutant receptors resulted in a loss of affinity greater than that of the T3.37A mutant receptor for all test compounds, DA, DNS, DHX and A77636 were markedly affected. All test compounds lost the ability to activate adenylate cyclase at the D_1-T3.37A(108)/S5.46A(202) mutant receptor. The loss of cyclase activation at this mutant receptor establishes a requirement for the T3.37 and S5.46 residues for receptor activation. Conversely, the D_5-T3.37A(125)/S5.46A(233) mutant receptor retained its ability to activate cAMP accumulation, suggesting the molecular interactions necessary for receptor activation may differ between the D_1 and D_5

receptors. These studies are helping to define why there is an absolute requirement, at least at present, for a catechol function in all full D_1 agonists.

INTRODUCTION

G protein-coupled receptors (GPCRs) are integral membrane proteins that mediate signal transduction in response to an array of extracellular stimuli. These proteins are important pharmaceutical targets as approximately 30% of approved therapeutics act selectively on members of the GPCR family (Hopkins and Groom, 2002). Based on sequence conservation, human GPCRs can be classified into five distinct subfamilies: rhodopsin, secretin, glutamate, adhesion, and frizzled-taste-2 (Fredriksson *et al.*, 2003). Rhodopsin-like GPCRs are further divided into several subclasses with particular ligand specificity, including the peptide, biogenic amine, opsin, and olfactory receptors.

The catecholamine-binding GPCRs represent a subset of the biogenic amine receptors consisting of the adrenergic, serotonergic, and dopaminergic receptors. These GPCRs share a highly conserved binding core in which the endogenous ligand is anchored in the binding pocket by two conserved polar regions: i) an aspartate at position 3.32 that makes direct contact with the protonated amine of aminergic ligands, and ii) two catechol hydroxyls that hydrogen bond with serine residues at position 5.42 and 5.46 (and 5.43 when present) in TM5 (Kristiansen, 2004). This network of hydrogen bonds in TM5 is critical for ligand recognition and receptor activation. The specific mode of ligand engagement with these serine residues is dependent on receptor type and structural features of the ligand.

Mutagenesis studies of the D_1 dopamine receptor have demonstrated that residues D3.32, S5.42, S5.46, as well as S5.43 are critical for binding and receptor activation (Pollock *et al.*, 1992). D3.32 is the ionic binding partner for the amine nitrogen of D_1 receptor compounds, S5.42 (and possibly S5.43) hydrogen bond with the *meta*-hydroxyl, while S5.46 forms a hydrogen bond with the *para*-hydroxyl of catechol-containing compounds. We have evidence that these molecular interactions are equally as important for binding and activation of the D_5 dopamine receptor (unpublished observations). Binding and functional data of the D_1-like receptors indicates that the exact nature of an interaction of ligand and receptor is dependent on the structural features of each ligand that can vary by structural class. The catechol requirement for full D_1 agonism makes it critical to fully understand the molecular interactions involved in catechol recognition at D_1-like receptors.

We hypothesize that a threonine in TM3 (T3.37) is positioned to interact with the *para*-hydroxyl of the catechol ring of D_1 receptor agonists. T3.37 is completely conserved across catecholamine receptors, yet to date no published studies have investigated the role of this residue in ligand recognition and receptor activation. In rhodopsin, the protonated carboxylate of E3.37 hydrogen bonds with the main chain carbonyl of H5.46, thus forming an important link between TM3 and TM5 (Palczewski *et al.*, 2000). Recent NMR studies have demonstrated that this interaction is disrupted upon receptor activation. Interestingly, SCAM studies by Javitch *et al.* (1995) indicated that T3.37 is not accessible in the D_2 dopamine receptor binding pocket.

To test the hypothesis that T3.37 interacts with the *para*-hydroxyl of D_1 agonists, we constructed a non-conservative point mutation of threonine to alanine in the D_1 and

D_5 dopamine receptors, and subsequently created D_1- and D_5- T3.37A/S5.46A double mutant receptors. We utilized an array of structurally and pharmacologically diverse D_1-like receptor test compounds to assess ligand-binding and activation of wild-type and mutant receptors. The results of this study support the hypothesis that T3.37 influences receptor interaction with the *para*-hydroxyl of D_1 receptor ligands. Furthermore, this study provides evidence of possible structural differences between the D_1 and D_5 receptor subtypes.

RESULTS

Effect of T.3.37A and T3.37A/S5.46A mutations on D_1-like receptor expression and ligand binding

The wild-type and mutant D_1-like dopamine receptors were transiently expressed in HEK293 cells and tested for their ability to bind [^{3}H]SCH23390. The dissociation constant (K_D) of [^{3}H]SCH23390 was 1.5 nM and 2.5 nM for the D_1-wt and D_5-wt receptors, respectively, and the receptors were expressed at 3,611 (D_1) and 3,042 (D_5) fmol/mg protein. The D_1- and D_5-T3.37A mutant receptors were expressed at 2,218 and 2,049 fmol/mg protein with K_D's slightly higher than that of the wild-type receptors (D_1= 2.1, D_5= 3.9). The D_1- and D_5-T3.37A/S5.46A mutant receptors were expressed at a somewhat lower density (1,166 and 1,352 fmol/mg protein for D_1 and D_5 receptors, respectively), and had similar lower K_D's (1.2 K_D for D_1 and 1.5 K_D for D_5) compared to the wild-type receptors (Figure 3.1 and Table 3.1).

Cell-surface radioimmunoassays (RIA) were performed to assess the cell-surface expression of each HA-tagged mutant receptor to that of wild-type. Figure 3.2

demonstrates that the D_1- and D_5-T3.37A and T3.37A/S5.46A mutant receptors were expressed at the cell surface at levels comparable to that of the wild-type receptors.

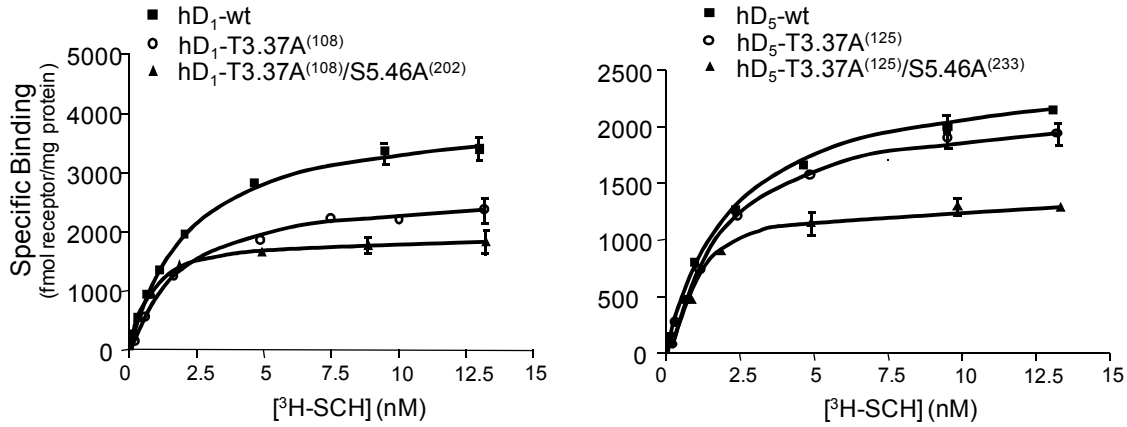


Figure 3.1. Saturation assays for D_1/D_5-WT and mutant receptors labeled with [^{3}H]SCH23390. HEK293 cell membranes transiently expressing wild-type or mutant receptors were tested in radioreceptor saturation isotherm experiments with increasing concentrations of [^{3}H]SCH23390. Non-specific binding was determined with 1μM cold SCH23390. Data were analyzed using a one-site hyperbolic curve fitting function (Prism 4.0) to obtain the K_D and B_{max} for [^{3}H]SCH23390 at wild-type and mutant receptors. Data are mean ± S.E.M. ND= Not Determinable.

Table 3.1. K_D and B_{max} of D_1/D_5-WT and mutant receptors labeled with [^{3}H]SCH23390.

Receptor Type	N	K_D	B_{max}
D_1		*(nM)*	*(fmol/mg protein)*
Wt	12	1.5 ± 0.2	3,600 ± 560
T3 .37A(108)	2	2.1 ± 0.9	2,200 ± 260
T3.37A(108)/S5.46A(202)	3	1.2 ± 0.2	1,200 ± 600
D_5			
Wt	13	2.5 ± 0.5	3,040 ± 740
T3.37A(125)	2	3.9 ± 0.5	2,050 ± 70

T3.37A(125)/S5.46A(233)	2	1.5 ± 0.1	1,350 ± 120

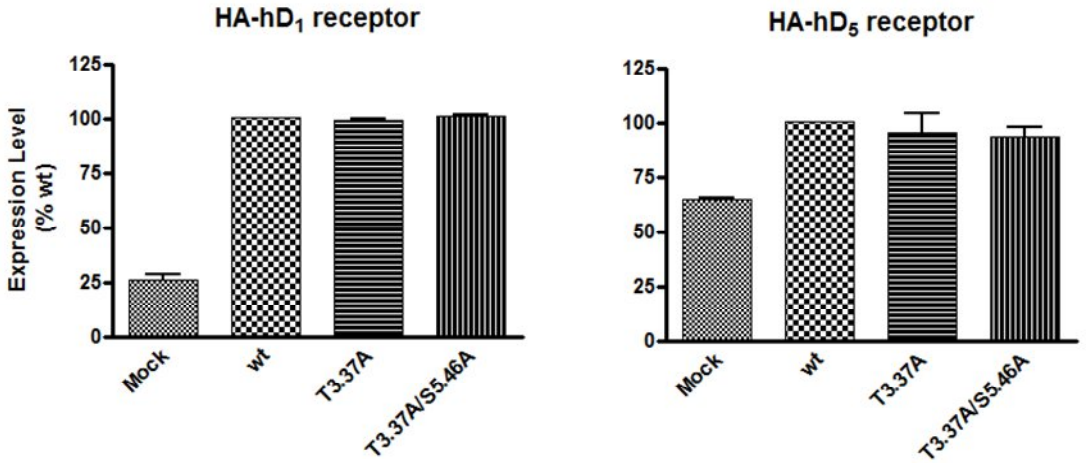


Figure 3.2. Cell surface expression of D_1-like mutant receptors. HEK293 cells transiently expressing HA tagged D_1-like mutant receptors, exhibiting decreased [^{3}H]SCH23390 binding, were tested for cell surface expression relative to each respective wild-type receptor via RIA. Data are representative of 2-3 independent experiments run in quadruplicate.

Effect of D_1 and D_5 T3.37A (D_1-T108A & D_5-T125A) on agonist affinity

The affinity of each probe ligand for the wild-type and mutant receptors was determined in cell membranes using competition radioreceptor assays with [^{3}H]SCH23390 as the radioligand. To determine apparent affinity constant, $K_{0.5}$, experimental IC50 values were corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). The rank order of affinities for compounds at the wild-type receptors was as follows, **D_1**: SCH23390 > A77636 $\geq$ SKF82958 > SKF82526 > DNS = DHX > SKF38393 > DA; **D_5**: SCH23390 > A77636 > SKF82958 $\geq$ DNS = DHX > SKF38393 > DA. These data are consistent with previous reports of for the D_1 and D_5 receptors, but include ligands not reported in those earlier studies (Sunahara *et al.*, 1991; Dearry *et al.*, 1990). Binding assays revealed a decrease in affinity for all agonists tested (Figures 3.3 and 3.4, and Table 3.2).

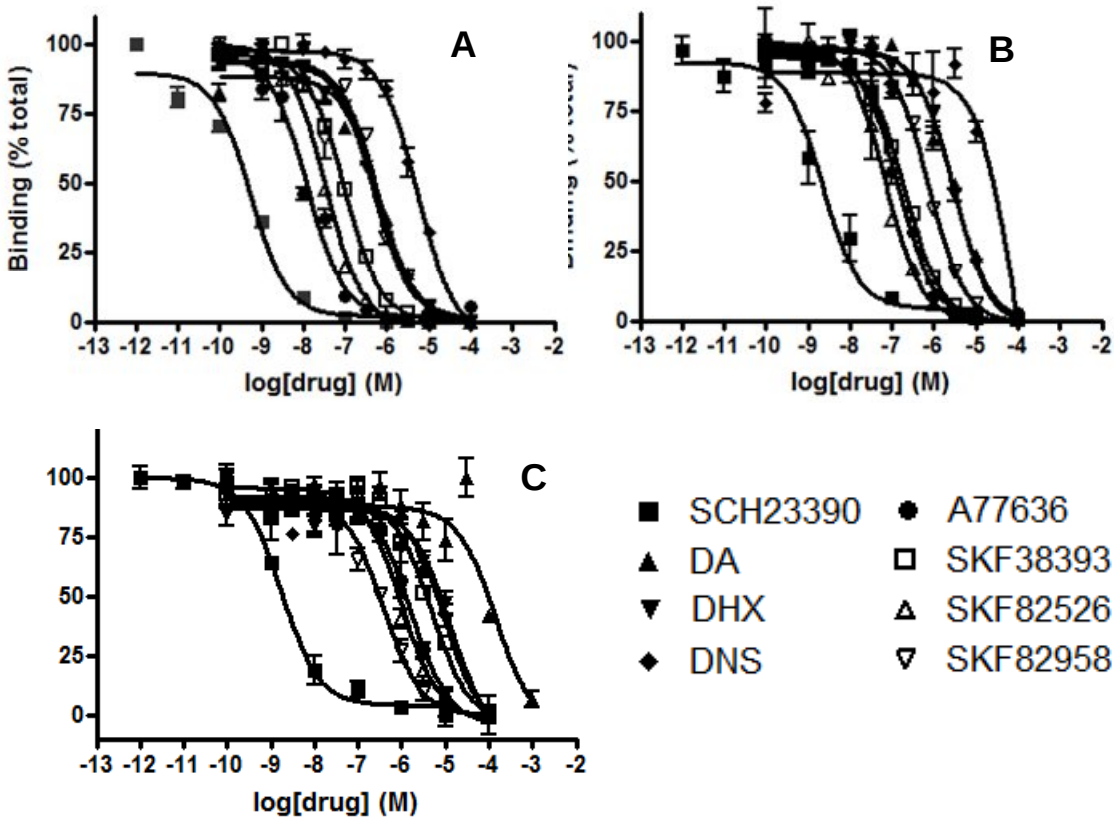


Figure 3.3. Binding of probe ligands to the D_1-WT and single and double-mutant receptors. Membrane preparations of D_1-wild type ***[A]****, T3.37A* ***[B]****, and T3.37A/S5.46A* ***[C]*** *mutant receptors were incubated with [^{3}H]SCH23390 for 15 min with varying concentrations of test compound. 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 duplicate or triplicate and data represents 2-3 independent experiments.*

At the D_1- and D_5-T3.37A mutant receptors, affinity of the phenylbenzazepine (SKF38393, SKF82526, & SKF82958) compounds were affected minimally, whereas there were dramatic decreases in the affinity of DA, A77636, DHX, and DNS, particularly at the D_5 mutant receptor. The affinity of dopamine was most severely altered (40-fold) at the D_1-T.3.37A$^{(108)}$ mutation, whereas the decreases for DHX, DNS, and A77636 were more modest. At the D_5-T3.37A$^{(125)}$ mutant receptor, losses in affinity were most dramatic for DA, DHX, and DNS, with decreases of 100-, 140-, and 220-fold, respectively. Binding of the antagonist SCH23390, a compound with a *para*-Cl instead of a hydroxyl, was unaffected at the D_1-like T3.37A mutant receptors, consistent with the

data from the saturation studies (Figure 3.1). The rank order of binding affinities at the T3.37A mutant receptors was as follows, $\mathbf{D}_1$: SCH23390 > SKF832958 > SKF82526 > A77636 > SKF38393 > DHX $\geq$ DNS > DA; $\mathbf{D}_5$: SCH23390 > SKF82958 > A77636 $\geq$ SKF82526 > SKF38393 > DHX = DNS > DA.

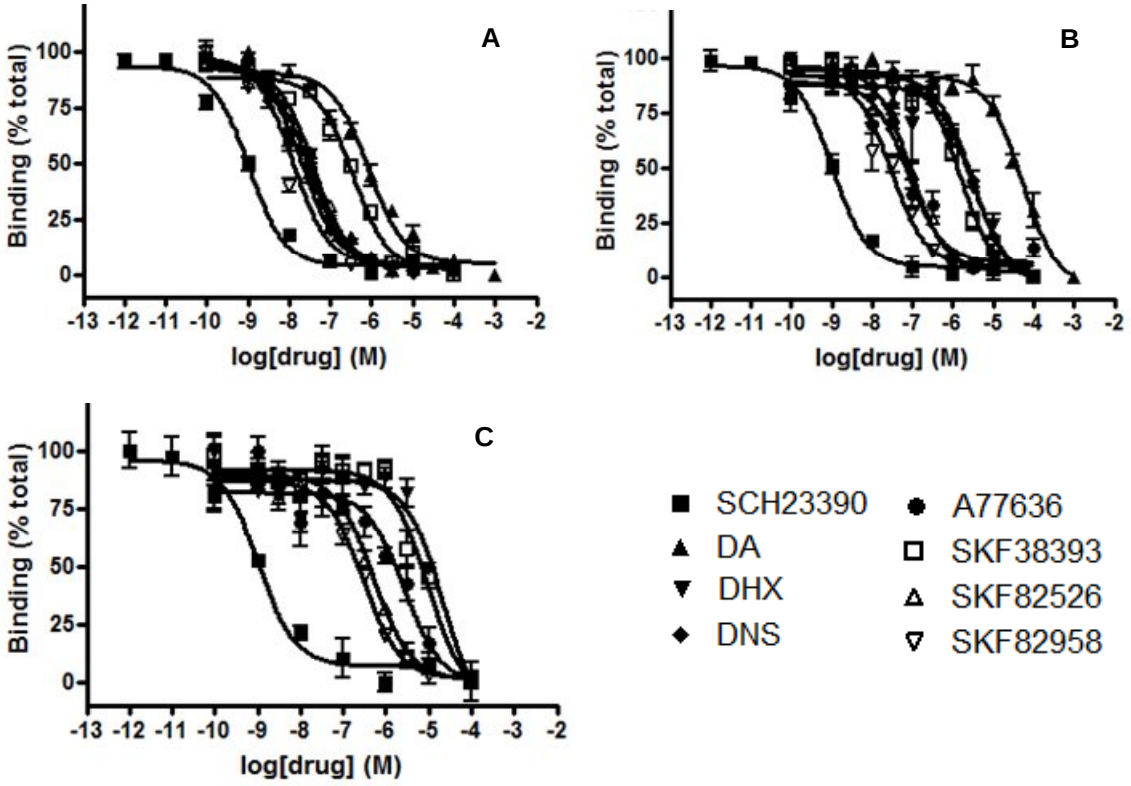


Figure 3.4. Binding of probe ligands to the D_5-WT and single and double-mutant receptors. Membrane preparations of D_5-wild type ***[A]****, T3.37A* ***[B]****, and T3.37A/S5.46A* ***[C]*** *mutant receptors were incubated with [^{3}H]SCH23390 for 15 min. with varying concentrations of test compound. 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 duplicate or triplicate and data represents 2-3 independent experiments.*

Table 3.2. Affinity of test ligands for the D_1- and D_5-WT and mutant receptors labeled with [^{3}H]SCH23390.

	D_1		
Ligand	**wt**	**T3.37A(108)**	**T3.37A(108)/S5.42A(202)**
SCH23390	0.24±0.03	0.24± 0.02 (1)	0.2± 0.05 (1)
Dopamine	2,550 ±390	103,000± 27,000 (40)	1,850,000 ± 72,000 (60)
DHX	177 ±125	847 ± 26 (5)	3,240 ± 620 (18)
DNS	148 ±7.5	1,110 ± 116 (7)	2,790 ± 480 (16)
A77636	5.2 ±2.1	46.3 ± 3.4 (9)	428 ± 68 (75)
SKF38393	220 ±18.6	284 ± 52 (1)	1110 ± 190 (5)
SKF82526	35.3 ±3.4	72 ± 7.9 (2)	271 ± 24 (7)
SKF92958	7.5 ±1.8	21.8 ± 2.6 (3)	103 ± 7 (13)
		D_5	
	Wt	**T3.37A(125)**	**T3.37A(125)/S5.42A(233)**
SCH23390	0.35±0.07	0.44± 0.02 (1)	0.42 ±0.17 (1)
Dopamine	238 ±49	124,000± 1,600 (100)	N.D.
DHX	10.2±2.0	1,430 ± 340 (140)	5,650 ± 1150 (552)
DNS	6.1±0.8	1,360 ± 1,200 (220)	N.D.
A77636	1.8±0.01	21 ± 4.5 (12)	694 ± 32 (397)
SKF38393	100 ±15.7	657 ± 27 (7)	2,330 ± 290 (23)
SKF82526	10.2±1.5	35 ± 7.1 (3)	130 ± 14 (13)
SKF92958	4.1±0.8	7.6 ± 1.4 (2)	74 ± 5.2 (18)

HEK293 cell membranes containing D_1/D_5-wt or mutant receptors were incubated with one concentration of [^{3}H]SCH23390 and 7-12 concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) to obtain estimates for apparent affinity ($K_{0.5}$) using the Cheng-Prusoff equation. Values are expressed as mean ± S.E.M. (nM). In parentheses is the fold change value as compared to the $K_{0.5}$ of each drug at the wild type receptor. Data are representative of 3-10 experiments run in triplicate.

Effect of D_1 and D_5 T3.37A/S5.46A (D_1-T108A/S202A & D_5-T125A/S233A) on agonist affinity

As with the D_1-like T3.37A mutant receptors, the alterations in agonist binding affinity (Table 3.2 and Figures 3.3 and 3.4) at the D_1- and D_5-T3.37A/S5.46A double-mutants can be grouped according to structural class (Figure 1.3). As expected from the saturation assays (Table 3.1), binding of SCH23390 was unaffected at the D_1-

T3.37A$^{(108)}$/S5.46A$^{(202)}$, whereas dopamine and A77636 had large affinity decreases of 60- and 75-fold, respectively, and the phenylbenzazepines (SKF38393, SKF82526, & SKF82958) displayed modest decreases in affinity. As with the D_5-T3.37A$^{(125)}$ mutant, the decreases in affinity at the D_5-T3.37A$^{(125)}$/S5.46A$^{(233)}$ mutant receptor were more dramatic than those at the D_1-T3.37A$^{(108)}$/S5.46A$^{(202)}$ mutant receptor. The losses in binding affinities at the D_5 double mutant receptor ranged from no effect for SCH23390, to 552- and 397-fold for DHX and A77636 respectively. The severe decrease in the affinities of DA and DNS at D_5-T3.37A/S5.46A precluded accurate assessment of affinity constants for these compounds. The rank order of binding affinities was as follows, $\mathbf{D_1}$: SCH23390 > SKF82958 > SKF82526 > A77636 > SKF38393 > DNS > DHX > DA; $\mathbf{D_5}$: SCH23390 > SKF82958 > SKF82526 > A77636 > SKF38393 > DHX > DNS = DA.

Functional effects of D_1/D_5-T3.37A and T3.37A/S5.46A mutant receptors

We utilized a number of structurally and pharmacologically diverse agonists to assess the mutational effect on receptor activation. Wild-type and mutant receptors were stimulated with a range of agonist concentrations to stimulate adenylate cyclase. The cAMP accumulation was produced by treatment with agonist and expressed as % maximal stimulation produced by dopamine at each receptor type. The effects on the action of dopamine are summarized in Table 3.3. These data highlight the fact that T3.37A alone had little effect on maximal efficacy, whereas the double-mutant T3.37A/A5.46A had small significant effects on the D_5 double mutant yet abolished dopamine induced stimulation for the D_1.

Table 3.3. Dopamine-induced stimulation of cAMP synthesis for the D_1-like WT and mutant receptors.

D_1	Activity *(fmol cAMP/mg/min)*		D_5	Activity *(fmol cAMP/mg/min)*	
	Basal	**Dopamine**		**Basal**	**Dopamine**
wt	2966 ± 309	6431± 447	**wt**	2977 ± 179	6409 ± 366
T3.37A	2371 ± 497	5211± 603	**T3.37A**	2135 ± 139	6579 ± 121
T3.37A/S5.46A	901 ± 51	793± 32	**T3.37A/S5.46A**	1388 ± 173	4832 ± 322

The rank order of potency at the D_1 and D_5 wild-type receptors was as follows, **D_1**: SKF82526 ≥ SKF82958 > A77636 > DHX ≥ DNS > SKF38393 > DA; **D_5**: A77636 ≥ SKF82526 = SKF82958 > DHX = DNS > SKF38393 > DA. At the D_1 receptor dopamine, DHX, DNS, and A77636 were all full agonists produced cAMP accumulation equal to that of dopamine. SKF38393 was a partial agonist producing approximately 36% of the maximal activity of dopamine, while SKF82526 and SKF82958 were partial agonists of high intrinsic activity of approximately 87% and 90% of dopamine. Similar to the D_1 receptor, at the D_5-wt receptor DNS, DHX, and A77636 were full agonists. SKF38393 displayed partial agonist activity with 40% activity of that of DA. SKF82526 and SKF82958 were high intrinsic activity partial agonists producing approximately 73% and 86% of dopamine.

T3.37A mutant receptors (D_1-T108A & D_5-T125A)

Mutation of T3.37 to alanine in both D_1-like receptors resulted in changes in the intrinsic activity of all test compounds. SKF82958 and SKF82526 also exhibited intrinsic activity greater than that of dopamine at the D_1- and D_5- T3.37A mutant receptors, while A77636, DNS (excluding the D_5 mutant receptor), and DHX were reduced from full to partial agonists (relative to dopamine). Specifically, the intrinsic activity of SKF82526 and SKF82958 at the D_5-T3.37A$^{(125)}$ mutant receptor increased dramatically from 73%

and 86% of dopamine in the wild-type receptor to 156% and 159% of dopamine in the mutant receptor. Changes in potency were modest at both the D_1- and D_5-T3.37A mutant receptors. At the D_1 mutant receptor, dopamine, DHX, and DNS (from 11- to 7-fold) displayed the greatest decreases in potency, whereas A77636 (22-fold) and DHX (14-fold) were most affected at the D_5-T3.37A$^{(125)}$ receptor. The potency and intrinsic activity of SKF38393 was affected modestly at both D_1-like mutant receptors.

Table 3.4. Agonist potency and intrinsic activity at the D_1/D_5-WT and mutant receptors.

D_1	**wt**		**T3.37A**		**T3.37A/S5.46A**	
Ligand	**EC50 (nM)**	**IA**	**EC50 (nM)**	**IA**	**EC50**	**IA**
Dopamine	545 ± 300	100	5972 ± 751 (11)	100	N.D.	N.D.
DHX	48 ± 20	99	332 ± 56 (7)	41	N.D.	N.D.
DNS	70 ± 26	101	454 ± 56 (7)	70	N.D.	N.D.
A77636	24 ± 8	97	49 ± 4.5 (2)	65	N.D.	N.D.
SKF38393	298 ± 37	36	728 ± 356 (2)	29	N.D.	N.D.
SKF82526	3 ± 0.4	87	4.9 ± 2.4 (1)	117	N.D.	N.D.
SKF82958	25 ± 11	90	103 ± 35 (4)	112	N.D.	N.D.

D_5	**wt**		**T3.37A**		**T3.37A/S5.46A**	
Ligand	**EC50 (nM)**	**IA**	**EC50 (nM)**	**IA**	**EC50 (nM)**	**IA**
Dopamine	390 ± 34	100	1630 ± 442 (4)	100	5676 ± 757 (15)	100
DHX	12 ± 1.8	100	172 ± 43 (14)	69	556 ± 212 (45)	81
DNS	13.1± 2.4	98	105 ± 9 (8)	102	538 ± 109 (40)	85
A77636	2.2 ± 0.2	102	48 ± 24 (22)	65	357 ± 106 (162)	84
SKF38393	52.3± 8.6	40	268 ± 122 (5)	43	92 ± 21 (2)	27
SKF82526	9.6± 4.4	73	23 ± 7.6 (2)	156	77 ± 20 (8)	206
SKF82958	9.7± 2.8	86	56 ± 13 (6)	159	507 ± 71 (53)	167

IA = Intrinsic activity, based on dopamine.
N.D.= not determinable due to a lack of measurable intrinsic activity.
HEK293 cell membranes containing D_1/D_5-wt or mutant receptors were incubated with 7 concentrations of test ligand. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) for best fit to obtain EC50 values. Values are expressed as mean ± S.E.M. (nM). Fold change value as compared to the EC50 of each drug at the wild-type receptor is listed in parentheses.

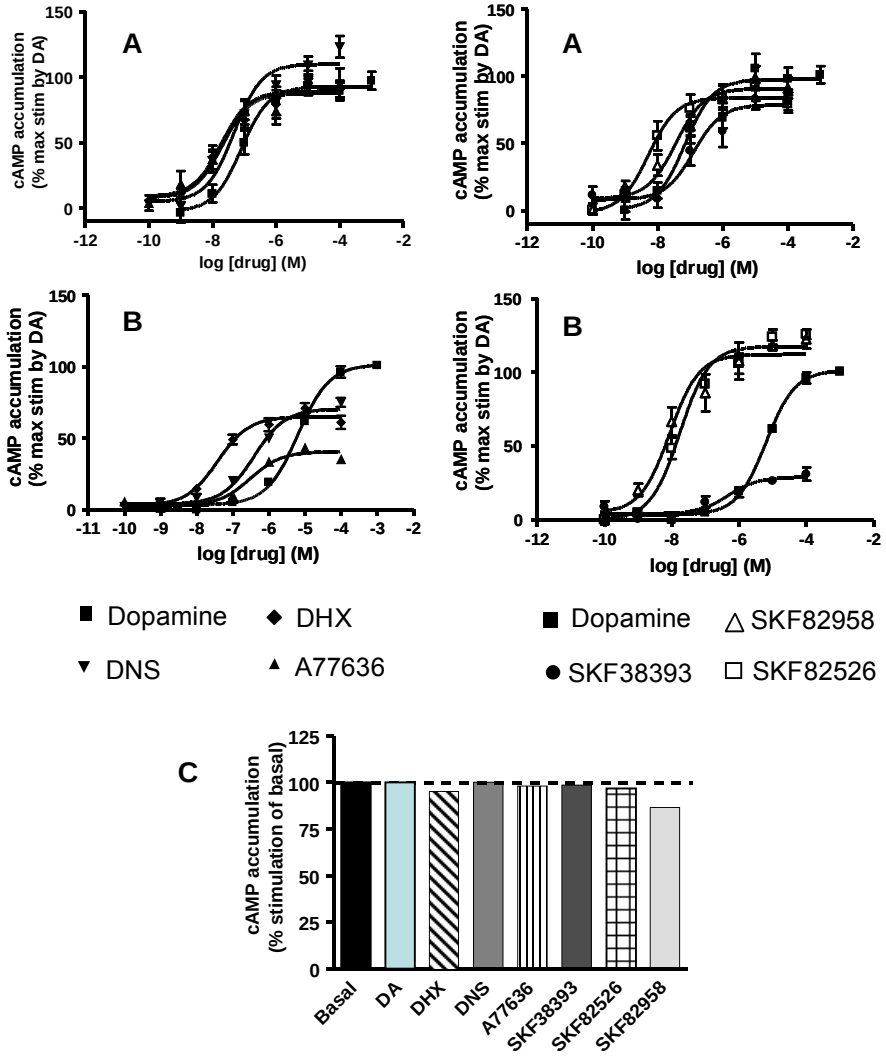


Figure 3.5. Ligand effects on cAMP accumulation at D_1-WT, T3.37A, and T3.37A/S5.46A mutant receptors. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37°C. Dose-response curves were generated using 7 concentrations of test compound at the:* ***[A]*** *D_1- wild-type;* ***[B]*** *T3.37A; and* ***[C]*** *T3.37A/S5.46A, mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

T3.37A/S5.46A mutants (D_1-T108A/S202A & D_5-T125A/S233A)

Figure 3.5 shows that a double mutation of T3.37 and S5.46 to alanine in the D_1 dopamine receptor resulted in a complete loss in the activation of adenylate cyclase for all test compounds (Figure 1.3). As shown in Table 3.3, the basal level of cAMP was

reduced from 2966 fmol/mg/min at the D_1 wild type receptor to 901 fmol/mg/min at the mutant receptor.

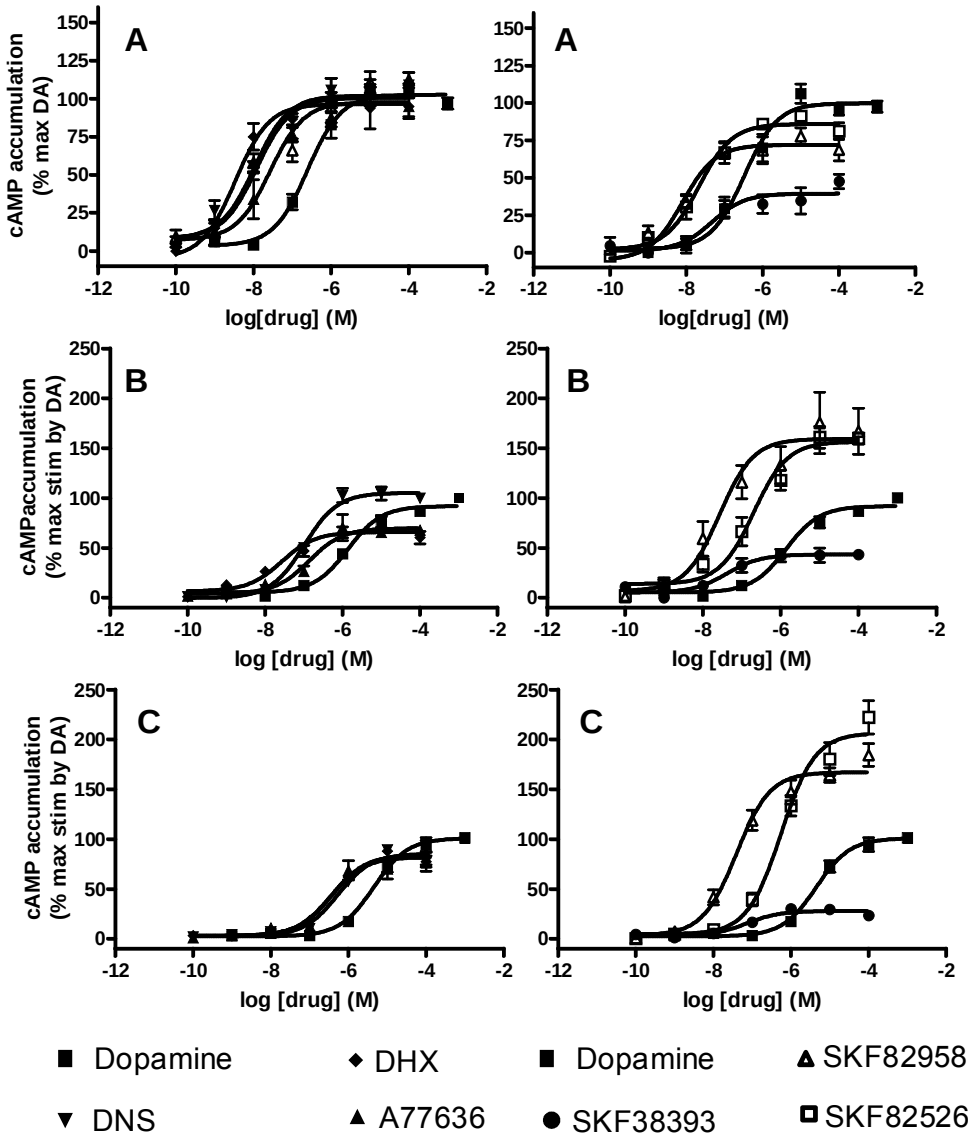


Figure 3.6. Ligand effects on cAMP accumulation at D_5-WT, T3.37A, and T3.37A/S5.46A mutant receptors. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37° C. Dose-response curves were generated using 7 concentrations of test compound at the D_5- wild-type* ***[A]****, T3.37A* ***[B]****, T3.37A/S5.46A* ***[C]****, mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

Based on the results for the D_1 mutant receptor, we anticipated a complete loss of cAMP accumulation at the D_5-T3.37A/S5.46A mutant receptor. However, all test compounds stimulated cAMP accumulation above basal at the D_5 mutant receptor (Table 3.3, Figure 3.6). As observed at the D_5-T3.37A mutant receptor, A77636, DHX, and DNS exhibited decreases in intrinsic activity at the D_5-T3.37A/S5.46A mutant receptor, while the intrinsic activity of SKF82526 and SKF82958 was increased greatly (206% and 167% of dopamine). All test compounds exhibited reduced potency at the mutant receptor with A77636 experiencing the most dramatic loss (162-fold). The rank order potency of test compounds at the D_5-T3.37A/S5.46A mutant receptor was as follows, SKF38393 $\geq$ SKF82526 > A77636 > SKF82958 = DHX = DNS > DA.

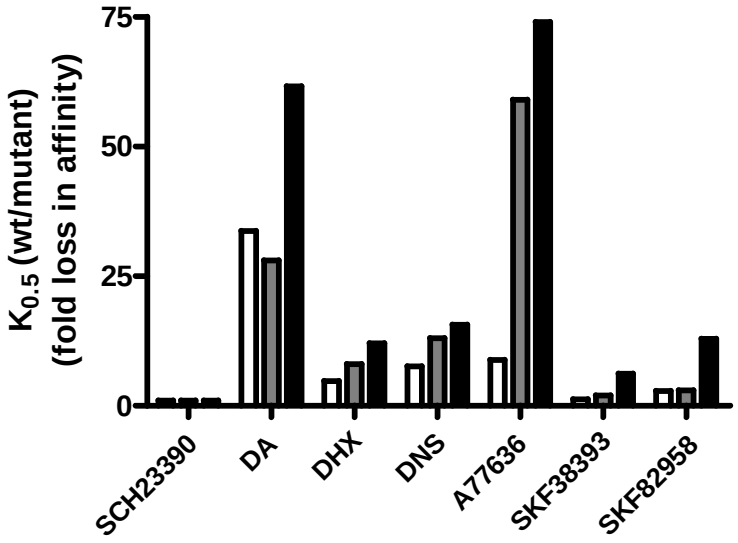


Figure 3.7. Fold loss in affinity of ligands at the D_1- T3.37A, S5.46A, and T3.37A/S5.46A mutant receptors*. HEK293 cells transiently expressing HA tagged D_1-like mutant receptors, exhibiting decreased [^{3}H]SCH23390 binding, were tested for cell surface expression relative to each respective wild-type receptor via RIA. Data are representative of 2-3 independent experiments run in quadruplicate.*

DISCUSSION

Despite the highly conserved nature of T3.37 among catecholamine GPCRs, to our knowledge the involvement of this residue in ligand binding and receptor activation has not been explored experimentally in any aminergic receptors. We hypothesized that T3.37 is positioned to influence the binding of agonists by interacting with the *para*-OH of the catechol ring of D_1 receptor ligands. The work in this Chapter provides evidence that T3.37 plays an important role in ligand-binding and activation of the D_1-like receptors.

Strader *et al*. (1988; 1989), using the β_2-adrenergic receptor, was the first to demonstrate a critical role for catechol interaction with TM5 serine residues (S5.42 and S5.46) in ligand interaction of catecholamine GPCRs. Specifically, the authors demonstrated that S5.42 interacts with the *meta*-hydroxyl and S5.46 with the *para*-hydroxyl of isoproterenol. Since then, these TM5 serines residues (including S5.43) have been shown to be critical for recognition of the endogenous ligand in most Class A GPCRs, including the D_1-like dopamine receptors. These serine residues form a network of H-bonds that serve to anchor the endogenous ligand in the receptor binding pocket.

Previous mutagenesis studies in our lab, using the structurally diverse D_1 agonists utilized in this study, have shown that ligands of different structural classes have differential dependence on the TM5 serines (unpublished observations). A77636 is most dependent on S5.46 for binding and receptor activation, likely due to the adamantly substituent that positions the ligand in a slightly different mode from other D_1 receptor full agonists. DHX and DNS are highly dependent on S5.46, whereas the phenylbenzazepine compounds are less reliant on S5.46 for receptor interaction than the other D_1 agonists. Despite sharing a similar structural backbone, the pattern of interaction

with the TM5 serines is different for SKF38393 than SKF82526 and SKF82958. The 6-chlorine substituent on the catechol ring of SKF82526 and SKF82958 is thought to orient these ligands in the binding pocket in a manner that greatly reduces their reliance on S5.46. These studies have greatly aided in refinement of the D_1 agonist pharmacophore, leading to a more comprehensive understanding of how structurally diverse agonists dock, invaluable data for the experiments described in this Chapter.

A T3.37 mutation was shown to abolish ligand-receptor interactions without affecting global receptor conformation (Fersht, 1987). The structurally diverse agonists used in the current study caused differential affects between ligand classes, indicating that the mutation caused regional, rather than global, changes in receptor conformation. Based on our hypothesis, I expected ligand-binding and activation at the D_1-like T.3.37A mutant receptors to yield results similar to those observed at the D_1- and D_5-S5.46A mutant receptors. Indeed, as illustrated in Tables 3.2 and 3.4, the trend in ligand affinity and potency paralleled those changes observed at the S5.46A mutant receptors. The affinity and potency of phenylbenzazepines compounds- which have minimal dependence on S5.46 for binding and receptor activation- were less affected than the more rigid compounds (i.e. DHX, DNS, and A77636) at both the D_1- and D_5- T3.37A and T3.37A/S5.46A mutant receptors. As shown in Figure 3.7, the fold change (vs. wild-type) decrease in ligand affinity at the D_1-T3.37A, D_1-S5.46A, and D_1-T3.37A/S5.46A mutant receptors are progressively more dramatic. The decrease in agonist affinity for the experiments in this work exhibit a strikingly similar trend to the decreases in ligand binding observed at the S5.46A mutant receptor suggesting that similar ligand-receptor contacts were affected in each mutation.

The changes exhibited in the intrinsic activity of test compounds at the D_1-like mutant receptors further support our hypothesis T3.37 is positioned in the region of the receptor binding *para*-OH of the D_1 compounds. The relative intrinsic activity of the rigid compounds at the D_1- and D_5-T3.37A receptors was decreased compared to wild-type. The intrinsic activity of A77636 and DNS was decreased ~30%, whereas that of DHX was reduced more than 50%. Conversely, the high efficacy partial agonists SKF825626 and SKF82958 were altered from high efficacy partial agonists at wild-type to greater than full agonists at the D_1-T3.37A, D_5-T3.37A and T3.37A/S5.46A mutant receptors. These changes were greatest at the D_5-T3.37A/S5.46A mutant receptor, as the relative intrinsic activity of SKF82526 and SKF82958 increased to 206 and 167 percent respectively of that of the D_5 wild type receptor. Increases in the intrinsic activity of SKF82958 and SKF82526, and a decrease for DHX, were also observed at the D_1- and D_5-S5.46A mutant receptors in previous mutagenesis experiments. The change in the intrinsic activity of SKF82526 and SKF82958 at the D_1 and D_5 mutant receptors is relative to dopamine. These changes reflect a decrease in absolute cAMP accumulation caused by dopamine not an increase in cAMP formation by SKF82526 and SKF82958. In fact, the fmol of cAMP/mg/min elicited by SKF82526 and SKF82958 was minimally altered between the wild-type and mutant receptors.

The most remarkable result of this study was the complete loss of cyclase activation for all test compounds at the D_1-T3.37A/S5.46A mutant receptor. Despite normal cell-surface expression and ligand-binding, D_1 agonists did not stimulate cAMP formation at the D_1-T3.37A/S5.46A mutant receptor. This result was entirely unexpected given the ability of all test compounds to stimulate adenylate cyclase activation at both

the D_1-T3.37A and S5.46A mutant receptors. Given this finding, I anticipated similar results for the D_5-T3.37A/S5.46A mutant receptor. Unexpectedly, the D_5 double mutant receptor retained the ability to stimulate cAMP accumulation upon agonist binding. The loss of cyclase activation at the D_1-T3.37A/S5.46A mutant receptor establishes a requirement for the T3.37 and S5.46 residues for receptor activation, at least at this signaling pathway. Furthermore, this finding provides insight into possible structural differences between the D_1 and D_5 dopamine receptors.

Whether T3.37, along with S5.46, interacts directly with the *para*-OH of D_1 receptor ligands or with the main-chain carbonyl of S5.46 remains unclear. Threonine residues often form intra- and interhelical hydrogen bonds in α-helical environments (Ballesteros *et al.*, 2000). It is possible that T3.37 stabilizes the carbonyl group of S5.46 for interaction with the *para*-OH group, thereby forming a functional link between TM3 and TM5 important for receptor activation. Alternatively, our D_1 molecular model indicates that T3.37 may hydrogen bond with S5.42. This hypothesis requires additional investigation (discussed in the future studies section in Chapter 7) and is the subject of current modeling studies. While the exact mechanism is unclear, the experiments in this Chapter provide convincing evidence that T3.37 contributes to a complex network of H-bonds involved in stabilizing the catechol hydroxyls in binding to the D_1-like dopamine receptors.

T3.37 is highly conserved in aminergic GPCRs and is likely to be involved in the binding and activation process of many other aminergic receptors. To our knowledge, the role of T3.37 in ligand-binding and activation in catecholamine receptors has yet to be examined experimentally. Several molecular modeling studies, however, have predicted

an important role for T3.37 in ligand recognition. Docking studies with the 5-HT_{2A} receptor have suggested that the 5-OH substituent of the serotonin analog 3-(2-(methylamino)ethyl)-1H-indol-5-ol (termed N-ω-methyl-5-HT by the authors) forms a hydrogen bond with the hydroxyl group of T3.37 (Shapiro *et al.*, 2000). A computational study using the β_2-adrenergic receptor predicted that T3.37 forms a hydrogen bond with T4.56 in the inactive state; docking simulations show that this bond is broken by salbutamol (Bhattacharya *et al.*, 2008a). Xhaard *et al.* (2006) predicted that T3.37 is part of a large conserved "binding core" present in catecholamine-binding GPCRs. Recent computational studies of bovine rhodopsin have proposed a central role for 3.37 (glutamine in bovine rhodopsin) in formation of the active receptor state (Bhattacharya *et al.*, 2008b). In addition to the hydrogen bond between E3.37 and backbone carbonyl of H5.46 demonstrated in the crystal structure of inactive rhodopsin, Bhattacharya *et al.* (2008b) observed another hydrogen bond between E3.37 and C4.56 on TM4. In the predicted active receptor state of rhodopsin, the interaction of E3.37 with the carbonyl of H5.46 is disrupted when TM5 rotates in a clockwise fashion, and forms a new hydrogen bond with the nitrogen of H5.46. NMR experiments have demonstrated that the hydrogen bond between E3.37 and the carbonyl of H5.46 is broken upon receptor activation (Patel *et al.*, 2005). Bhattacharya *et al.* (2008b) propose that movement of the β-ionone ring towards TM5 causes a conformational change in M5.42 that disrupts the interaction between E3.37 and H5.46. The loss of two hydrogen bond contacts, E3.37 and E3.28, causes TM3 to rotate clockwise 15º thus breaking the important salt bridge between R3.50 and E6.30.

In conclusion, the current data supports the hypothesis that T3.37 participates in ligand-binding and receptor activation in the D_1-like dopamine receptors by influencing binding of the *para*-OH group of D_1 agonists. In light of the requirement of catechol moieties for full agonism at the D_1 receptor, this study is important for further understanding this constraint. While the exact nature of the interaction (i.e. whether T3.37 interacts with the carbonyl of S5.46, with the *para*-OH of D_1 agonists, or possibly S5.42) remains to be determined, it is clear that T3.37 plays an important role in positioning the D_1 agonists in the binding pocket and is required for high affinity full agonism. Understanding the nature of this interaction will impact our general understanding of D_1 receptor activation, as well as aid in the design of novel D_1-like agonists. Furthermore, given the highly conserved nature of T3.37 in catecholamine receptors we anticipate that these findings can be extended to other aminergic GPCRs.

CHAPTER 4: DETERMINE MORE SPECIFICALLY THE ROLE OF PHENYLALANINE 6.51 IN THE INTERACTION OF D_1-LIKE DOPAMINE RECEPTORS WITH STRUCTURALLY DIVERSE D_1 AGONISTS.

For publication in:
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PREFACE:

F6.51 resides in the cluster of aromatic amino acid residues (i.e. 'rotamer toggle switch') located around the proline kink in TM6. Studies of several aminergic receptors have shown that this switch region plays a key role in transducing ligand binding to receptor activation. Prior studies in our lab, involving the mutation of F6.51 and F6.52 to A, suggested a key role for F6.51, but not F6.52, in the activation of D_1-like receptors. Employing the structurally diverse set of D_1 agonists used in Chapter 3, the experiments in this chapter explore in more detail how F6.51 contributes to formation of an active receptor state.

ABSTRACT

The molecular mechanisms by which agonists bind and activate GPCRs in general, and the D_1 and D_5 dopamine receptors in particular, is largely unknown. Previous studies of F6.51 using non-conservative mutations (i.e. F6.51A) provided evidence that this residue influences receptor activation by interacting with the catechol ring of D_1 agonists. In this chapter, conservative (to tryptophan and isoleucine) and non-conservative mutations (to leucine and isoleucine) of F6.51 were made to examine further the intermolecular forces involved in ligand binding and receptor activation. After site-directed mutagenesis, selected probe ligands [e.g., SCH23390, dopamine, dihydrexidine (DHX), SKF38393] were used to characterize the mutant receptors [i.e., hD_1-F6.51(288)I/W]. The mutant receptors bound [^{3}H]SCH23390 with decreased affinity. Although all three agonists had decreased potency at the mutant vs. WT receptors, SKF38393 had a large increase in intrinsic activity at the hD_1-F6.51(288)I and L mutant receptors. In general, the potency of rigid compounds at each mutant receptor (i.e. DHX, DNS, and A77636) was decreased to a far greater extent than were phenylbenzazepines. Although the ligands stimulated cAMP accumulation at the D_1-F6.51W, D_1-F6.51Y, and the D_5-F6.51Y mutant receptors, the D_5-F6.51W mutation resulted in a complete loss of cAMP accumulation for all test compounds. To our knowledge, this is the single greatest difference seen between these two receptors. These data may offer insight into the structural requirements that play a role in D_1-like receptor activation, and may possibly assist in the design of novel D_1-like ligands.

INTRODUCTION

Despite enormous efforts, knowledge of the molecular requirements involved in GPCR activation remains inadequate, particularly for the D_1-like dopamine receptors. In an inactive state GPCRs are constrained by non-covalent interactions between helical side chains that act to stabilize a conformational state favoring an inactive state. Agonist binding to the transmembrane region disrupts the intramolecular interactions that stabilize the inactive state, thereby facilitating the formation of interactions that favor an active receptor conformational state. In order to better understand the mechanism by which agonist binding elicits the conformational changes that lead to receptor activation, it is imperative to elucidate the ligand-receptor interactions responsible for creating and stabilizing an active receptor state.

The current activation model for rhodopsin-like GPCRs proposes the involvement of several molecular switches that act in concert to restrict the receptor to a basal state in the absence of ligand-induced activation. These non-covalent interactions are disrupted upon agonist binding, and new intramolecular interactions are formed that favor active receptor conformations. One prominent molecular switch -- coined the 'rotamer toggle switch'-- is comprised of a cluster of aromatic residues (i.e. Phe6.44, Trp6.48, Phe6.51, Phe6.52) surrounding a highly conserved proline in the TM6 helix. Agonist binding to a residue or residues in this cluster induces a coordinated movement of these residues around the proline kink resulting in the counterclockwise rotation of the cytoplasmic end of TM6 away from TM3 (Farrens *et al.*, 1996; Shi *et al.*, 2002). The agonist-induced rotation of the cytoplasmic portion of TM6 away from the receptor core and upwards towards the membrane bilayer is thought to be an important step in GPCR activation

(Ghanouni *et al.*, 2001b; Hamdan *et al.*, 2002; Jensen *et al.*, 2001). The nature of the interaction of an agonist with the aromatic residues in the switch region is believed to be an important determinant of the degree of receptor activation.

In a recent study of D_1-like dopamine receptors (unpublished results), we examined the role of two TM6 phenylalanine residues (F6.51 & F6.52) that are hypothesized to reside in the toggle switch region. Both of these residues were mutated to alanine, and ligand-binding and receptor activation examined using the group of probe compounds that have been used throughout this Dissertation (see Figure 1.3). These experiments indicated a key role for F6.51, but not F6.52, in the activation of D_1-like receptors. The phenylalanine residue at position 6.51 is highly conserved across aminergic receptors and has been demonstrated to play a role in receptor activation in several GPCRs. Substituted cysteine accessibility studies in the D_2 dopamine receptor provided evidence that F6.51 is solvent accessible in the binding site crevice, suggesting its potential involvement in ligand binding and receptor activation (Javitch *et al.*, 1998). Molecular modeling and mutagenesis studies in the 5-HT_{2A} (Braden *et al.*, 2006; Choudhary *et al.*, 1993; Roth *et al.*, 1997), muscarinic acetylcholine (Ward *et al.*, 1999), adrenergic (Chen *et al.*, 1999; Peltonen *et al.*, 2003), histamine (Wieland *et al.*, 1999), and D_2/D_3 dopamine receptors (Boeckler *et al.*, 2005; Cho *et al.*, 1995) also are consistent with a role for F6.51 in ligand-binding and activation. Our D_1 molecular modeling studies suggest that F6.51 is positioned to form aromatic interactions with the catechol ring of D_1 agonists.

The current experiments were designed to explore further the role of F6.51 in the activation of D_1-like receptors. I constructed non-conservative (Ile, Leu) and conservative

(Trp, Tyr) point mutations in both the D_1 and D_5 dopamine receptors and used an array of structurally and pharmacologically diverse test compounds to probe binding and subsequent activation. These findings provide evidence that F6.51 is a key residue in ligand-induced activation of the D_1-like receptors, and also unearthed a major difference between the D_1 and D_5 receptors..

RESULTS

Binding characteristics of F6.51 mutant receptors in HEK293 Cells.

The D_1/D_5 wild-type and mutant dopamine receptors were transiently expressed in HEK293 cells, and then tested for their ability to bind the D_1-selective antagonist [^{3}H]SCH23390. The characteristics of the D_1-wt (K_D = 1.5 ± 0,2; B_{max} = 3,600 ± 600 fmol/mg) and D_5-wt (K_D = 2.5 ± 0,5; B_{max} = 3,040 ± 740 fmol/mg) were consistent with data reported in earlier chapters. As can be seen in the left panel of Figure 4.1, the saturation experiments indicated that the affinity of specific binding of [^{3}H]SCH23390 for the D_1- and D_5- F6.51I/L/Y/W mutants was decreased to such a degree that it was impossible to determine values for K_D and B_{max}.

To determine whether the loss of binding was due to a decrease in cell-surface expression of the mutant receptors or effects on the interaction of SCH23390 with these receptors, I performed cell surface radioimmunoassay (RIA) experiments with HA-tagged receptors, and assessed the cell-surface expression of mutant receptors relative to each wild-type receptor. The results of these experiments indicate that despite the apparent loss of binding sites (Figure 4.1 left), there was no significant decrease in cell-surface expression for any of the D_1 or D_5 mutant receptors compared to wild type (Fig. 4.1, right panel).

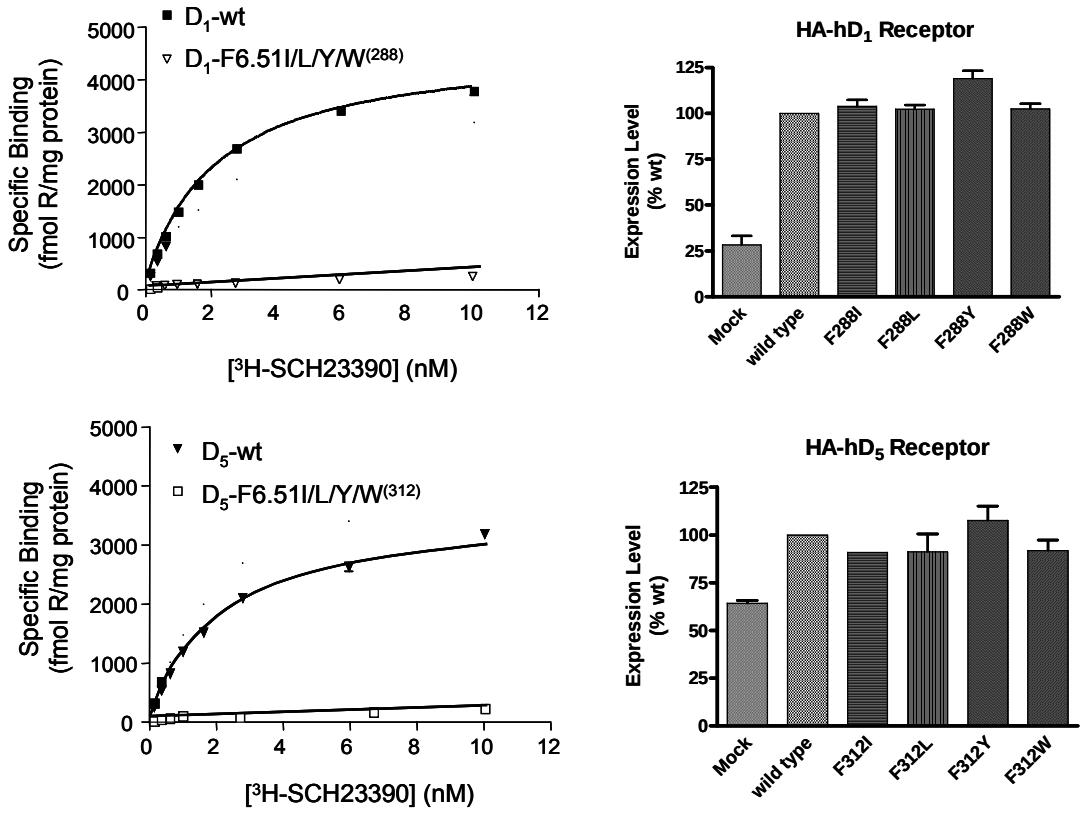

Figure 4.1. D_1/D_5-F6.51 mutations result in a loss of [^{3}H]SCH23390 binding but do not alter cell-surface expression*. HEK293 cell membranes transiently expressing wild-type or mutant receptors were tested in radioreceptor saturation isotherm experiments with increasing concentrations of [^{3}H]SCH23390. Non-specific binding was determined with 1 μM unlabeled SCH23390. Data were analyzed using a one-site hyperbolic curve fitting function (Prism 4.0) to obtain the K_D and B_{max} for [^{3}H]SCH23390 at wild-type and mutant receptors. Data are mean ± S.E.M. HA-tagged mutant receptors were subsequently tested for cell-surface expression relative to wild-type receptors via RIA. Data are representative of 2-3 independent experiments.*

Functional effects of D_1-like mutant receptors

I next examined the ability of agonists to stimulate adenylate cyclase at the wild-type and mutant D_1 and D_5 dopamine receptors. cAMP accumulation was measured using membrane preparations expressing wild-type or mutant receptors, and full dose-response curves for adenylate cyclase activation were generated for all test compounds. These data are summarized in Table 4.1.

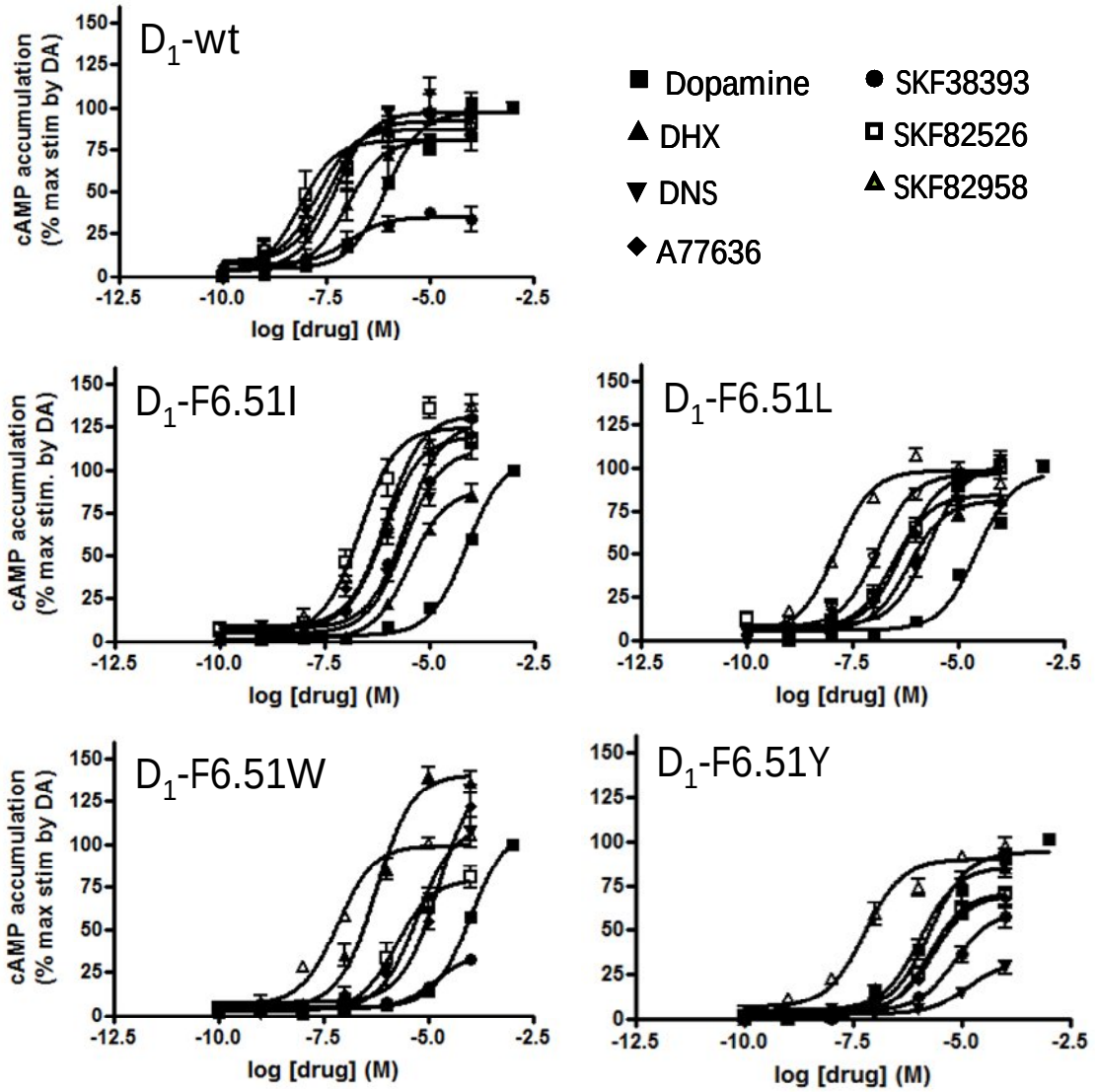

Figure 4.2. Agonist-mediated cAMP accumulation at the D_1-WT and F6.51 mutant receptors. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37° C. Dose-response curves were generated using 7 concentrations of test compound at the D_1- wild-type and mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

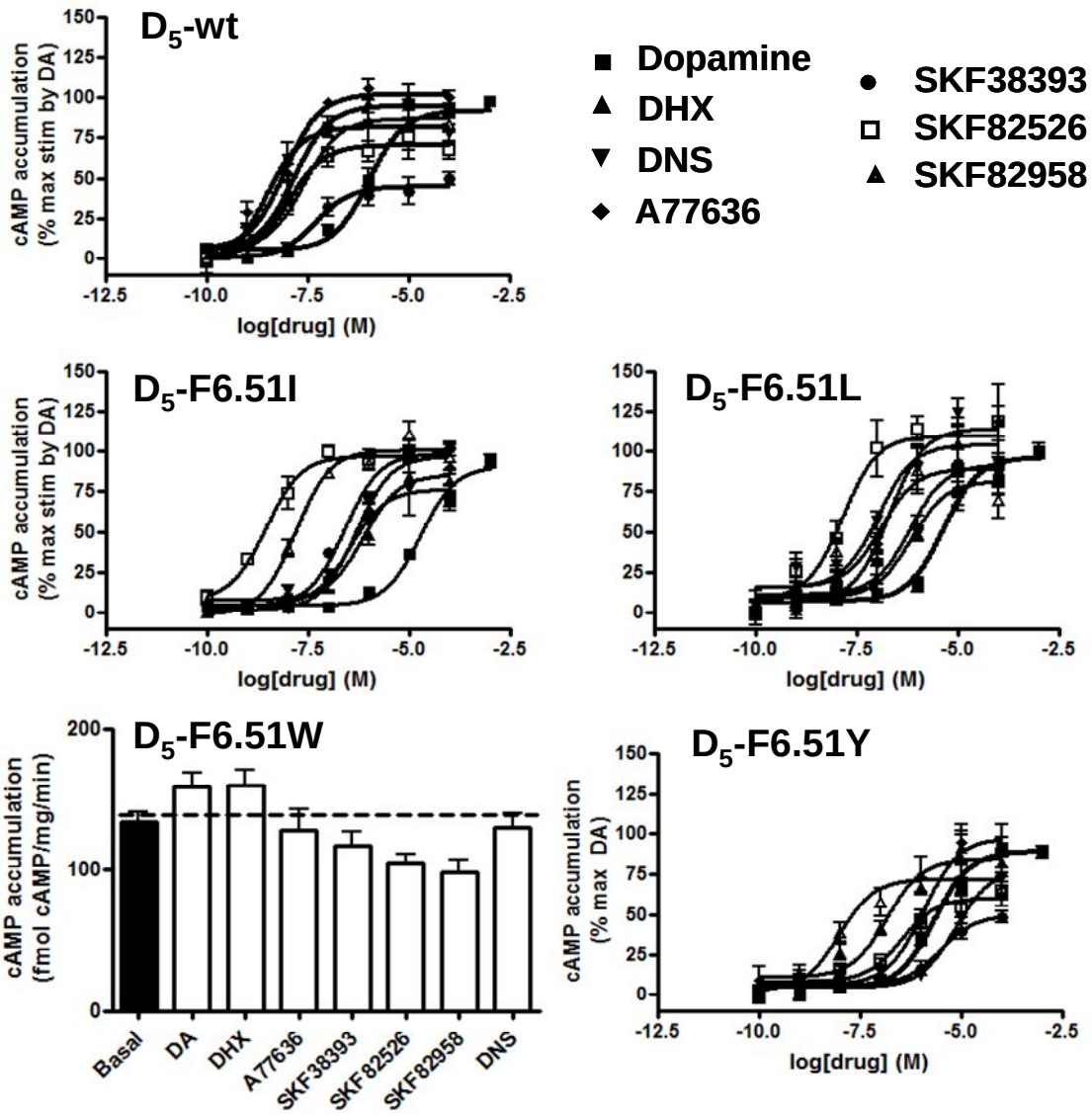

Figure 4.3. Agonist-mediated cAMP accumulation at the D_5-WT and F6.51 mutant receptors. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37^o C. Dose-response curves were generated using 7 concentrations of test compound at the D_5- wild-type and mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean $\pm$ S.E.M.*

Table 4.1. Agonist potency and intrinsic activity at the D_1-WT and mutant receptors.

	hD5-wt		*hD5-F6.51I*			*hD5-F6.51L*			*hD5-F6.51Y*			*hD5-F6.51W*		
Ligand	**EC50 (nM)**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***
Dopamine	550 ± 120	100	15,000 ± 1.3	*27*	100	4,000 ± 1,000	*8*	100	3,000 ± 800	*6*	100	N.D.	N.D.	N.D.
DHX	10.6 ± 0.3	97	560 ± 127	*52*	85	915 ± 58	*75*	81	280 ± 130	*23*	93	N.D.	N.D.	N.D.
DNS	12.5 ± 3.2	102	540 ± 85	*43*	97	273 ± 67	*21*	114	14,000 ± 4,000	*1,060*	105	N.D.	N.D.	N.D.
A77636	2 ± 0.2	97	133 ± 20	*66*	76	117 ± 16	*64*	104	3,100 ± 1,000	*1,700*	107	N.D.	N.D.	N.D.
SKF38393	46 ± 7	44	277 ± 57	*4*	98	71 ± 20	*2*	91	1,170 ± 160	*25*	54	N.D.	N.D.	N.D.
SKF82526	9.6 ± 4.4	70	17.4 ± 4.2	*1*	97	12 ± 2	*1*	109	433 ± 155	*45*	67	N.D.	N.D.	N.D.
SKF82958	3.6 ± 0.7	87	31 ± 11	*10*	100	152 ± 24	*16*	90	11 ± 5	*1*	80	N.D.	N.D.	N.D.
	hD1-wt		*hD1-F6.51I*			*hD1-F6.51L*			*hD1-F6.51Y*			*hD1-F6.51W*		
Ligand	**EC50 (nM)**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***
Dopamine	870 ± 180	100	85,000 ± 12,000	*98*	100	35,000 ± 15,000	*40*	100	7,200 ± 2,900	*8*	100	103,000 ± 20,000	118	100
DHX	115 ± 43	99	3,000 ± 735	*10*	88	743 ± 120	*5*	81	1,350 ± 390	*9*	86	711 ± 207	2	141
DNS	82 ± 49	97	3,800 ± 1,600	*46*	112	2,700 ± 1,100	*28*	102	15,000 ± 6,000	*156*	32	5,300 ± 1,300	65	110
A77636	24 ± 9	100	1,080 ± 317	*38*	119	405 ± 156	*17*	84	6,700 ± 3,100	*283*	70	11,500 ± 5,900	415	141
SKF38393	440 ± 340	35	2,750 ± 550	*6*	127	498 ± 55	*1*	96	9,500 ± 2,400	*17*	60	16,900 ± 5,400	38	36
SKF82526	32 ± 10	81	311 ± 142	*10*	124	14 ± 4	*1*	98	3,100 ± 1,200	*96*	71	1,780 ± 680	54	80
SKF82958	30 ± 15	90	122 ± 56	*4*	132	232 ± 160	*8*	96	555 ± 400	*18*	90	51 ± 18	2	99

IA = Intrinsic activity.
Fold = the fold change value as compared to the EC50 of each drug at the wild type receptor. Fold changes were calculated from raw data, not from rounded values in Table.
HEK293 cell membranes containing D_1-wt or mutant receptors were incubated with 7 concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) for best fit to obtain EC50 values. Data are representative of 3-6 individual experiments run in quadruplicate. Values are expressed as mean $\pm$ S.E.M.

The rank order of potency at the wild-type D_1 receptor (Figure 4.2 and Table 4.1) was: **D_1**: SKF82958 > SKF82526 = A77636 > DHX $\geq$ DNS > SKF38393 > DA. For the **D_5**, there were differences in the rank order, with A77636 > SKF82958 > DHX = DNS > SKF82526 $\geq$ SKF38393 > DA (Figure 4.3 and Table 4.1).

I also assessed the intrinsic activity (relative to dopamine) of each ligand at each receptor. At the D_1-wt receptor, DHX, DNS, and A77636 were full agonists. The phenylbenzazepine SKF38393 was a partial agonist (46% intrinsic activity), whereas SKF82526 and SKF82958 had higher intrinsic activity (84% and 91%, respectively). At the D_5-wt receptor, as with the D_1-wt, DHX, DNS, and A77636 were full agonists. SKF38393 was a partial agonist (52% intrinsic activity), and SKF82526 and SKF82958 were partial agonists with somewhat higher intrinsic activity (62% and 86%, respectively). With both receptors, the D_1-like-selective antagonist SCH23390 completely attenuated the functional effects of the highest concentration of each agonist (data not shown).

Leu and Ile mutation of F6.51 provide evidence of a structural basis for efficacy.

Point mutations of Leu and Ile in the D_1 and D_5 receptors were constructed as these residues eliminated aromaticity, but still provided steric bulk and hydrophobicity. The alterations in potency and intrinsic activity were comparable at the D_1 and D_5 receptors in both the Ile and Leu mutant receptors. The potency of the phenylbenzazepine compounds (SKF38393, SKF82526, and SKF82958) was less affected by either of these mutations than was that of the rigid compound. The decrease in potency of DA at the D_1 Ile (98-fold decrease) and Leu (40-fold decrease) mutants, however, was greater than that of the D_5 mutant receptors (27-fold decrease at Ile, 8-fold at Leu). Additionally, the

decreases in potency of DHX for the D_1-F6.51I/L mutant receptors were much less than that of the D_5-F6.51I/L mutants. With one exception, the intrinsic activity of the probe ligands was not significantly affected.

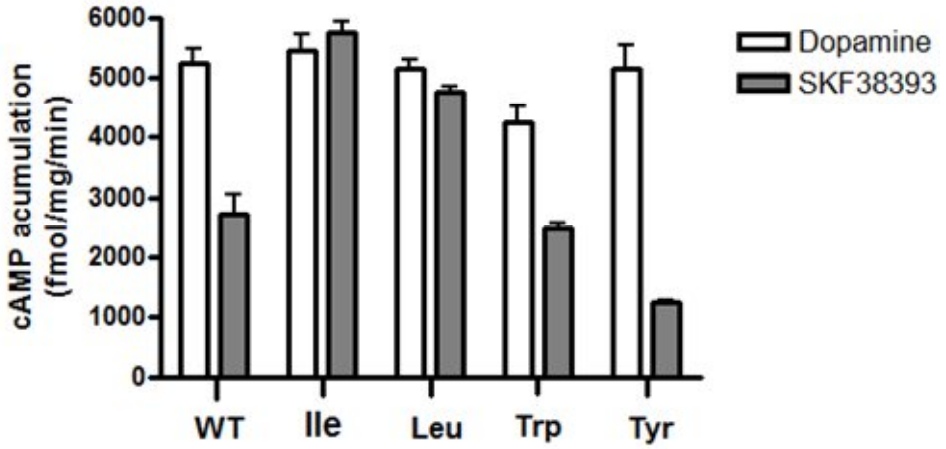

Figure 4.4. SKF38393 exhibits a dramatic increase in intrinsic activity at the D_1-F6.51I and L mutant receptors. Data are expressed as fmol of cAMP produced per mg per minute in response to 100 μM drug treatment. Basal levels of cAMP have been subtracted out.

As shown in Figure 4.4, the exception was SKF38393 whose intrinsic activity was altered from that of poor partial agonist at both the D_1/D_5 wild-type receptors to a full agonist at both the Ile and Leu mutants in the D_1 and D_5 receptors. Its potency at both the D_1 and D_5 receptors was affected only minimally, the ability of SKF38393 to stimulate cAMP synthesis, however, increased from 35% intrinsic activity at D_1 and 40% at D_5 in the wild-type receptor to levels equal to dopamine in the D_1/D_5 mutant receptors (98% at D_1-Leu mutant, 91% and 98% at Leu and Ile respectively at the D_5 receptor), and even greater than dopamine (128% intrinsic activity) with the D_1-Ile mutant. As can be seen in Figures 4.2 and 4.3, the increase in cAMP accumulation was both relative (i.e., vs. dopamine) and absolute. The intrinsic activity of two other phenylbenzazepines used in

this study, SKF82526 and SKF23958, was not significantly affected at either the Ile or Leu mutants in the D_1 and D_5 dopamine receptors.

Conservative mutations of F6.51 reveal structural differences in D_1-like receptors.

Tyrosine mutation

Unlike the Leu and Ile mutant receptors, mutational effects of F6.51 to Tyr were not dependent on the structural class of the compound. A77636 displayed dramatic losses of potency (283-fold decrease at D_1 receptor, 1,700-fold at D_5 receptor), but less pronounced changes in intrinsic activity. The potency of dopamine was minimally affected at both the D_1 (8-fold decrease) and D_5 (6-fold decrease) receptors. There was no change in the absolute cAMP accumulation of dopamine at the Tyr mutant receptor versus the wild-type receptor. The potencies of SKF38393 and SKF82526 were modestly affected at both the D_1 and D_5 receptors, whereas SKF82958 had much lower potency (95-fold decrease at D_1, 45-fold at D_5). The intrinsic activity of the phenylbenzazepines at D_1- and D_5-F6.51Y mutant receptors was affected only minimally.

DHX and DNS, despite their structural similarity, were differentially affected at the Tyr mutant receptor in the D_1 and D_5 receptors. The potency of DNS (155-fold decrease at D_1 receptor, >1,000-fold at hD_5 receptor) for the Tyr mutant receptor decreased dramatically, whereas the decrease for DHX (9-fold decrease at D_1 receptor, 23-fold at D_5 receptor) was modest. Additionally, the intrinsic activity of DNS decreased from a full agonist to partial agonist, particularly in the D_1 mutant receptor (33% of maximal dopamine stimulation), whereas DHX retained full intrinsic activity.

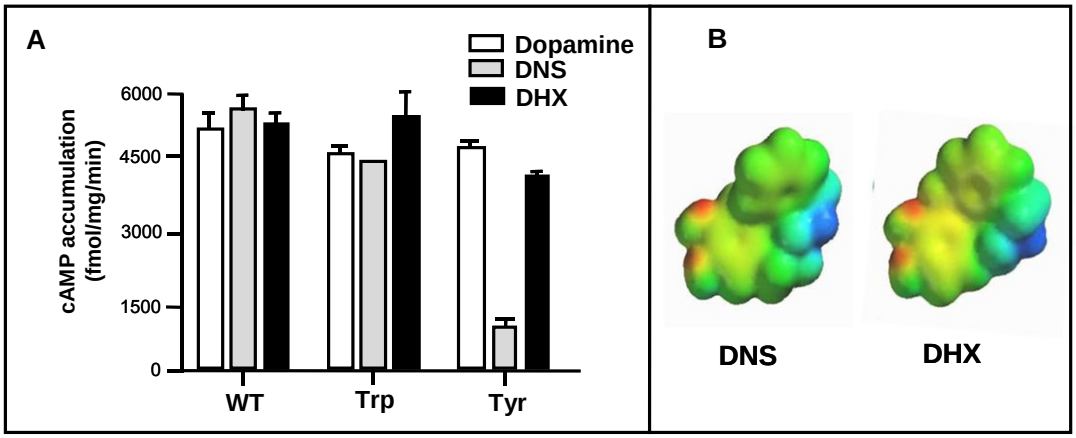

Figure 4.5. Dinapsoline and dihydrexidine are affected differentially at the Tyr mutant receptor*. The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37° C. [Panel A] Data shows the fmol cAMP produced per milligram of protein per minute by 100 μM DNS, DHX, and DA at wild-type and mutant receptors. All data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean + S.E.M. [Panel B] Energy-minimized structures of DHX and DNS.*

Trp mutation

Unexpectedly, mutation of F6.51 to Trp in the D_5 dopamine receptor resulted in the failure of all test compounds to stimulate cAMP accumulation above basal (Figure 4.3). Conversely, all test compounds elicited cAMP accumulation at the D_1-F6.51Y mutant receptor. Unlike the Leu and Ile mutant receptors, the phenylbenzazepines were differentially affected yielding 44-fold, 2-fold, and 54-fold decreases in potency for SKF38393, SKF82958, and SKF82526 respectively. The intrinsic activity of the phenylbenzazepines at the Trp mutant receptor was also affected minimally. A77636 resulted in the greatest loss in potency compared to the wild type receptor (634-fold). The intrinsic activity of A77636, however, was increased above that of dopamine.

As observed for the Tyr mutant receptor, the potency of DHX (2-fold decrease) and DNS (56-fold decrease) was differentially affected in the Trp mutant receptor; however DNS retained full agonist activity. The loss of potency for dopamine at the Trp

(188-fold decrease) was much greater than at the Tyr mutant receptor (8-fold) while the intrinsic activity was unaffected.

DISCUSSION

In a prior study we demonstrated that mutation of F6.51 to alanine affected the functional potency and efficacy of an array of structurally dissimilar D_1 agonists according to chemical class. Specifically, the receptor interactions of dopamine, DHX, DNS and A77636 were markedly affected by the F6.51A mutant receptor whereas the phenylbenzazepines (SKF38393, SKF82958, & SKF82526) were affected minimally by this mutation. The goal of this chapter was to probe further the role of F6.51 in the transduction of ligand binding to receptor activation. This was accomplished by constructing conservative (Trp and Tyr) and non-conservative (Leu and Ile) mutations of F6.51 in the D_1 and D_5 dopamine receptors.

Based on our studies of the F6.51A mutant receptor we anticipated that the D_1 antagonist SCH23390 would have significantly decreased affinity at many of the mutant D_1-like receptors. Indeed, all mutant receptors resulted in a dramatic loss in affinity for SCH23390, thus precluding the assessment of agonist binding affinities. Cell surface RIA experiments in Figure 4.1 demonstrated protein folding and membrane expression was not affected by any of the D_1 or D_5 receptor mutants. Concerns about mutational affects on global structure of the receptor were addressed by using several different chemical classes of D_1 agonists.

Mutation of 6.51 in a variety of GPCRs has suggested that the residue is solvent accessible (Chen *et al.*, 1999; Javitch *et al.*, 1998) and may form a π-π interaction with both agonists and antagonists (Braden *et al.*, 2006; Cho *et al.*, 1995; Huang *et al.*, 1995;

Nardone and Hogan, 1994). Studies of catecholamine receptors indicate that F6.51 is located in the cluster of aromatic residues that comprises the rotamer toggle switch (ref). Javitch *et al.* (1998) found that F6.51 was accessible in the D_2 receptor binding site crevice. Chen *et al.* (1999) showed that F6.51 is not only solvent accessible but is a key switch residue in α_{1B}-adrenergic receptor activation. Based on these findings and D_1 molecular modeling studies, we hypothesized that F6.51 forms an aromatic interaction (π-π or CH-π interaction) with the catechol ring of D_1 agonists.

Semi-conservative mutations of phenylalanine to leucine and isoleucine were made to eliminate aromaticity while conserving steric bulk and hydrophobicity. Similar to the findings observed at the F6.51A mutant receptor, agonist interactions with the D_1- and D_5-F6.51I/L mutant receptors were affected according to chemical class. The potency and intrinsic activity of the rigid compounds (DHX, DNS, and A77636) was less affected at the D_1-like F6.51I and L receptors than the phenylbenzazepine compounds (SKF38393, SKF82526, and SKF82958). This finding suggests that mutational effects are not due to global changes in receptor structure, thus assuaging concerns about global structural changes that affect all ligand classes. Remarkably, the intrinsic activity of SKF38393 at the D_1/D_5-F6.51I/L mutant receptors was altered from that of a low efficacy partial agonist (at wild-type) to a full agonist (or greater). A lesser increase in intrinsic activity was observed for SKF82526 and SKF82958 but not for the rigid agonists. The removal of aromaticity appears to orient SKF38393 in a position that favors a receptor active state, by altering the nature of the interaction of the pendant phenyl ring with the so-called "hydrophobic accessory region" of the D_1 receptor (Mottola *et al.*, 1996).

Figure 4.6 shows the bound conformations of the full agonist dopamine and partial agonist SKF38393. In the dopamine bound conformation, the side chain of F6.51 makes an aromatic contact with the catechol ring of dopamine, while in the SKF38393 bound conformation F6.51 interacts with the catechol ring and also the azepine moiety of the ligand. Cysteine scanning experiments involving rhodopsin, and also from previous simulation studies (Bhattacharya *et al.*, 2008a), show that TM6 rotates in the clockwise direction in order to fully activate the receptor. Therefore, on full activation F6.51 should move closer to TM5.

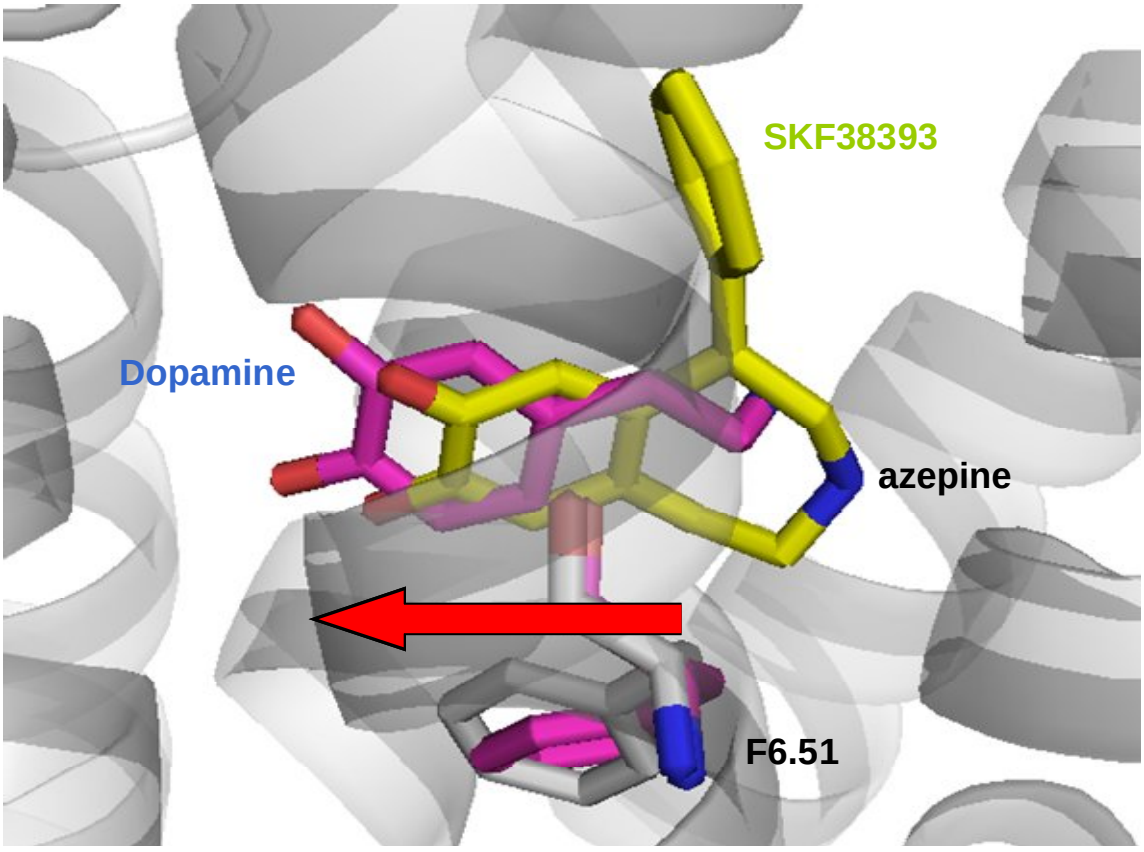

Figure 4.6. Modeling of the bound conformations of the full agonist dopamine and partial agonist SKF38393 .

In the dopamine bound conformation, F6.51 could move towards TM5 and at the same time maintain favorable contact with the catechol ring. In SKF38393 bound conformation, F6.51 shows favorable contact with the azepine ring in the partially active state. This interaction is lost when F6.51 moves towards TM5, and SKF38393 stabilizes a partially active state of the receptor. Aromatic interaction with the azepine moiety is lost when F6.51 is substituted with non-aromatic residues such as Leu and Ile. As a result, TM6 shows less preference for the partially active state and is free to move to the fully active state. Therefore SKF38393 behaves as a full agonist in the F6.51L/I mutants. This finding is particularly important as full agonism is required for D_1 receptor-mediated therapeutic effects in Parkinson's disease (Taylor *et al.*, 1991).

To evaluate further the molecular interactions between F6.51 and the test compounds we constructed conservative mutations of phenylalanine to tryptophan and tyrosine. Mutation of F6.51 to tyrosine, which has similar steric bulk and an aromatic side chain, led to dramatic potency losses for DNS and A77636 but not for DHX and dopamine. At the D_5-F6.51Y mutant receptor, the fold change decrease in potency for A77636 and DNS was ~50- to 200-fold greater than that of dopamine and DHX; however these compounds exhibited only minor changes in intrinsic activity. Tryptophan, although slightly larger than phenylalanine, preserves its hydrophobicity and aromaticity. The complete loss of cAMP accumulation for all test compounds at the D_5-F6.51W mutant receptor was entirely unexpected. All test compounds elicited activation at D_1-F6.51W and Y mutant receptors, as well as at the D_5-F6.51Y mutant receptor. Cell surface expression of the D_5-F6.51W mutant receptor was unaffected compared to the D_5 wild-type receptor indicating that the mutant receptor is properly folded and expressed. These

data indicate that, in the D_5 receptor, the F6.51W mutation is disrupting the molecular interactions necessary for formation of an active receptor state. Additionally, the loss in the ability of the D_5-F6.51W mutant receptor but not the D_1-F6.51W mutant receptor provides evidence of possible structural differences between the receptor subtypes. The transmembrane regions (TMs 3, 5, 6 and 7) with which D_1 agonists interact are highly conserved among D_1 and D_5 receptors. An alternatively, the EC-II loop between TMs 4 and 5, which our D_1 model predicts to interact with all the test compounds, is not conserved among D_1 and D_5 receptors. Therefore, the interaction of the EC-II loop with the ligands could be the contributing factor to the difference among the two mutant receptors. However, accurate conformation of the EC-II loop is difficult to determine given the lack of homology of the loops among the GPCR crystal structures. Further examination of these structural differences may aid in the design of a D_1 vs. D_5 selective compound.

DNS and DHX, structurally similar compounds that have almost identical binding and functional profiles at the D_1 wild-type receptor, were affected differently at D_1 Trp and Tyr mutant receptors. Specifically, the potency and intrinsic activity of DNS was altered dramatically at the F6.51Y mutant receptor while that of DHX was affected minimally (Figure 4.5). Also, at the D_5-F6.51Y mutant receptor we observed a 1060-fold potency loss (vs. wild-type) for DNS while that of DHX was decreased only 23-fold. Unlike phenylalanine and tryptophan, tyrosine is unlikely to be planar and has H-bonding potential. Docking studies show that the interaction of F6.51 with DNS was 2Kcal/mol stronger than with DHX. This may explain the larger loss of potency of DNS compared to that of DHX in the F6.51Y/W mutant receptors. Docking of DHX and DNS into a D_1

receptor model that has Tyr and Trp substituted for Phe will provide important details of the molecular interactions responsible for this phenomenon. These findings demonstrates that minor structural changes can have profound effects on receptor activation (even between two structurally similar compounds) and underscore the importance of understanding the structural features responsible for ligand recognition and receptor activation.

The phenylalanine at position 6.51 is highly conserved in aminergic GPCRs and is part of a cluster of aromatic residues critical for ligand recognition and receptor activation in catecholamine receptors. This residue is located in a motif known as the 'rotamer toggle switch', which is comprised of a cluster of aromatic residues (F6.44, W6.48, F6.51, F6.52) surrounding a highly conserved proline kink in the sixth transmembrane helix (TM6) (Shi *et al.*, 2002). Agonist interaction with a residue or residues in this switch region has been proposed to trigger a coordinated rearrangement of the other aromatic residues of the 'toggle switch' that act to modulate the bend angle of TM6 around the proline kink, subsequently resulting in the movement of the cytoplasmic end of TM6 away from TM3 (Bissantz *et al.*, 2003; Visiers *et al.*, 2001). The intracellular loop of TM3 has been implicated as an important region G-protein interaction and thus second messenger activation (Wess *et al.*, 1989). Weinstein (Weinstein, 2006) proposed that the degree of interaction with residues in the toggle switch determines it efficacy of a ligand. Shi *et al.* (2002) showed that the rotameric positions of F6.52 and W6.48 are coupled in the β_2-adrenergic receptor and act in a coordinated fashion to modulate configuration of the conserved proline kink at 6.50. Molecular modeling and docking studies of the β_2-adrenergic receptor predict that the

interaction between ligand and W6.48 is mediated by F6.52, which is thought to reside between the ligand and W6.48 rotamer (Bhattacharya *et al.*, 2008a). The authors propose that upon ligand binding F6.52 alters its rotameric conformation to form a π-π interaction with the ligand, which in turn leads to alteration of the W6.48 rotamer and subsequent movement of TM3 away from TM6. The role of F6.51 and F6.52 in rotamer toggle switch appears to vary across catecholamine GPCRs, prior studies in our lab have demonstrated that F6.52 does not directly interact with D_1 agonists (unpublished observations).

Based on the findings in this chapter, as well as studies of the cognate residue in other catecholamine receptors, we propose that F6.51 serves as a key switch residue in the D_1 receptor that can trigger the transduction of agonist binding to W6.48. Our findings support the notion that F6.51 and W6.48 are coupled in the D_1-like receptors, and play a key role in coupling ligand binding to receptor activation. The molecular interactions between an agonist and F6.51 appear to be a key determinant efficacy. We hypothesized that F6.51 resides lower in the binding pocket, positioned to form aromatic interactions with the catechol ring of D_1 agonists, and the findings in this study can be interpreted based on this hypothesis. Alternatively, a few homology modeling and docking studies of catecholamine receptors predict that F6.51 is positioned higher in the binding crevice (Peltonen *et al.*, 2003; Xhaard *et al.*, 2006), in a position to interact with the accessory ring system of D_1 agonists. The orientation of the pendant phenyl ring is thought to be important for D_1 receptor activation (Mottola *et al.*, 1996). This hypothesis is intriguing as it may reconcile the differences between DHX and DNS observed at the D_1-F6.5Y and W mutant receptors. The azepine ring of DNS allows the β-phenyl moiety

to twist orthogonally by about 18^{o} with respect to the catechol ring, and 28^{o} with respect to the β-phenyl of dihydrexidine (Negash *et al.*, 1997). The twist may orient the pendant phenyl moiety such that DNS interacts with the F6.51Y and W mutant receptors differently than DHX. D_1 receptor molecular modeling and docking studies will lend additional insight to the location of F6.51.

In conclusion, the results of this study provide convincing evidence that, in the D_1-like dopamine receptors, F6.51 plays a key role in coupling agonist binding to the TM helical movement that lead to G-protein activation. These data demonstrate that subtle changes in ligand-receptor interactions can have large, unanticipated effects on functional activity, and underscore the importance of understanding the molecular interactions governing functional effects. Detailed knowledge of the nature of the conformational changes leading to receptor activation is critical for the design of effective therapeutics. This study provides important insight into the structural mechanisms responsible for full agonism at the D_1-like receptors. Furthermore, we demonstrate what we believe to be the largest difference ever seen between the D_1 and D_5 receptors.

CHAPTER 5: DETERMINE THE ROLE OF TWO TM6 RESIDUES (W6.48 & N6.55) HYPOTHESIZED TO PLAY AN IMPORTANT ROLE IN THE TRANSDUCTION OF LIGAND-BINDING TO RECEPTOR ACTIVATION OF THE D_1-LIKE RECEPTORS.

PREFACE:

The work presented in Chapter 4 provided a detailed investigation of the role of F6.51 in D_1-like receptor activation. Previous studies of other aminergic receptors have demonstrated the importance of W6.48 and N6.55 in ligand-binding and receptor activation. This chapter will focus on these two TM6 residues that have been hypothesized to make important contributions to the formation of an active receptor conformational state.

ABSTRACT

Studies of numerous GPCRs have suggested that the residues surrounding the proline kink in TM6 play a central role in the transduction of agonist binding to receptor activation. W6.48 has been shown to serve as a key residue in this process in several catecholamine receptors while N6.55 has an important role in ligand recognition and receptor activation of the β_2-adrenergic receptor. The work in this chapter is the first effort to understand the role of W6.48 and N6.55 in D_1-like receptor activation. "Non-conservative" (i.e. Ala) mutations of N6.55 and W6.48 were constructed in both D_1 and D_5 receptors. Mutant receptors were characterized using an array of functionally and pharmacologically diverse test compounds in an adenylate cyclase assay to examine the functional consequences of each mutation. All mutant receptors exhibited a dramatic loss in affinity for the D_1 receptor antagonist SCH23390. The N6.55A mutation led non-specific changes in the D_5 receptor but not the D_1 receptor, suggesting possible structural differences between the two receptor subtypes. The D_1- and D_5-W6.48A mutant receptors exhibited greatly decreased basal (and ligand stimulated) levels of cAMP accumulation. Additionally, the intrinsic activity of two phenylbenzazepine compounds (SKF82526 & SKF82958) was greatly reduced at the D_1 and D_5 mutant receptors. These findings support the notion that W6.48 plays a central role in formation of the active receptor state of D_1-like receptors. The data in this chapter provide important knowledge of how N6.55 and W6.48 contribute to activation of the D_1-like receptors, and will aid in understanding the structural mechanisms required for D_1 receptor activation.

INTRODUCTION

The experiments in this Chapter continue our efforts to understand how agonist binding triggers the conformational changes that lead to the active state of D_1-like dopamine receptors. As discussed in the Introduction of this Dissertation, numerous studies have shown that TM6 amino acids play a key role in the transduction of agonist binding to GPCR activation. In particular, the aromatic residues that comprise the rotamer toggle switch (i.e. W6.48, F6.51, and F6.52) are thought to function as sensors for agonist binding (Jongejan *et al.*, 2005; Shi *et al.*, 2002; Singh *et al.*, 2002). In the experiments performed in Chapter 4, I investigated the role of one of these residues- F6.51- in D_1-like receptor activation. My findings demonstrate that the nature of agonist interaction with F6.51 is a key determinant of agonist efficacy. In addition to this detailed study of F6.51, I have previously shown that F6.52 does not directly interact with D_1 agonists (unpublished results). To explore the role of TM6 residues in ligand-binding and activation of D_1-like receptor further, I targeted two amino acids (W6.48 and N6.55) hypothesized to make important contributions in this region of the D_1-like receptor agonist binding site.

The tryptophan residue at position 6.48 is completely conserved across all catecholamine receptors and has been implicated as a key residue for the transduction of ligand binding to receptor activation (Bissantz *et al.*, 2003; Roth *et al.*, 1997; Shi *et al.*, 2002). In rhodopsin, the β-ionone ring of 11-*cis*-retinal interacts with the indole of W6.48, restricting the receptor in an inactive conformational state (Lin and Sakmar, 1996). Upon photoisomerization of retinal, the β-ionone ring moves away from W6.48, whose enhanced freedom permits the structural rearrangements necessary for receptor

activation (Lin and Sakmar, 1996). SCAM studies of the D_2 dopamine receptor found that W6.48 faces the binding crevice, and predicted this residue to reside in the aromatic cluster of residues that comprises the rotamer toggle switch (Shi and Javitch, 2004). Studies of the β_2-adrenergic and α_{1B}-adrenergic receptors indicate that agonist binding affects the rotameric configuration of W6.48, thereby modulating the bend of TM6 around the proline kink that subsequently results in receptor activation (Chen *et al.*, 1999; Gentili *et al.*, 2004; Shi *et al.*, 2002).

The amino acid at position 6.55 varies across catecholamine receptors: tyrosine (α_2-adrenergic), histidine (D_2-like receptors) and asparagine (β-adrenergic and D_1-like receptors). Studies of bovine rhodopsin suggest that W6.55 forms a direct interaction with retinal (Nakayama and Khorana, 1991). SCAM studies predict that H6.55 is accessible in the hD_{2L} receptor binding pocket (Javitch *et al.*, 1998), and experimental data suggests an interaction between H6.55 and dopamine antagonists (Woodward *et al.*, 1994). In the β_2-adrenergic receptor, N6.55 has been implicated in agonist recognition by interacting with the β-OH group in the aliphatic side chain of isoproterenol (Wieland *et al.*, 1996; Zuurmond *et al.*, 1999).

To examine the role of W6.48 and N6.55 in the D_1-like receptors, we constructed non-conservative point mutations to alanine and assessed binding and subsequent activation of wild-type and mutant receptors using a diverse group of D_1 agonists. We used a battery of ligands (Figure 1.3) that include full and partial agonists from the phenylbenzazepine class (SKF38393, SKF82526, SKF82958), an isochroman (A77636), and rigid D_1 agonists from two different chemical classes (DNS and DHX). The study is

the first to examine the role of W6.48 and N6.55 in binding and activation of the D_1-like dopamine receptors.

RESULTS

Binding characteristics of TM6 mutant receptors in HEK293 cells

Wild-type and mutant HA-tagged hD_1/hD_5 dopamine receptors were expressed in HEK293 cells and tested for their ability to bind the D_1 antagonist [^{3}H]SCH23390. The characteristics of the D_1-wt (K_D = 1.5 ± 0,2; B_{max} = 3,600 ± 600 fmol/mg) and D_5-wt (K_D = 2.5 ± 0,5; B_{max} = 3,040 ± 740 fmol/mg) were consistent with data reported in earlier chapters. Binding of [^{3}H]SCH23390 was significantly decreased for both D_1- and D_5-W6.48A and N6.55A mutant receptors such that K_D and B_{max} was unable to be determined (Figure 5.1). These saturation binding experiments (see Figure 5.1) indicated that there was a marked decrease in the affinity of [^{3}H]SCH23390 for the D_1- and D_5-F6.51I/L/Y/W mutants such that receptors such that the K_D and B_{max} values could not be calculated.

To determine whether this loss of binding was due to a decrease in expression of the mutant receptors or a loss of SCH23390 binding, we conducted cell surface RIA experiments with the HA-tagged receptors to assess the cell surface expression of mutant receptors relative to the wild-type receptors. The results of these experiments revealed a significant decrease in cell-surface expression of the D_1-W6.48A mutant receptor to 37% of wild-type receptor expression (Figure 5.2). There was no significant decrease in cell-surface expression for any of the other D_1 and D_5 mutant receptors compared to wild type. The loss of [^{3}H]SCH23390 binding for mutant receptors precluded assessment of the ligand-binding characteristics for all D_1 and D_5 mutant receptors.

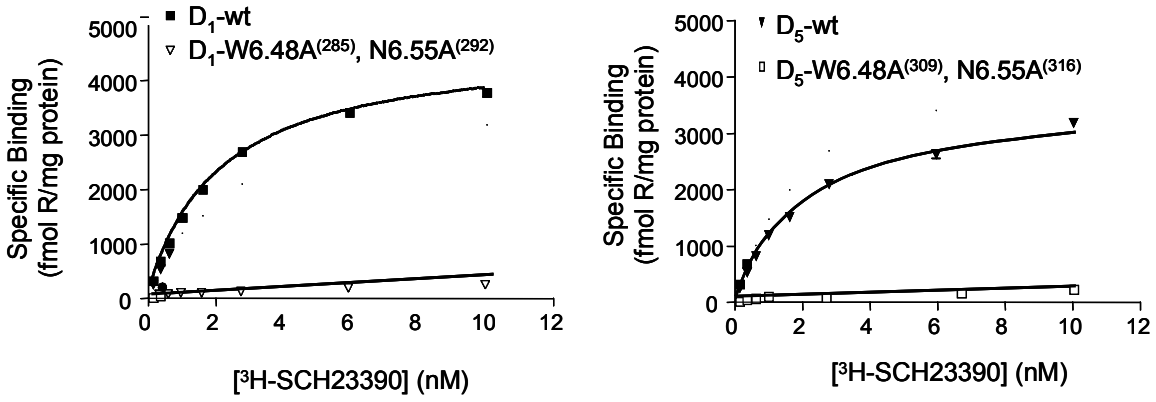

Figure 5.1. K_D and B_{max} of D_1/D_5-WT and mutant receptors labeled with [^{3}H]SCH23390. *HEK293 cell membranes transiently expressing wild-type or mutant receptors were tested in radioreceptor saturation isotherm experiments with increasing concentrations of [^{3}H]SCH23390. Non-specific binding was determined with 1μM cold SCH23390. Data were analyzed using a one-site hyperbolic curve fitting function (Prism 4.0) to obtain the KD and B_{max} for [^{3}H]SCH23390 at wild-type and mutant receptors. Data are mean ± S.E.M. ND= Not Determinable.*

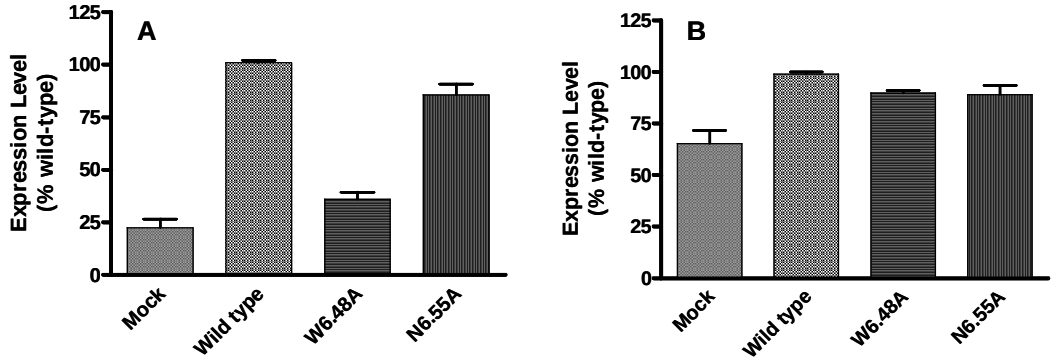

Figure 5.2. D_1-W6.48A mutant receptor cell surface expression is decreased compared to the D_1-wild type receptor. *HEK293 cells transiently expressing HA tagged mutant receptors, showing decreased [^{3}H]SCH23390 binding, were tested for cell surface expression relative to each respective wild-type receptor via RIA. [A] D_1 dopamine receptor, [B] D_5 dopamine receptor. Data are representative of 2-3 independent experiments run in quadruplicate.*

Effect on W6.48A and N6.55A mutations on potency and efficacy of dopamine at the D_1 and D_5 receptors

I next examined if agonists were capable of stimulating adenylate cyclase at the mutant D_1 and D_5 dopamine receptors. cAMP accumulation was measured using membrane preparations expressing wild-type or mutant receptors, and full dose-response curves for adenylate cyclase activation were generated for dopamine. The stimulation of

cAMP synthesis caused by dopamine was completely inhibited by the D_1-selective antagonist SCH23390 (data not shown). As can be seen in Table 5.1, dopamine as expected caused nearly a five-fold increase in cAMP production in the D_1-wt and a three-fold increase in the D_5-wt. The N6.55A mutation of both D_1-like receptors had no appreciable effect on the maximal fold-stimulation induced by dopamine, although it did seem to decrease the constitutive activity of the D_5-N6.55A mutant receptor. The W6.48A mutation also decreased the basal activity significantly, but markedly reduced the fold-stimulation, especially relative to the D_1-wt (Table 5.1)

Table 5.1. Basal and dopamine stimulated cAMP levels for D_1-like wild-type, W6.48, and N6.55 mutant receptors.

	cAMP Production (fmol cAMP/mg/min)	
	Basal	**DA**
D_1-wt	2,020 ± 230	9,510 ± 830
W6.48A	1,200 ± 100	2,600 ± 340
N6.55A	2,210 ± 550	11,400 ± 1030
D_5-wt	3,390 ± 370	9,560 + 330
W6.48A	1,050 ± 80	2,600 + 210
N6.55A	1,610 ± 100	7,380 + 300

Basal cAMP levels as fmol/mg protein/min are compared to cAMP levels over basal in response to 1 mM DA treatment. Values are expressed as mean ± S.E.M.

Effect of W6.48A and N6.55A mutations on ligand potency and efficacy at the D_1 and D_5 receptors

Full dose-response curves were generated for all of the test ligands. The cAMP accumulation produced by treatment with agonist was expressed as % maximal stimulation produced by dopamine at each receptor type. The rank order of potencies at the wild-type receptors was as follows: **D_1**: A77636 ≥ SKF82958 = SKF82526 > DHX ≥ DNS > SKF38393 > DA;

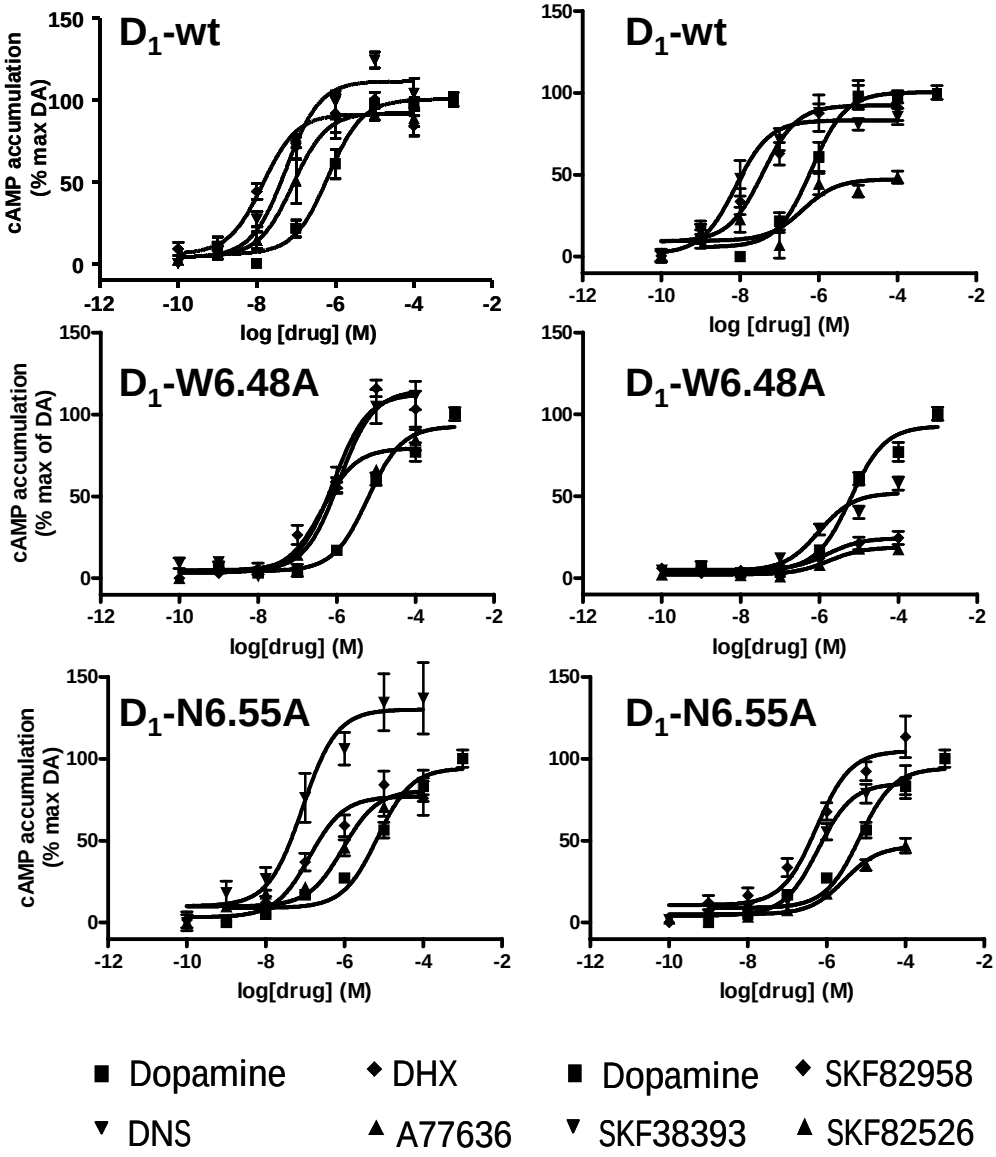

Figure 5.3. SKF82958 & SKF82526 have decreased relative intrinsic activity at the D_1-W6.48A$^{(285)}$ mutant receptor. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37^o C. Dose-response curves were generated using seven concentrations of test compound at the D1- wild-type and mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

Table 5.2. Agonist potency and intrinsic activity at the D_1 -WT and mutant receptors.

	hD_1-wt		*hD_1-W6.48A*			*hD_1-N6.55A*		
Ligand	**EC50 (nM)**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***
Dopamine	868 ± 175	100	5,720 ± 650	*6*	100	3,430 ± 908	*4*	100
DHX	84 ± 30	99	715 ± 309	*9*	111	844 ± 164	*10*	78
DNS	94 ± 28	100	1,180 ± 90	*13*	112	90 ± 24	*1*	129
A77636	27 ± 12	97	417 ± 200	*18*	79	121 ± 27	*4*	80
SKF38393	229 ± 95	46	2,080 ± 570	*11*	51	5,720 ± 1.13	*25*	44
SKF82526	32 ± 10	84	760 ± 340	*23*	18	702 ± 188	*22*	85
SKF82958	30 ± 15	91	152 ± 410	*50*	23	845 ± 362	*28*	103

HEK293 cell membranes containing D_1-wt or mutant receptors were incubated with 7 concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) for best fit to obtain EC50 values. Values are expressed as mean $\pm$ S.E.M. (nM). In parentheses is the fold change value as compared to the EC50 of each drug at the wild-type receptor. Data are representative of 3-6 individual experiments run in quadruplicate.

D_5: A77636 > SKF82958 > DHX = DNS > SKF82526 $\geq$ SKF38393 > DA. At the D_1-wt receptor DHX, DNS, and A77636 were full agonists, producing cAMP accumulation equal to dopamine. SKF38393 was a partial agonist producing ~ 46% of the maximal intrinsic activity of dopamine, while SKF82526 and SKF82958 were high efficacy partial agonists producing ~ 84% and 91% of maximal dopamine respectively. At the D_5-wt receptor, as in the D_1-wt receptor, DHX, DNS, and A77636 were all full agonists. SKF38393 was a partial agonist of ~ 52% of the maximal efficacy produced by dopamine; SKF82526 and SKF82958 were both high efficacy partial agonists producing ~ 62% and 86% of the maximal efficacy produced by dopamine. Receptor activation at the highest concentration of each agonist was inhibited using the D_1-selective antagonist SCH23390 (data not shown).

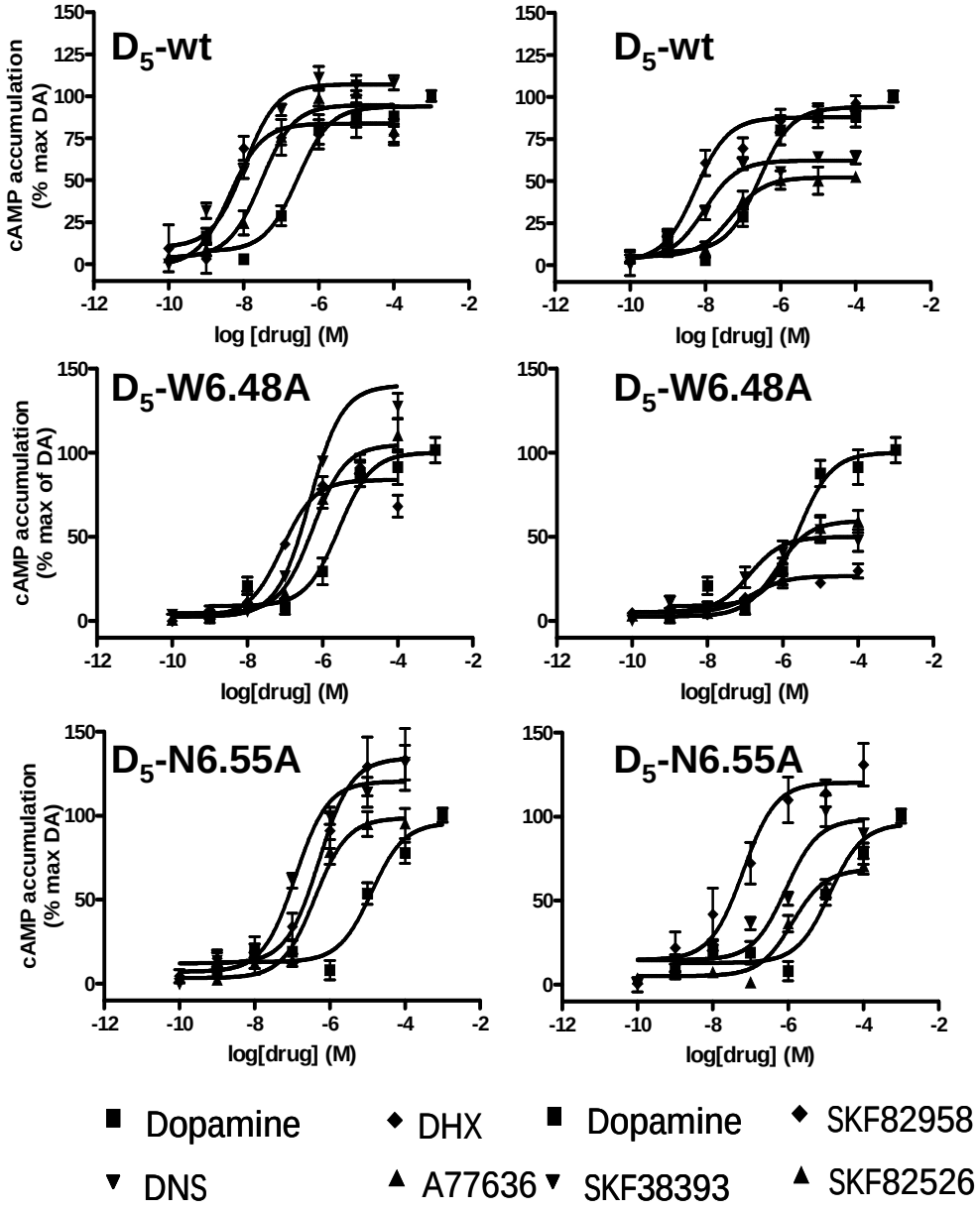

Figure 5.4. D_5-N6.55A$^{(316)}$ results in an increase in relative intrinsic activity for all test compounds*. The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37^o C. Dose-response curves were generated using seven concentrations of test compound at the D_5- wild-type [A], W6.48A [B], and N6.55A [C] mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

Table 5.3. Agonist potency and intrinsic activity at the D_5 -WT and mutant receptors.

	hD_5-wt		*hD_5-W6.48A*			*hD_5-N6.55A*		
Ligand	**EC50 (nM)**	***IA***	**EC50 (nM)**	**Fold**	***IA***	**EC50 (nM)**	**Fold**	***IA***
Dopamine	471 ± 85	100	1,310 ± 141	*3*	100	6,820 ± 934	*15*	100
DHX	12.2 ± 1.6	99	552 ± 155	*45*	88	482 ± 82	*40*	132
DNS	13.1 ± 2.4	100	459 ± 56	*35*	136	178 ± 68	*14*	120
A77636	1.8 ± 0.4	97	124 ± 33	*68*	103	459 ± 101	*251*	97
SKF38393	52.3 ± 8.6	52	607 ± 160	*12*	59	3,150 ± 1,680	*60*	66
SKF82526	52.3 ± 8.6	62	491 ± 190	*51*	48	869 ± 26	*90*	97
SKF82958	9.7 ± 2.8	86	144 ± 40	*15*	27	218 ± 48	*23*	120

HEK293 cell membranes containing D_5-wt or mutant receptors were incubated with 7 concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) for best fit to obtain EC50 values. Values are expressed as mean ± S.E.M. (nM). In parentheses is the fold change value as compared to the EC50 of each drug at the wild type receptor. Data are representative of 3-6 individual experiments run in quadruplicate.

N6.55A Mutant Receptor

Mutation of N6.55 to Ala in the D_1 receptor resulted in a 20-30 fold decrease in the potency of the phenylbenzazepines (i.e. SKF38393, SKF82958, and SKF82526) while DA, DHX, DNS, and A77636 were affected minimally (Figure 5.3). As shown in Figure 5.3 and Table 5.2, the maximal activity produced in response to DNS and SKF82958 was greater than that produced by dopamine while the intrinsic activity of all other test compounds was relatively unaffected. Unlike that of the D_1 mutant receptor, changes in the potency of test compounds at the D_5-N6.55A mutant was not dependent on compound structural class. A77636 displayed the greatest loss in potency (251-fold decrease relative to wild-type), followed by SKF82526, SKF38393 and DHX with 90-, 60-, and 45-fold respectively. The potency of SKF82958, DNS, and DA at the D_5-N6.55A mutant receptor was affected minimally. The maximal activity of all test compounds at the D_5-N6.55A mutant receptor increased to levels greater than that at the D_5-wild-type receptor. The intrinsic activity of DNS, DHX, and SKF82958 at the D_5

mutant receptor increased to levels above that of dopamine (120-, 132-, and 120-fold respectively).

W6.48A Mutant Receptor

The data with the W6.48A mutant D_1-like receptors indicate that this tryptophan residue is critical for receptor activation. The cAMP accumulation produced in response to 1 mM dopamine at the W6.48A mutant receptors was a fraction of that produced by the wild-type receptors (Table 5.1). Interestingly, the intrinsic activity (both relative and absolute) of SKF82958 and SKF82526 was most affected by this mutation. As shown in Table 5.2, the D_1-W6.48A mutant receptor showed modest decreases in potency for DA, DHX, DNS, A77636 and SKF38393, and more severe potency losses for both SKF82526 and SKF82958 (23- and 50-fold respectively). The intrinsic activity of SKF82526 and SKF82958 dramatically decreased from 84% and 91% (of maximal DA stimulation) respectively at the wild-type receptor to 50% and 23% at the mutant receptor. The maximal activity of DNS and DHX at the D_1-W6.48A mutant receptor increased ~10% above that of their levels at the D_1 wild-type receptor while the intrinsic activity of A77636 decreased from 100% to 79%.

Table 5.3 indicates that mutation of W6.48 to alanine at the D_5 receptor resulted in 30-70 fold losses in potency for DHX, DNS, A77636, and SKF82526. The potency of SKF82958 and SKF38393 was decreased by 15- and 12-fold respectively, while dopamine was unaffected. As with the D_1 mutant receptor, the intrinsic activity of SKF82526 and SKF82958 at the D_5-W6.48A mutant receptor decreased dramatically, from 62% and 86% respectively at the wild-type receptor to 49% and 25% at the mutant receptor.

DISCUSSION

This chapter examined the role of two TM6 amino acid residues- N6.55 and W6.48- in binding and activation of the D_1-like receptors. Several studies have suggested a central role for W6.48 in formation of the active receptor state. Studies of the β_2-adrenergic receptor (Wieland *et al.*, 1996; Zuurmond *et al.*, 1999), bovine rhodopsin (Sakmar, 1998), and D_2 dopamine receptor (Javitch, 1998; Woodward *et al.*, 1994) provide support for agonist-receptor residue interactions at 6.55. The role of these residues in ligand recognition and receptor activation has yet to be explored in D_1-like receptors. To explore the role of these TM6 residues, we made non-conservative point mutations of N6.55 and W6.48 to alanine and subsequently probed the mutant receptors with an array of structurally and pharmacologically diverse compounds.

Mutation of N6.55 to Ala resulted in a dramatic decrease the affinity of the antagonist SCH23390 for the D_1 and D_5 receptors, precluding competition binding studies that could provide detail into the contribution of this residue to agonist binding. Cell-surface radioimmunoassays demonstrated that the D_1- and D_5-N6.55A mutant receptors were expressed on the cell membrane at levels comparable to the wild-type receptors, and cAMP assays revealed that the mutant receptors retained functional activity.

The D_1-like receptors are highly similar to the β_2-adrenergic receptor and share an asparagine residue at position 6.55. Studies of the β_2-adrenergic receptor show that N6.55 interacts with the β-hydroxyl group of norepinephrine (Wieland *et al.*, 1996; Zuurmond *et al.*, 1999). Our D_1 molecular model predicts that N6.55 participates in interhelical hydrogen bonds that could be the driving force for the rotation of TM6 leading to rotamer toggle and hinge bending motion of TM6. We anticipated compounds would be affected

by the N6.55A mutation according to structural class. Indeed, at the D_1-N6.55A mutant receptor we observed a modest decrease (22- to 28-fold loss) in the potency of all compounds with the phenylbenzazepines (SKF38393, SKF82526, and SKF82958) and slight decreases (1- to 10-fold loss) in the potency of the more rigid compounds (DHX, DNS, and A77636). Conversely, a ligand-specific trend in potency and/or intrinsic activity changes was not observed at the D_5-N6.55A mutant receptor. These non-specific changes indicate that the N6.55A mutation in the D_5 receptor is causing more global changes in receptor activation, and may provide hydrophobic packing that supports ligand-receptor interaction. For example, N6.55 is predicted to reside one helical turn above F6.51 and may influence the interaction of F6.51 with agonists through helical packing (Xhaard *et al.*, 2006).

As with the N6.55A mutant receptor, the D_1- and D_5-W6.48A mutant receptors displayed a dramatic decrease for SCH23390. Cell surface RIAs revealed a decrease in the expression of the D_1-W6.48 mutant, but not for the analogous D_5 mutant receptor, indicating that, at the D_1 receptor, W6.48 may have an important role in stabilizing the receptor for proper folding and membrane expression. Shi *et al*. (2002) proposed that W6.48 is a central residue in the putative 'rotamer toggle switch' of amine receptors. The rotamer configuration of W6.48- in coordination with other aromatic amino acid residues surrounding the TM6 proline kink- has been proposed to modulate the bend of TM6, leading to the movement of TM6 away from TM3 that is necessary for receptor activation (Kobilka and Deupi, 2007; Shi *et al.*, 2002). In rhodopsin, the rotamer configuration of W6.48 changes from *g*+ to *t* during the transition from the inactive to active state (Chabre and Breton, 1979). Lin and Sakmar (1996) demonstrated that photoisomerization of

retinal moves the β-ionone ring away from W6.48, leading to the rearrangement necessary for activation. Vilardaga *et al.* (2006) suggest that the rotamer configuration of W6.48 changes upon the activation of rhodopsin. Alteration of the rotamer conformation of W6.48 from *g*+ to *t* in β_2-adrenergic receptor has been proposed to cause a corresponding change of F6.52 to *g*+ from *t* to avoid steric clash. Conversely, alteration of F6.52 from *t* to *g*+ is accompanied by a change of W6.48 to *t* (Shi *et al.*, 2002). This coordinated rearrangement of rotamer configurations is thought to transduce agonist binding, and trigger the movement of TM6 away from TM3 (Visiers *et al.*, 2002). Thus, I expected that mutation of this tryptophan to alanine, a residue incapable of forming the active rotamer, would lead to a decrease in receptor activation regardless of the agonist bound. Mutation of phenylalanine to alanine should disrupt the ability of W6.48 to participate in the cascade of rotamer reconfigurations that occurs in the toggle switch region thus affecting receptor activation. Indeed, basal and ligand stimulated levels of cAMP accumulation were greatly reduced at the D_1- and D_5-W6.48A mutant receptors. Bhattacharya *et al.* (2008a) showed in rhodopsin that water molecules mediate rotamer toggling of W6.48. This rotamer toggling process is connected to hinge bending around the highly conserved TM6 proline kink, an important part process in the creation of an active receptor state. When W6.48 is mutated to alanine the water assisted rotamer toggling process is lost, thereby decreasing modulation of hinge bending of TM6, resulting in decreased activation of the D_1 and D_5 dopamine receptors.

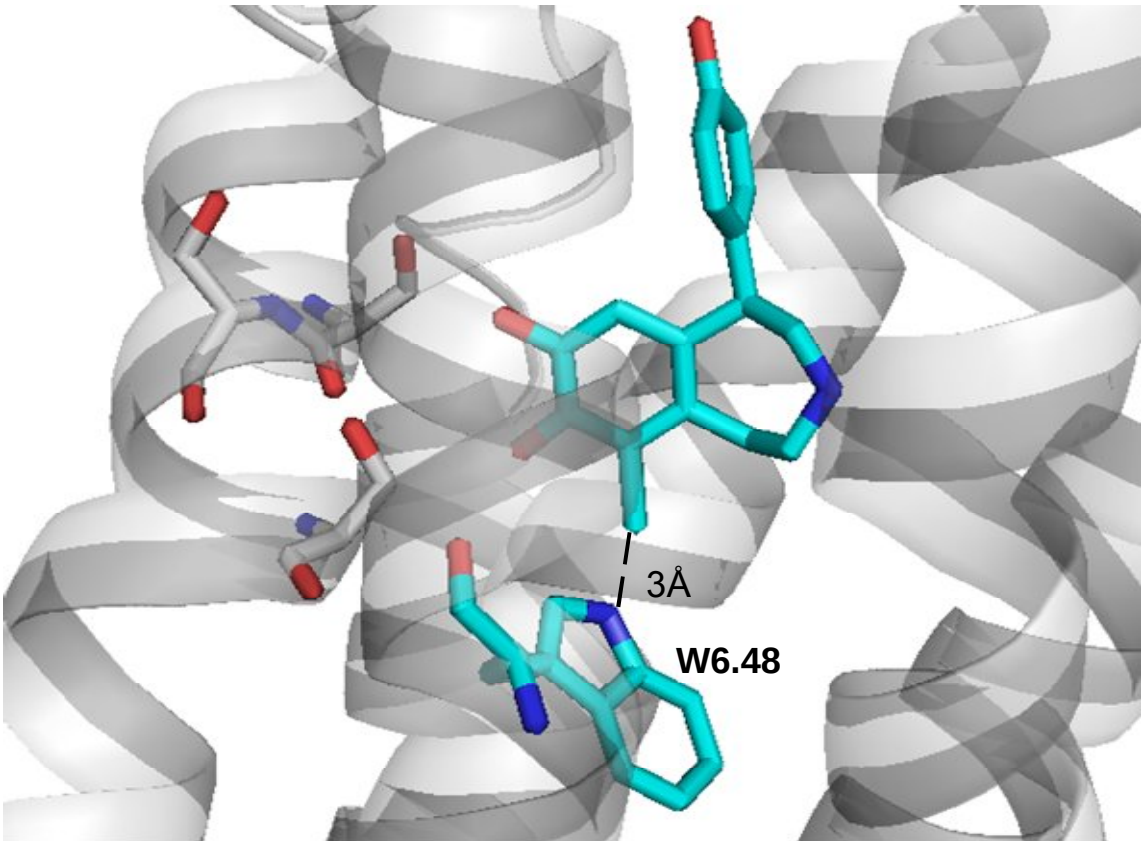

Figure 5.5. Bound conformation of SKF82526 in D_1 receptor showing the hydrogen bond between the chlorine group and W6.48.

The decreases in basal and agonist-stimulated levels of cAMP accumulation provide evidence that W6.48 does indeed serve a central role in formation of an active receptor state. Our D_1 molecular model predicted that SKF82526 and SKF82958, but not the other compounds used in this study, interact directly with W6.48 (Figure 5.5). The dramatic reduction in cAMP accumulation mediated by SKF82526 and SKF82958 at the D_1-like mutant receptors supports this hypothesis. Previous studies in our lab provided evidence of a unique binding position for SKF82526 and SKF82526 due to the 6-Cl substituent appended to the catechol ring. In the predicted docked conformations, this chlorine group hydrogen bonds with the indole nitrogen of W6.48. Mutating W6.48 to Ala removes the hydrogen bond, leading to dramatic losses in the intrinsic activity of the two ligands.

In conclusion, these data suggest a key role for W6.48 in the transduction of ligand-binding to receptor activation. W6.48 appears be a key residue in the putative toggle switch region and is highly involved in creation of an active receptor conformation. The exact role of N6.55 in the D_1-like receptors is less clear, but our findings suggest that this residue plays an indirect role in D_5 receptor activation, perhaps by contributing to hydrophobic binding core. These findings provide important insight into the molecular mechanisms involved in activation of the D_1-like dopamine receptors.

CHAPTER 6: INVESTIGATION OF THE ABILITY OF LIGAND-RECEPTOR INTERACTIONS TO AFFECT AGONIST-SPECIFIC DIFFERENCES IN RECEPTOR INTERNALIZATION AND TRAFFICKING.

PREFACE:

A prior study in our lab found that two structurally dissimilar agonists (A77636 & DNS) cause different degree of D_1 receptor internalization and display divergent patterns of long-term receptor trafficking. Experiments revealed a slow dissociation rate of A77636 from the D_1 receptor and modeling studies predicted that the adamantly group of A77636 is stabilized by V159, W163 on TM4 and L291, L295 on TM6. These contacts are predicted to be sufficient to eliminate the slow dissociation of A77636. This chapter examined the role of each of these residues in ligand-binding, receptor activation, and internalization of the D_1 receptor.

ABSTRACT

Recently, we demonstrated that dopamine, DNS, and A77636 cause different levels of D_1 receptor internalization and also target the receptor to divergent intracellular trafficking pathways, thereby demonstrating functional selectivity at the D_1 receptor. Experiments revealed that A77636 persists on the receptor for long periods of time, indicating that ligand dissociation may influence receptor trafficking. Docking studies of A77636 in the D_1 receptor model predict that the accessory adamantly group of A77636 is stabilized by residues distal to the binding pocket sufficient to eliminate dissociation. The experiments in this Chapter tested our hypothesis that differences in major effects on internalization and receptor trafficking can be produced by specific ligand-receptor interactions distal from the binding site and not critical for binding or activation. Four amino acid residues were mutated to alanine and the effects of dopamine, A77636, and DNS on binding, activation, and receptor internalization were examined at the D_1 wild type and mutant receptors. Mutant receptors did not cause dramatic losses in affinity, potency, or efficacy for any of the test compounds. Unexpectedly, the mutant receptors exhibited non-specific effects on receptor internalization 1 h after agonist removal. The work in this Chapter provides the foundation for future studies to explore whether these residues influence long-term receptor trafficking of the D_1 dopamine receptor.

INTRODUCTION

The experiments in this Chapter build on recent studies in our lab concerning differences in ligand-induced receptor internalization and long-term trafficking. Receptor trafficking is a major mechanism by which GPCRs are regulated, allowing for the fine-

tuning of signal magnitude (Hicke, 1999; von Zastrow, 2003). As discussed in the Introduction of this Dissertation, internalization is an important mechanism in the regulation of D_1 receptor responsiveness and is likely to have important physiological relevance. Recently, we explored the relationship between agonist structure, receptor affinity, and efficacy of adenylate cyclase activation and receptor internalization for 13 agonists from three different chemical families (Ryman-Rasmussen *et al.*, 2005). This study revealed interesting disparities in the ability of synthetic D_1 agonists to regulate receptor trafficking, indicating that functional selectivity cannot be predicted by simple structural examination. These findings demonstrated clearly that D_1 agonists can cause functional selectivity at the endpoints of adenylate cyclase and receptor internalization.

In a subsequent study we further investigated D_1 receptor functional selectivity by examining the ability of structurally dissimilar agonists to regulate receptor trafficking following internalization (Ryman-Rasmussen *et al.*, 2007). We compared the ability of two structurally distinct agonists, A77636 (an isochroman) and DNS (an isoquinoline), to induce receptor internalization with that of dopamine. Our study revealed that, in the HEK293 cell line, steady state levels (1h) of receptor internalization differ significantly between dopamine, DNS, and A77636. We next investigated post-endocytic agonist effects on receptor trafficking and discovered that these agonists were functionally selective in regulating long-term receptor trafficking. Dopamine caused the D_1 receptor to recycle back to the cell surface within 1h of removal. The D_1 receptor was retained intracellularly up to 48 h after removal of A77636, whereas DNS caused the receptor to recycle back to the membrane after 48 h. Additional experiments revealed a slow dissociation rate of A77636 from the D_1 receptor. To explore possible structural

differences in binding modes we examined the predicted binding site of dopamine, A77636, and DNS. The D_1 model indicates that the accessory hydrophobic adamantyl group of A77636 is stabilized by V159, W163 on TM4 and L291, L295 on TM6. These contacts are predicted to be sufficient to eliminate the slow dissociation of A77636. The results of this study indicate that ligand-receptor interactions distal to the binding pocket may dictate the ability of an agonist to induce receptor internalization and regulate long-term receptor trafficking.

The work in this chapter tested the hypothesis that differences in major effects on receptor internalization can be produced by specific ligand-receptor interactions distal from the binding site and not critical for binding or activation**.** To test this hypothesis, amino acid residues V159, W163, L295, and L291 were mutated to alanine and assessed for effects on receptor binding, adenylate cyclase activation, and receptor internalization. The work in this Chapter indicates these residues do not dramatically influence ligand-binding or receptor activation, but have a non-specific effect on receptor internalization.

RESULTS

Expression of Mutant Receptors

To assess the effects of point mutations on antagonist binding affinity and B_{max} wild-type and mutant D_1-like dopamine receptors were expressed in HEK293 cells and tested for their ability to bind [^{3}H]SCH23390. The K_D and B_{max} of [^{3}H]SCH23390 was 1.5 nM and 3,600 fmol receptor/mg protein at the D_1-wt receptor. The K_D and B_{max} for the D_1-L295A and L291A mutant receptors were very similar from that of the wild-type receptor. Conversely, the W163A and V159A mutant receptors bound [^{3}H]SCH23390 with an affinity approximately 5-6 fold lower than the D_1-wt receptor and were expressed

at approximately 830 fmol receptor/mg protein (Table 6.1). However, as shown in Figure 6.1, cell-surface radioimmunoassays (RIA) indicate that the cell surface expression of all D_1 mutant receptors did not diverge significantly from that of the D_1-wild type receptor.

Table 6.1. K_D and B_{max} of D_1-like wild-type and mutant receptors labeled with [^{3}H]SCH23390.

Receptor Type	N	KD (nM)	B_{max} (fmol R/mg protein)
D_1-wild type	12	1.5 ± 0.2	3,600 ± 600
D_1-W163A	4	5.2 ± 0.2	820 ± 7
D_1-V159A	4	6.8 ± 1.3	870 ± 120
D_1-L295A	3	0.9 ± 0.1	3,900 ± 600
D_1-L291A	3	2.2 ± 0.5	3,700 ± 600

HEK293 cell membranes transiently expressing wild-type or mutant receptors were tested in radioreceptor saturation isotherm experiments with increasing concentrations of [^{3}H]SCH23390. Non-specific binding was determined with 1μM cold SCH23390. Data were analyzed using a one-site hyperbolic curve fitting function (Prism 4.0) to obtain the K_D and B_{max} for [^{3}H]SCH23390 at wild-type and mutant receptors. Data are mean ± S.E.M.

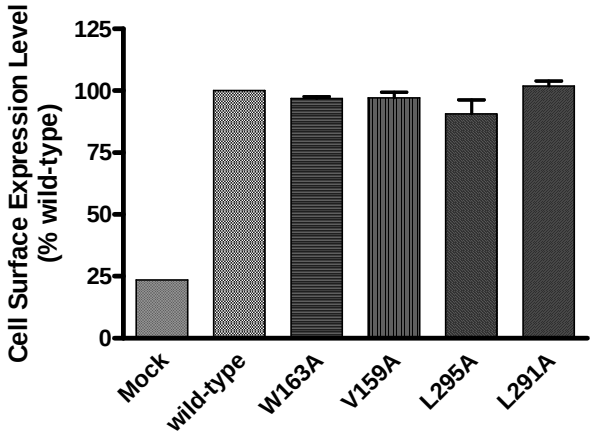

***Figure 6.1. Cell surface expression of D1-WT and mutant receptors**. HEK293 cells transiently expressing HA tagged D_1-WT and mutant receptors, exhibiting decreased [^{3}H]SCH23390 binding, were tested for cell surface expression relative to each respective wild-type receptor via RIA. Data are representative of 2-3 independent experiments run in quadruplicate.*

Effects of D_1 Receptor Mutants on Ligand Affinity

The affinity of dopamine, A77636, and DNS for the wild-type and mutant receptors was determined by competition radioreceptor assays versus [^{3}H]SCH23390 using cell membranes. To determine apparent affinity constant, $K_{0.5,}$ experimental IC50 values were corrected for radioligand K_D and concentration using the bimolecular Cheng-Prusoff relationship (Cheng and Prusoff, 1973). The rank order of affinities for compounds at the D_1 wild-type receptor was as follows, **D_1**: SCH23390 > A77636 > DNS > DA.

As shown in Table 6.2 and Figure 6.2, there were modest alterations (4-5 fold) in the affinity of DNS and dopamine for the V159A mutant receptor, and the binding of SCH23390 was reduced 23-fold at the V159A mutant receptor. No notable changers in affinity were observed for DNS, A77636, or dopamine at any of the other D_1 receptor mutants.

Table 6.2. Affinity of test ligands for the D1-WT and mutant receptors labeled with [^{3}H]SCH23390.

	SCH23390	Dopamine	A77636	DNS
hD1-wt	0.1 ± 0.01	2600 ± 146	11.7± 2.3	215 ± 15
hD1-L291A	0.02 ± 0.03 (1)	4655 ± 138 (2)	8.7± 1.2 (1)	259 ± 24 (1)
hD1-L295A	0.07 ± 0.03 (1)	3086 ± 153 (1)	9.6± 1.4 (1)	219 ± 22 (1)
hD1-V159A	2.2 ± 0.12 (23)	11674 ± 1282 (4)	31 ± 4.2 (3)	957 ± 289 (4)
hD1-W163A	0.72 ± 0.27 (7)	7271 ± 1373 (3)	9.9± 3.5 (1)	1022 ± 493 (5)

HEK293 cell membranes containing D_1-wt or mutant receptors were incubated with one concentration of [^{3}H]SCH23390 and 7-12 concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) to obtain estimates for apparent affinity (K0.5) using the Cheng-Prusoff equation. Values are expressed as mean $\pm$ S.E.M. (nM). In parentheses is the fold change value as compared to the $K_{0.5}$ of each drug at the wild type receptor. Data are representative of 3-10 experiments run in triplicate.

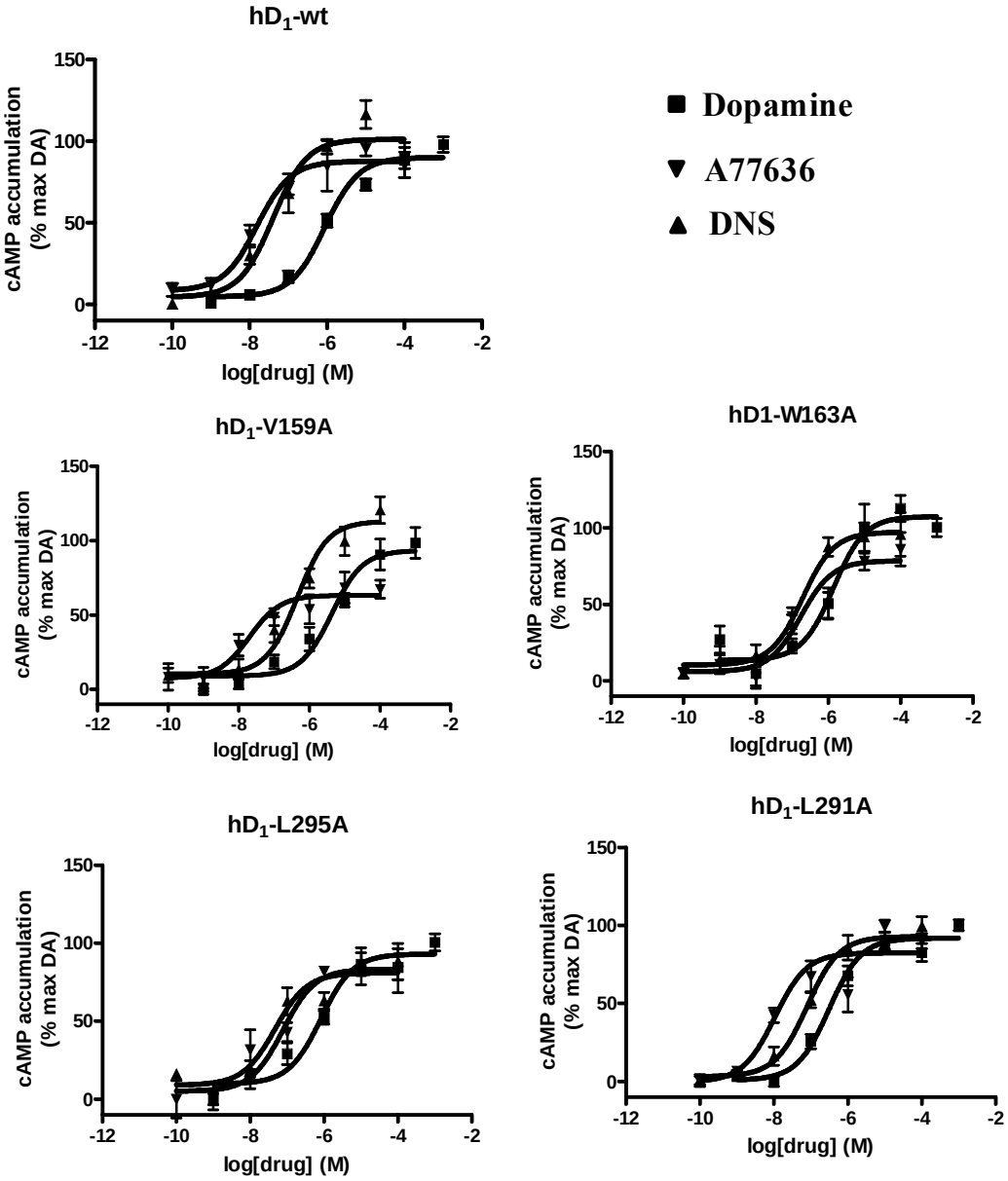

Figure 6.2. Ligand-induced cAMP accumulation at D_1-WT and mutant receptors. *The ability of test compound to stimulate cAMP production was measured using HEK293 cell membranes transiently expressing mutant receptors. Membranes were incubated with drug for 15 min at 37° C. Dose-response curves were generated using 7 concentrations of dopamine, A77636, or DNS at the D_1- wild-type and mutant receptors. Results were analyzed by non-linear regression using a sigmoidal equation for best fit to obtain potency (EC50) and intrinsic activity values (Prism 4.0). Data are expressed as % maximal stimulation by 1 mM DA at each receptor type. Data are representative of 3-5 independent assays run in quadruplicate and each value represents the mean ± S.E.M.*

Effects of D_1 Mutant Receptors on ligand-induced cAMP accumulation

Next, we examined the effect mutant receptors had on the ability of test compounds to elicit receptor activation. Wild-type and mutant receptors were incubated for 15 min with a range of agonist concentrations and subsequent cAMP accumulation measured by RIA and expressed as % maximal stimulation produced by dopamine at each receptor type. The rank order of potencies at the D_1 wild-type receptor was as follows, A77636 > DNS > DA. At the D_1 receptor DNS and A77636 were full agonists producing cAMP accumulation equal to that of dopamine. Receptor stimulation was blocked using the antagonist SCH23390 (data not shown).

Table 6.3. Agonist potency and intrinsic activity of test compounds at the D_1-WT and mutant receptors.

	Drug	Dopamine	A77636	DNS
hD1-wt	**EC50 (nM)**	640 ± 150	23 ± 9	87 ± 24
	Intrinsic Activity	100	100	97
hD1-W163A	**EC50 (nM)**	1,040 ± 440 (1)	112 ± 28 (5)	457±319 (5)
	Intrinsic Activity	100	80	97
hD1-V159A	**EC50 (nM)**	1060 ± 230 (2)	35 ± 19 (1)	453 + 208 (5)
	Intrinsic Activity	100	63	112
hD1-L295A	**EC50 (nM)**	790 ± 290 (1)	4.6± 3.4 (0.19)	237±135 (3)
	Intrinsic Activity	100	85	84
hD1-L291A	**EC50 (nM)**	430 ± 130 (1)	15 ± 5.0 (1)	84 + 21 (5)
	Intrinsic Activity	100	85	100

HEK293 cell membranes containing D_1 -wt or mutant receptors were incubated with seven concentrations of test compound. Dose-response curves were analyzed by non-linear regression using a sigmoidal equation (Prism 4.0) for best fit to obtain EC50 values. Values are expressed as mean + S.E.M. (nM). In parentheses is the fold change value as compared to the EC50 of each drug at the wild type receptor. Data are representative of 3-6 individual experiments run in quadruplicate.

A77636, DNS, and DA exhibited slight losses in potency at the D_1 receptor mutants (Table 6.3). Modest decreases in the intrinsic activity of A77636 occurred at the D_1-W163A, L295A, and L291A mutant receptors, while a more dramatic decrease in

maximal activity was observed for A77636 at the V159A mutant (63% of dopamine) (Table 6.3).

D_1 Mutants Resulted in Non-specific Effects on Ligand Internalization

To examine the ability of test compounds to cause receptor internalization, wild-type and mutant receptors were incubated with agonist for 1 h at 37 C. Following the incubation period cells were fixed with PFA and treated with HA-antibodies to measure the degree of receptor internalization. Receptor internalization was measured by RIA and expressed as % of control. The rank order of receptor internalization efficacy at the D_1 wild-type receptor was as follows, A77636 > DNS > dopamine. Receptor internalization caused by each agonist was blocked by the antagonist SCH23390.

Figure 6.3 and Table 6.4 show the changes in receptor internalization at each D_1 mutant receptor. There was a significant decrease (compared to internalization of the D_1-wt receptor) in the receptor internalization elicited by dopamine at the L291A and V159A mutant receptors, for A77636 at the L291A, L295A, and V159A receptors, and for DNS at the V159A mutant receptor.

Table 6.4. Recovery of cell surface D1-WT and mutant receptors after 1 h agonist removal.

	SCH23390	Wild-type	L291A	L295A	V159A	W163A
Dopamine	99	80	89*	89	98*	85
A77636	100	66	76*	78*	81*	68
DNS	100	74	86	84	90*	79

*HEK293 cells expressing HA-hD_1-WT or mutant receptors were treated with 10 μM dopamine, A77636, or DNS for 1 h. A cell surface HA tag was used to measure cell surface HA-hD_1 1 h after agonist removal. Data are expressed as % control and are mean + S.E.M. *p>0.01*

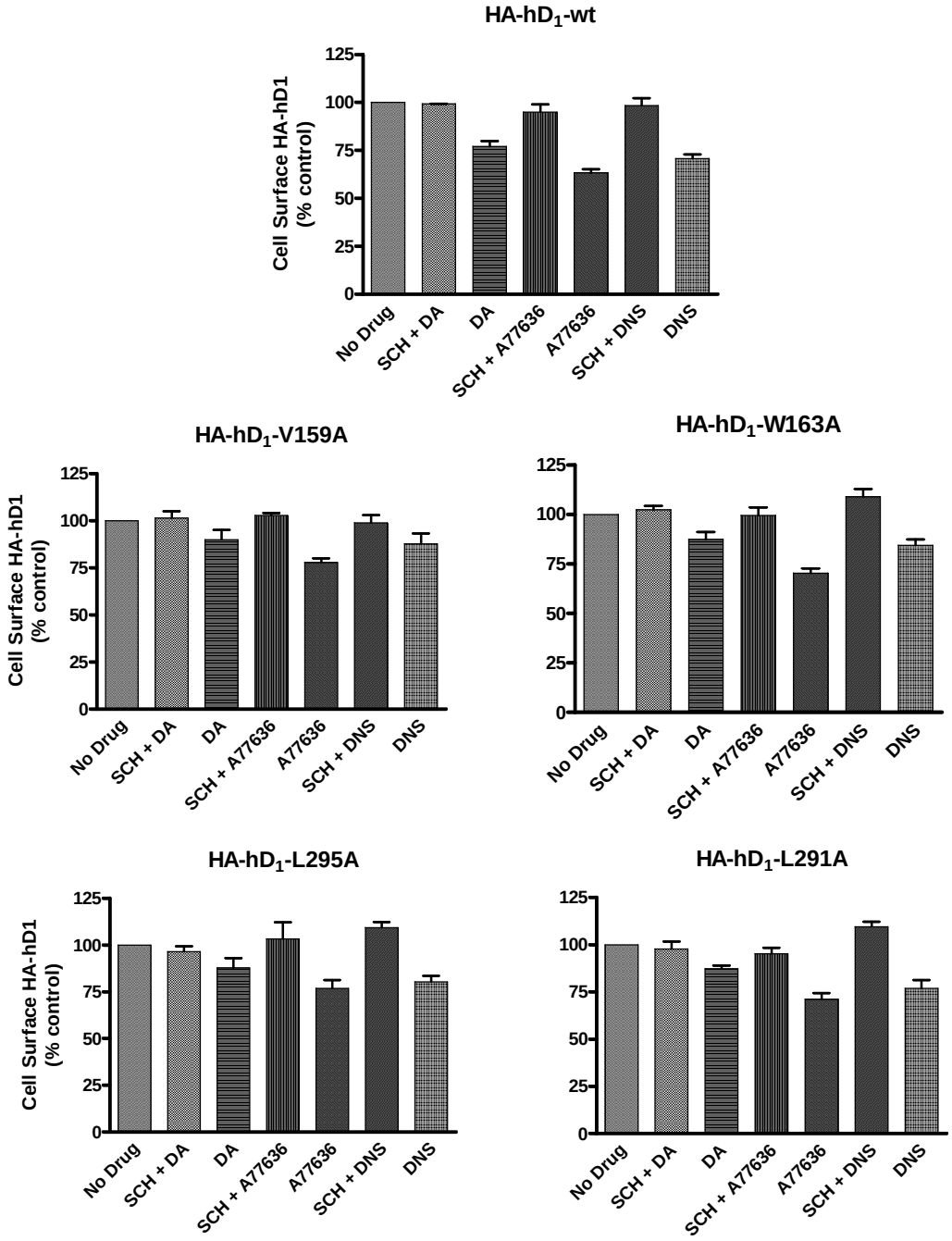

Figure 6.3. Recovery of cell surface HA-hD$_1$ receptors 1 h after agonist removal*. HEK293 cells transiently expressing HA-tagged D$_1$-WT and mutant receptors were treated with 10 μM dopamine, A77636, or DNS for 1 h in quadruplicate. Data are expressed as a percentage of no drug and are the means and standard errors of three to four independent experiments.*

DISCUSSION

Dinapsoline and A77636 were utilized for these studies because they are structurally dissimilar full agonists that are of therapeutic interest. Previous studies have shown that A77636 induces profound behavioral tolerance *in vivo* within 24 h, thereby precluding its use in Parkinson's disease therapy (see Background) (Lin *et al.*, 1996). Dinapsoline, however, does not elicit tolerance in a rat model of Parkinson's disease (Gulwadi *et al.*, 2001). Several promising D_1 agonists such as A77636 demonstrate rapid and profound tolerance when administrated in human and non-human primates, thereby prohibiting their use as therapeutics (Asin and Wirtshafter, 1993; DeNinno *et al.*, 1991; Kebabian *et al.*, 1992a; Lin *et al.*, 1996). Understanding the cellular events that contribute to tolerance may be important in the development of clinically relevant D1 agonists that are less prone to the development of tolerance. Although the nature of the relationship between agonist-induced receptor internalization and the development of tolerance is unclear, early regulatory responses to continuous D1 receptor activation may be important steps leading to motor tolerance.

Mutational effects on ligand binding, adenylate cyclase activation, and receptor internalization for dopamine, A77636, and DNS were examined for each D_1 mutant receptor. The B_{max} for the W163A and V159A mutant receptors were reduced ~ 3-fold, however cell surface RIAs showed that all mutant receptors were expressed at levels comparable to that of the wild-type receptor. This result suggests that the reduction in B_{max} is likely due to the decreased affinity of SCH23390 for the W163A and V159A mutant receptors and not a decrease in cell surface expression. Modest alterations in the binding affinity of DNS, A77636, and dopamine occurred at the D_1 receptor mutants. The decrease in affinity of the antagonist SCH23390 at the V159A (23-fold) mutant

receptor was unexpected, however, due to the lack of another radiolabeled D_1 antagonist the interactions involved in binding of SCH23390 at the D_1 receptor is ill-defined. These findings support our hypothesis that the targeted residues do not play an important role in agonist binding.

Test compounds did not exhibit dramatic changes in the potency or intrinsic activity of compounds at any of the D_1 mutant receptors. However, the decrease in intrinsic activity of A77636 at the D_1-V159A mutant receptor suggests that this valine residue might be positioned in a region of the D_1 receptor binding pocket important for receptor activation by A77636, presumably by interacting with the adamantly group appended to A77636. While unexpected, the decrease in intrinsic activity of A77636 at the V159A mutant receptor is not surprising as receptor activation is inherently dynamic, confounding our ability to predict residues important for creating an active receptor state from an inactive receptor model.

As with the stably transfected HA-hD_1 cell line used our previous study, the time course of internalization for the transiently expressed wild-type receptor reached steady-state by 30 min and was constant through 2 h. The rank order of agonist internalization efficacy of the HA-hD_1-wild type receptor was identical at the stably and transiently expressed receptors. The non-specific changes in the degree of receptor internalization for dopamine, A77636 and DNS at the mutant D_1 receptors were unexpected given the predicted location of the amino acid residues targeted in this study. This result indicates that these residues- indirect of agonist interaction- contribute to formation of the conformational state required for D_1 receptor phosphorylation by GRKs or PKA and/or receptor interaction with β-arrestin.

Although previous studies suggest that receptor internalization and long-term trafficking might be interrelated (i.e. at the wild type receptor A77636 exhibited the greatest degree of receptor internalization and long-term trafficking), these mechanisms may in fact be distinct and dependent on different molecular interactions. The conformational changes that lead to receptor internalization are largely unknown, and it is feasible that any residues that may be responsible for creating the conformational state that may be responsible for the unexpected endocytic trafficking of the A77636 bound-D_1 receptor come into contact with the adamantly group of A77636 only upon D_1 receptor internalization. Numerous studies have demonstrated that slight changes in receptor conformation- due to mutation of an amino acid residue or interaction with intracellular signaling partners- can produce receptor conformational changes that cause dramatic effects on receptor function. Thus, it is feasible that binding of β-arrestin may produce alterations in receptor conformation, thereby creating new molecular interactions between the ligand and receptor.

Preliminary experiments were performed to confirm that the results of long-term receptor trafficking studies in our previous study using HEK293 cells stably expressing the D_1 receptor could be reproduced, and expanded, with the mutant receptors, using transiently transfected HEK cells. Initial experiments showed that the degree of D_1-wt receptor internalization at 48 h was roughly equivalent to that of receptor internalization following 1 hr incubation (data not shown). While preliminary, these results suggest that the use of transiently expressed mutant receptors may not be amenable for examining the effects of long-term receptor trafficking, however time constraints did not permit definitive conclusions to be drawn. Additional troubleshooting and assay optimization

will be necessary to determine the feasibility of trafficking studies using transiently transfected wild-type and mutant receptors. The hypothesis of this study was that mutation of one or more of the targeted amino acid residues would selectively affect receptor internalization and/or receptor trafficking and not agonist affinity or efficacy. Despite the unexpected non-specific effects of the mutant receptors on agonist internalization, it is possible that long-term receptor trafficking is affected. Future studies will explore the effects of each of these mutations on long-term receptor trafficking of dopamine, dinapsoline, and A77636. These studies will reveal whether these residues are indeed responsible for differences in receptor trafficking between A77636, DNS, and dopamine.

In our previous study, scanning of the D_1 receptor model resulted in the identification of a much weaker but perhaps significant binding site for dopamine in the intracellular portion of TMs 3, 4, 5, and 6 (Ryman-Rasmussen *et al.*, 2007). This led us to formulate an alternative hypothesis, that this secondary site serves as an allosteric binding site for the endogenous ligand to regulate reformation of the receptor. Other agonists (such as dinapsoline and A77636) may not bind to this site, thus leading to the low degree of dissociation and subsequent effects on receptor trafficking. An additional direction is to explore whether differential phosphorylation by PKA and GRK may be responsible for the differences in endocytic trafficking of dopamine, DNS, and A77636. A study of the β_1-adrenergic receptor demonstrated that the pathway selected for receptor internalization is primarily dictated by the kinase that phosphorylates the receptor (Rapacciuolo *et al.*, 2003). The authors found that PKA-mediated phosphorylation directs internalization via the caveolae pathway, whereas GRK-mediated phosphorylation

occurs through clathrin-coated pits. Studies indicate a role for both PKA- and GRK-mediated phosphorylation in D_1 receptor desensitization (Bates *et al.*, 1991; Black *et al.*, 1994; Tiberi *et al.*, 1996; Zhou and Fishman, 1991; Zhou and Fishman, 1991).

In summary, the experiments in this Chapter demonstrate that V159, W163, L291, and L295 have minimal effects on the affinity, potency, and intrinsic activity of dopamine, dinapsoline, and A77636, and appear to play a role in creating the conformational state required for D_1 receptor internalization. These interactions, however, appear to be agonist independent. These findings provide the foundation for important experiments that will explore the effects of these mutations on long-term receptor trafficking of dopamine, dinapsoline, and A77636. Results of the trafficking experiments will help guide future studies to understand the basis of long-term receptor trafficking and the development of tolerance. Such studies may provide evidence of a structural basis for functional selectivity at the D_1 receptor and aid in the design of clinically useful D_1 agonists that are less likely to lead to tolerance.

CHAPTER 7. SUMMAY, CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY OF STUDIES CONDUCTED

Overview of original goals

Recent advances in neurobiology have demonstrated the potential utility of D_1-like dopamine receptor agonists as therapeutic compounds. Multiple disorders have been linked to dopaminergic dysfunction, and many of these disorders may be ameliorated with D_1 agonists. Despite immense promise, there are no D_1 receptor agonists currently available as therapeutic compounds. To develop such compounds, it is necessary to understand the structural basis for ligand-binding and activation of D_1-like receptors. Very few studies have attempted to determine ligand-receptor interactions in the D_1 receptor, and virtually none have been conducted for the D_5 receptor. Consequently, the structural mechanisms governing ligand-binding and activation of D_1-like receptors are poorly understood. The work described in this Dissertation investigates key molecular interactions underlying binding, activation, and internalization of the D_1-like dopamine receptors.

The data collected in these studies relied on the use of a group of structurally and pharmacologically diverse probe ligands [phenylbenzazepines (SKF38393, SKF82526, & SKF82958), an isochroman (A77636), rigid compounds (DHX & DNS), and the endogenous ligand (dopamine)]. The resulting data greatly extend our knowledge of the structural mechanisms involved in binding and activation of the D_1-like receptors. Furthermore, these findings provide evidence of structural differences between the D_1

and D_5 receptor subtypes. This work lays the foundation for future studies that will investigate further the molecular interactions mediating binding and activation of the D_1-like dopamine receptors.

Improvement of cAMP assay

Chapter 2 described improvements to our method of assessing cAMP accumulation. The cAMP pathway is the most studied D_1 receptor signaling pathway and was central to the work conducted for this Dissertation. Modifications of the original cAMP assay included coupling the primary antibody directly to magnetic beads (opposed to the secondary antibody) and separating the antibody-bound magnetic beads from unbound marker using filtration on microplates. These alterations markedly improved speed and costs while retaining high sensitivity.

Analysis of TM3 threonine residue

Chapter 3 addressed the role of a TM3 threonine residue (3.37) in governing binding and activation of the D_1-like dopamine receptors. T3.37 is highly conserved across aminergic GPCRs yet no published studies have examined the role of this residue in receptor activation. A study of the D_2 dopamine receptor utilizing SCAM techniques indicated that T3.37 is not water-accessible in the binding site crevice (Javitch *et al.*, 1998), however we hypothesized that T3.37 is positioned to influence receptor interaction with the *para*-OH of D_1 receptor agonists. Results from this study showed that mutation of T.3.37 to alanine had more dramatic effects on the affinity and functional effects of the rigid D_1 agonists (DA, DHX, DNS, and A77636) than phenylbenzazepine compounds (SKF38393, SKF822526, and SKF82958). These findings closely parallel studies of the D_1- and D_5-S5.46A mutant receptors, a residue that has been shown to interact with the

para-OH of D_1 compounds (unpublished observations), indicating that these two residues have similar ligand-receptor interactions. A double mutant receptor (i.e. D_1- and D_5-T3.37A/S5.46A) resulted in even greater changes in binding and function, further supporting our hypothesis. While the exact nature of the interaction of T3.37 with D_1 agonists is unclear, these findings provide strong evidence that this residue influences the interaction of the *para*-OH group of D_1 agonists.

Analysis of TM6 residues

Chapters 4 and 5 focused on three amino acid residues located in sixth transmembrane helix. Residues in TM6 have been shown to play a key role in the activation of several GPCRs (Liapakis *et al.*, 2000; Strader *et al.*, 1989) as several studies have suggested the highly conserved cluster of aromatic residues (coined the 'rotamer toggle switch') surrounding the proline-kink in TM6 serves a key role in coupling agonist binding to receptor activation (Kroeze *et al.*, 2003; Shapiro *et al.*, 2000; Shi *et al.*, 2002; Bissantz *et al.*, 2003; Kroeze *et al.*, 2003; Shapiro *et al.*, 2000; Shi *et al.*, 2002). Agonist interaction with one or more of the residues located in the switch region is thought to cause a coordinated change in the rotamer configurations of these aromatic residues, modulating the bend angle of TM6 around the proline kink and leading to the movement of the cytoplasmic end of TM6 (Shi *et al.*, 2002).

Analysis of D_1- and D_5-F6.51 mutant receptors

Studies have shown that the residue at position 6.51 can play a role in ligand binding and receptor activation (Chen *et al.*, 2002a; Ward *et al.*, 1999). Prior studies in our lab demonstrated that mutation of F6.51 to alanine affected the potency of D_1 agonists according to structural class. The potency and intrinsic activity of the

structurally rigid full agonists (DNS, DHX, and A77636) decreased while the phenylbezazepines (SKF38393, SKF82958, and SKF82526) were affected minimally (unpublished results). To explore further the role of F6.51, I constructed non-conservative (Leu and Ile) and conservative (Trp and Tyr) mutations of the D_1 and D_5 receptors and characterized each mutation with the structurally and functionally diverse test compounds utilized in our studies of the F6.51A mutant receptor. The results support the notion that F6.51 plays a critical role in the activation of D_1-like receptors by interacting with the catechol ring of D_1 agonists. Similar to the F6.51A mutant receptors, the potency and intrinsic activity of test compounds at the D_1- and D_5-F6.51I/L mutant receptors was affected by structural class. SKF38393 exhibited striking increases in intrinsic activity at the F6.51I/L mutant receptors, increasing from partial agonist activity at the D_1 and D_5 wild-type receptors to full agonists at the mutant receptors. DHX and DNS, structurally similar full agonists with near identical potency at the D_1 wild-type receptor, exhibited dramatic differences in function at the D_1-F6.51Y and W mutant receptors. Additionally, data suggested that the D_5-F6.51W mutant receptor disrupts the molecular interactions necessary for stabilizing an active receptor conformational state. The data in this chapter demonstrated that F6.51 is a key switch in the activation of the D_1-like receptors, and provided evidence for ligand-specific receptor conformations.

Analysis of N6.55 and W6.48

Chapter 5 addressed the role of two TM6 residues, N6.55 and W6.48. The residue at position 6.55 varies across aminergic GPCRs but studies have demonstrated direct agonist interaction at this locus (Berthold and Bartfai, 1997; Wieland *et al.*, 1996). At the β_2-adrenergic receptor, a receptor that shares high homology with the D_1 receptor, N6.55

was shown to interact directly with the β-hydroxyl in the aliphatic side chain of isoproterenol (Wieland *et al.*, 1996; Zuurmond *et al.*, 1999). W6.48 is completely conserved across aminergic receptors is thought to be a key residue in the 'rotamer toggle switch' region of GPCRs (Bissantz *et al.*, 2003; Roth *et al.*, 1997; Shi *et al.*, 2002). The results of this chapter indicated that N6.55 might play a direct role in agonist-stimulated receptor activation of the D_1 receptor, as potency and intrinsic activity of test compounds at the D_1-N6.55A mutant receptor was affected according to structural class. Conversely, a class-specific trend in potency and intrinsic activity was not observed at the D_5-N6.55A mutant receptor, indicating possible global changes in receptor conformation.

Basal and ligand stimulated levels of cAMP accumulation were greatly reduced at the D_1- and D_5-W6.48A mutant receptors, supporting the notion of a critical role for W6.48 in the creating an active receptor state. As predicted by our D_1 modeling studies, SKF82958 and SKF82526 caused a dramatic reduction in cAMP accumulation at the D_1 and D_5-W6.48A mutant receptors. These data support our hypothesis that agonist interaction with W6.48 is a critical step in the formation of an active D_1-like receptor conformational state.

Analysis of D_1 receptor internalization and long-term trafficking

The experiments in Chapter 6 were based on prior studies in our lab concerning the divergent endocytic trafficking patterns exhibited by structurally dissimilar full agonists (DNS and A77636) (Ryman-Rasmussen *et al.*, 2007). This study found that the D_1 receptor was retained intracellulary up to 48 h after removal of A77636, whereas DNS caused the receptor to recycle back to the cell surface after 48 h. We hypothesized that structural differences in the binding modes of A77636 and DNS were responsible for the

differences in long-term trafficking. Alteration of several amino acids predicted to interact with the adamantly group of A77636, but not DNS or dopamine, did not cause dramatic differences in the affinity, potency, or intrinsic activity of the test compounds. Receptor internalization experiments 1 h after agonist removal indicated that the targeted residues may play a role in stabilizing the D_1 receptor for phosphorylation, indirect of agonist interaction. This work provides the foundation for future studies that will assess whether the targeted residues are responsible for the differences observed in agonist-induced long term trafficking.

Implications of this work

The work presented in this Dissertation provides invaluable insight into the structural mechanisms underlying D_1-like receptor binding and activation, and offers information that may be used in the design of novel D_1-like receptor compounds. This work addressed the structural features of the ligand and receptor that impart characteristics (e.g. efficacy, bioavailability, etc.) of clinical relevance, and therefore have implications for D_1 agonist design. The experiments in Chapters 4 and 5 provide insight into the structural basis of efficacy at the D_1-like receptors. These data provide clear evidence that subtle structural changes can have profound effects on receptor activation, underscoring the importance of understanding the interactions involved in creating an active receptor state. Effective amelioration of PD symptoms requires full D_1 agonism while evidence suggests that partial D_1 agonists may be more effective in treating cognitive dysfunction. It is therefore important, for the design of effective D_1 receptor drugs, to determine the structural features responsible for efficacy. Our studies of T3.37 provide convincing evidence that this residue influences ligand-binding and

receptor activation by contributing to the network of hydrogen bonds involved in stabilizing the catechol hydroxyls of D_1 agonists. These data reinforce the catechol requirement for D_1 full agonists, a feature that contributes greatly to the low bioavailability associated with most D_1 agonists. Lastly, this work elucidated possible structural differences between the D_1 and D_5 receptor subtypes that may aid in the design of the first D_1 subtype selective compound. Such an advance would be tremendously valuable for characterizing the *in vivo* role of D_1 and D_5 receptors and may prove to have clinical promise as well. The findings presented in this Dissertation will not only guide the design of future studies but may help direct the development of novel D_1 receptor agonists.

RELATED STUDIES

Structural changes involved in GPCR activation

Numerous studies have focused on understanding the conformational changes that occur from ligand binding to receptor activation. Methods such as x-ray crystallography (Schertler, 2005), site-directed mutagenesis (Chen *et al.*, 2002b; Gabilondo *et al.*, 1996), molecular modeling (Bhattacharya *et al.*, 2008b; Bhattacharya *et al.*, 2008a), site-directed spin labeling (Altenbach *et al.*, 1999; Altenbach *et al.*, 2001b) fluorescence spectroscopy (Gether, 2000; Ghanouni *et al.*, 2001b) and others have provided insight into the molecular interactions responsible for the conformational changes that lead to GPCR activation.

The crystal structure of bovine rhodopsin has served as a useful template for generation of homology-based GPCR models but, as discussed in the Introduction of this Dissertation, has significant limitations. Data from the recently crystallized β_2-adrenergic

receptor (Cherezov *et al.*, 2007) offers important insight that can be incorporated into GPCR homology models. While the x-ray structure of an inactive state of a GPCR does not provide insight into the conformational changes that occur during activation, resultant homology models, as well as *ab initio* models, has served to identify target residues for mutational studies and are equally as important in the interpretation of experimental results.

An iterative approach of receptor modeling and site-directed mutagenesis has lead to the identification of numerous molecular interactions that contribute to ligand recognition and receptor activation. For the catecholamine receptors, studies have shown that the endogenous ligand is anchored in the receptor binding pocket by an amine group on TM3 and a network of hydrogen bonds formed largely by serine residues in TM5 (Kristiansen, 2004). Studies have identified several conformational switches, common to all catecholamine GPCRs, important for receptor activation (Weinstein, 2006). Three of the most well-characterized molecular switches are: i) the DRY motif (D.349, R3.50, and E3.60), termed the "ionic lock". These residues form a salt bridge between TMs 3 and 6, this interaction is broken during receptor activation through protonation by the amine of catecholamine receptor ligands, ii) the "rotamer toggle switch" in TM6. This switch is comprised of a cluster of highly conserved aromatic residues that surround the proline kink in TM6. The rotamer configurations of these residues are thought to be coupled such that agonist interaction with one or more of these residues causes a coordinated movement of residues in the switch region thereby modulating the bend angle of the proline kink in TM6, iii) the NPxxY motif in TM7. Studies have shown that the interaction of this motif with helix 8 is important in regulating the interactions of the C-

terminal end of the GPCR with signaling partners (Kalatskaya *et al.*, 2004; Prioleau *et al.*, 2002). These motifs have a significant role in stabilizing the inactive receptor state as well as in the creation of the molecular interactions that stabilize an active receptor state. The nature of agonist interaction with these motifs, dependent the structural features of each agonist, dictates agonist affinity and efficacy.

Recent studies utilizing biochemical and biophysical approaches such as *in situ* disulfide cross-linking (Zeng *et al.*, 1999), fluorescent spectroscopy (Gether *et al.*, 1995; Swaminath *et al.*, 2005), and FRET (Vilardaga *et al.*, 2003) have provided direct insight into the conformational changes that occur upon receptor activation. Use of an *in situ* disulphide cross-linking strategy, largely utilizing the M3 muscarinic receptor, has provided insight about the rotational movement of TM6 upon receptor activation (Ward *et al.*, 2006) as well as other helical movements (Han *et al.*, 2005a; Han *et al.*, 2005b). A recent study of the M3 receptor found that agonists and inverse agonists cause opposite effects in the distance between the C-terminal part of TM8 and the cytoplasmic end of TM1 (Li *et al.*, 2007). A series of studies by Kobilka and co-workers have greatly advanced our understanding of the conformational changes that occur upon GPCR activation (Gether *et al.*, 1995; Gether *et al.*, 1997b; Gether *et al.*, 1997a; Ghanouni *et al.*, 2001b; Ghanouni *et al.*, 2001a; Swaminath *et al.*, 2004; Swaminath *et al.*, 2005). For these studies they labeled a modified β_2-adrenergic receptor with small cysteine-reactive fluorescent probes that provide information regarding intensity and lifetimes of the fluorophore (Gether *et al.*, 1997b). These studies have demonstrated that receptor activation is not, as believed previously, a bimodal process but occurs through a series of intermediate conformational states (Swaminath *et al.*, 2004; Swaminath *et al.*, 2005).

Recently, Yao *et al.* (2006) demonstrated that full agonists could induce conformational changes in TM3 and TM6 whereas a partial agonist induced conformational changes in only TM6. These studies suggest that agonists stabilize the receptor in a full or partial active conformational state by interacting with different combinations of molecular switches. A recently developed method using a cyan variant of GFP (CFP) and yellow variant (YFP), inserted into the third intracellular loop and C terminus, allows direct measurement of receptor activation in living cells (Vilardaga *et al.*, 2003). Using this approach, Nikolaev *et al.* (2006) showed that structurally distinct ligands induce kinetically distinct conformational states, confirming that ligand-induced conformations (Ghanouni *et al.*, 2001b) are not a product of reconstituted systems.

These studies underscore the need to understand the structural features of ligands responsible for stabilizing active receptor conformational states. If agonist binding results in several intermediate conformational states, it is intriguing to hypothesize that these intermediate states may have functional significance. Distinct conformational states may cause activation of specific G protein heterotrimers, favoring the activation of one or more effector pathways over others. For example, in one intermediate conformational state a ligand may activate G proteins that lead to partial activation of a specific effector pathway while another conformational state induced by the same ligand may activate a different set of G proteins that elicits full activation of a different effector pathway.

Crystal structures of GPCRs

Recent studies have produced high resolution crystal structures of the β_1- (Warne *et al.*, 2008) and β_2-adrenergic receptors (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007). These studies provide invaluable information concerning the structural features of

GPCRs that, prior to these findings, was available only from bovine rhodopsin. The ligand-binding site of the β_1-and β_2-adrenergic receptors is located in a position similar to that of retinal in rhodopsin, but key differences were observed from rhodopsin, particularly in two conserved regions thought to serve as key motifs regulating activation of aminergic receptors. In constrast to the inactive rhodopsin state, the "ionic lock" formed by a salt bridge between D131 of the DRY motif in TM3 and E268 at the bottom of TM6 is open. This indicates the ionic lock, proposed to have an essential role in maintaining GPCRs in an inactive state (Ballesteros *et al.*, 2001b), is not an essential feature of the inactive state of β_1-and β_2-adrenergic receptors. The "rotamer toggle switch", comprised of a cluster of highly conserved aromatic residues in TM6, has been proposed to play a key role in formation of an active receptor state. The crystal structure of the β_2-adrenergic receptor revealed extensive interaction between the bound inverse agonist carazolol and amino aid residues thought to comprise the toggle switch region (i.e. F6.51, F6.52, and W6.48) (Rasmussen *et al.*, 2007). Yet the switch region is closed in both the β_1-and β_2-adrenergic receptor structures, and can be overlayed with that of the dark (i.e., inactive) state of rhodopsin. Han *et al.* (2008) suggest that the molecular state of the β_2-adrenergic receptor is ambiguous, and is likely to be in some intermediate signaling state. Thus, they state that it is not surprising that some motifs are in an active-like state whereas as others are in an inactive-like state (Han *et al.*, 2008). Additionally, it is possible that the "ionic lock" and "rotamer toggle switch" regions are not interdependent, that one region can assume an active-like state and the other an inactive-like state that is dependent on the ligand bound. Future studies that utilize a variety of

experimental approaches are needed to reconcile apparent contradictions between newly obtained data derived from structural studies and current notions of GPCR activation.

Evidence for the existence of multiple D_1 effector pathways

The cAMP/PKA pathway is the most studied and therefore the best characterized D_1 receptor effector pathway. D_1 receptor-mediated stimulation of adenylate cyclase leads to accumulation of cAMP, activation of PKA, and a subsequent phosphorylation/dephosphorylation cycle of DARPP-32 (Greengard *et al.*, 1999). Activated PKA can phosphorylate other receptors in the cell (e.g. L-Ca^{2+}, NMDA) and also inhibit PP-1 that then dephosphorylates many substrates, including the same receptors phosphorylated by PKA (Snyder *et al.*, 1998), thereby creating a feedback loop that enables tight control over dopaminergic signaling. The apparent dependence of the D_1 receptor on the cAMP/PKA signaling pathway presents a challenge in identifying distinct effector pathways for examining functional selectivity.

Very few studies have investigated the ability of the D_1-like receptors to activate MAP kinases, and those that have are confounded by the promiscuity of ERK1/2. Phospho-ERK has been shown to form a stable heterotrimeric complex with the D_1 receptor and β-arrestin2 (Chen *et al.*, 2004) suggesting that MAP kinase activation may be mediated by a D_1 receptor- β-arrestin2 complex. Additionally, Nagai *et al.* (2007) showed dose-dependent D_1 receptor activation of ERK1/2 in the mouse prefrontal cortex that was unaffected by microinjection of a D_2 antagonist and blocked by a D_1 antagonist.

Several studies suggest that the D_1 receptor couples to PLC activation. However, the mechanism of D_1-mediated PLC activation remains ambiguous. Two studies using several phenylbenzazepine compounds in rat brain tissue lead the authors to hypothesize

the existence of a "D_1-like" phospholipase C-coupled receptor (Friedman *et al.*, 1997; Undie *et al.*, 1994). The potency values reported for PI hydrolysis were in the 10-100 μM range lending some uncertainty as to whether the effect was due to off-target effects or if D_1-mediated PLC signaling is a real phenomenon. Further evidence of a cAMP/PKA-independent PLC signaling pathway was shown in studies of adenylate cyclase V deficient mice (Iwamoto *et al.*, 2003; Lee *et al.*, 2002). In these studies, 85-90% of cyclase activity was abrogated in adenylate cyclase V deficient mice yet locomotion was enhanced. It is not clear whether the behavioral effects are due to a cyclase-independent PLC pathway, but these findings suggest a dependence on other signaling pathways for locomotion. A recent study that examined intracellular Ca^{2+} release supported the idea of D_1-$G\alpha_q$-mediated PLC activation; however, this mechanism was found to be co-dependent on a PKA-cAMP signal (Dai *et al.*, 2008).

Evidence for functional selectivity at the D_1 dopamine receptor

D_1 receptor functional selectivity has been difficult to demonstrate due to the lack of clear signaling pathways coupled to the receptor. As was discussed in the Introduction of this dissertation, the best evidence of functional selectivity at the D_1 receptor was shown in two recent studies comparing the functional endpoints of adenylate cyclase activation and receptor internalization. The first study examined the relationship between agonist structure receptor affinity, and efficacy of adenylate cyclase activation and receptor internalization in response to thirteen agonists from three different structural classes (Ryman-Rasmussen *et al.*, 2005). This study identified several D_1 agonists that activate adenylate cyclase with high efficacy but fail to cause receptor internalization. A subsequent study investigated the effects of two clinically relevant agonists (DNS and

A77636) on long-term receptor trafficking (Ryman-Rasmussen *et al.*, 2007). This study found that these agonists target the D_1 receptor to different intracellular trafficking pathways. Experiments revealed a slow dissociation rate of A77636 from the D_1 receptor, suggesting that ligand-receptor interactions distal to the binding pocket may dictate the ability of an agonist to cause receptor internalization and regulate long-term receptor trafficking. These findings served as the basis for the studies in Chapter 5. Characterization of additional D_1 receptor signaling pathways will greatly facilitate further investigation of functional selectivity at the D_1-like dopamine receptors.

***In vivo* D_1 receptor functional selectivity**

The clinical promise of functionally selective compounds lies in their postulated ability to selectively activate specific signaling pathways leading to decreased side effects while retaining therapeutic efficacy. SKF83822, a high affinity D_1 agonist, has been reported to selectively activate adenylate cyclase and not PLC (Undie *et al.*, 1994). Unlike typical D_1 agonists, SKF83822 does not induce intense grooming in rats (O'Sullivan 2004) or oral dyskinesia in non-human primates (Peacock and Gerlach, 2001). More intensive study must be performed to confirm the functionally selective actions SKF83822 in other systems, but these findings imply that differential behavioral effects can be induced by selective activation of signaling pathways.

Excessive stimulation of peripheral D_1 receptors can result in hypotension and tachycardia thereby precluding the use of high doses of D_1 agonists to treat disorders such as Parkinson's disease. Dopamine, via D_1-like receptors, can modulate blood pressure by regulating renal sodium excretion and controlling the resistance of arteries (Chatziantoniou *et al.*, 1995; Zeng *et al.*, 2004). A clearer understanding of D_1-like

signaling pathways in the central nervous system, kidney, and vascular tissues is an important step for the identification and design of functionally selective D_1 compounds. D_1-family receptors are expressed peripherally in many tissues including the adrenal glands, blood vessels, heart, the kidney and urinary tract, demonstrating the possible novel D_1 signaling pathways that have yet to be elucidated. Design of a D_1 agonist that is less efficacious at these transduction pathway(s), or others that have yet to be identified, could permit the use of high doses of drug in patients.

Additional work is required to elucidate the signaling pathway(s) underlying D_1-agonism induced side effects; however, the potential clinical utility of a functionally selective D_1 agonist is clear. Design of such compounds requires greater knowledge of D_1 signaling pathways as well as a better understanding of the structural characteristics underlying the functionally selective properties.

FUTURE DIRECTIONS

The goal of this Dissertation was to explore the structural mechanisms underlying binding and activation of the D_1-like dopamine receptors. The following major conclusions can be drawn from the data presented in this Dissertation: i) that subtle structural changes in the ligand-receptor interaction can have profound ramifications on receptor activation, ii) that agonist engagement with aromatic residues in TM6 of the D_1-like receptors is an important determinant of efficacy, iii) that structurally dissimilar- as well as structurally similar- D_1 receptor compounds have distinct modes of interaction with the D_1-like receptors. These conclusions suggest a variety of directions for future study.

Further mutagenesis studies

The data discussed in this Dissertation provides important information into D_1-like receptor structure-based function, but there is a plethora of future studies that would expand the work presented herein. Our data suggest that F6.51 and W6.48 are key components of the rotamer toggle switch in D_1-like dopamine receptor, coupling agonist binding to receptor activation. We propose that F6.51 serves as a "sensor" that interacts directly with the agonist. Agonist interaction with F6.51 may cause a change in the rotamer configuration of this residue that, in turn, alters the rotamer conformation of W6.48, leading to receptor activation. More detailed knowledge of the residues in, and around, the rotamer toggle switch will reveal important information concerning about the conformational changes that give rise to receptor activation.

The studies in Chapter 5 suggested that W6.48 plays a key role in the transduction of agonist binding to receptor activation in the D_1-like receptors. Shi *et al.* (2002) propose that agonist binding at the β_2-adrenergic receptor causes a change of W6.48 from its inactive, *g*+ ,conformation to an active conformation, *t*. To explore this hypothesis in the D_1-like receptors, W6.48 could be mutated to threonine, a residue essentially restricted to the *g*+ conformation. A Trp to Thr mutation should result in a largely inactive receptor. Additionally, mutation of F6.51 to threonine could provide further evidence that F6.51 influences the rotamer configuration of W6.48. A F6.51T mutant receptor, favoring the *g*+ rotamer, should force W6.48 into the *g*+ rotamer, promoting an inactive receptor state. Studies have also suggested that C6.47 influences the rotamer configuration of W6.48. In the inactive state of bovine rhodopsin, C6.47 appears to from an H-bond interaction with W6.48 (Palczewski *et al.*, 2000). Studies of the β_2-adrenergic receptor indicate that C6.47 is strongly correlated with the rotamer of W6.48 (Bhattacharya *et al.*, 2008a).

Additionally, studies of the histamine H_1 receptor and β_2-adrenergic receptor suggest that the residue at position 7.45 interacts with W6.48, and possibly C6.47. In histamine H_1 receptor, N7.45 restrains C6.47 and W6.48 in rotamer configurations, *t* and *g*+ respectively, that favor an inactive receptor state (Jongejan and Leurs, 2005). There is evidence that suggests N7.45 may hydrogen bond with C6.47 in β_2-adrenergic receptor (Bhattacharya *et al.*, 2008a). These studies indicate N7.45 could be an important link between ligand-binding and GPCR activation. Mutation to cysteine, glutamine, or leucine would provide insight into the role of this residue in the D_1-like receptors.

In rhodopsin, W6.48 is bounded above by Y6.51 and below by F6.44 (Palczewski *et al.*, 2000; Palczewski, 2006). Similarly, in the α_{1B}- and β_2-adrenergic receptors W6.48 is bounded by F6.52 and F6.51 above and F6.44 below (Chen *et al.*, 2002b; Chen *et al.*, 2002a; Han *et al.*, 1996). If this is true for the D_1-like receptors, F6.44 may influence the conformational freedom of W6.48 because the aromaticity of F6.44 stabilized the inactive state conformation of TM6 (Chen *et al.*, 2002b; Han *et al.*, 1996). Mutation of F6.44 to Leu, which should promote an active receptor conformation, may provide insight into the role of this residue in the D_1-like receptors.

The experiments in Chapter 3 provided evidence that T3.37 contributes to ligand-binding and activation by influencing receptor interaction with the *para*-OH of D_1 compounds. The nature of this interaction is unclear. T3.37 may interact directly with the *para*-OH of D_1 agonists or could hydrogen bond with the backbone carbonyl of S5.46. A reciprocal double mutant of S5.46 and T3.37 to threonine and serine respectively may help determine whether T3.37 and S5.46 H-bond. Substitution of the polar T3.37 with glutamine (more flexible and also polar side chain) and serine (shorter and less polar)

may provide additional insight into the role of T3.37. Bhattacharya *et al.* (2008b) suggest that T3.37 H-bonds with T4.56 and S5.46 in the inactive receptor state, forming a network of H-bonds involving TMs 3, 4, and 5. The role of T4.56 in the D_1-like receptors represents another intriguing direction to pursue. Additionally, our D_1 model suggests that T3.37 may hydrogen bond with S5.42, not S5.46. Future studies could explore this possibility by constructing a double mutant receptor (i.e. T3.37A/S5.42A).

Determination of multiple D_1-like signaling pathways

As was previously mentioned, several studies have indicated D_1-like receptors may be capable of activating other signaling pathways. However, the mechanism(s) by which D_1 receptors activate these pathways is unclear and requires additional study. For example, studies indicate that D_1 might be coupled to the activation of PLC (Friedman *et al.*, 1997; Undie *et al.*, 1994) but there is some uncertainty as to whether the effect was due to off-target effects, and the mechanism is poorly understood. Several studies have revealed the ability of the D_1 dopamine receptor to mediate the phosphorylation of each MAP kinase subfamily (Brami-Cherrier *et al.*, 2002; Gerfen *et al.*, 2002) but the mechanism by which D_1 receptors activate ERK1/2 has yet to be determined. More detailed knowledge of D_1-mediated signaling pathways will be useful for future studies relevant to the work in this Dissertation. Studies using receptor mutagenesis and structurally dissimilar agonists could be used to identify a structural basis for the selective activation of effector pathways.

D_1 receptor functional selectivity

Studies in our lab, using the endpoints of adenylate cyclase and long-term trafficking, have demonstrated functional selectivity at the D_1 dopamine receptor. The

mechanistic basis of this finding is unclear, but we hypothesize that structural differences in binding modes of the agonists are largely responsible. Future studies that examine whether mutation of V159, L291, L295, and W163 affects the long-term trafficking of A77636 or DNS will test our hypothesis. The data in this Dissertation demonstrate that structurally different- and even structurally similar- D_1 agonists interact with the receptor in a unique way. There is evidence that suggests the distinct conformational states induced by an agonist may have functional consequences (Swaminath *et al.*, 2004; Swaminath *et al.*, 2005; Yao *et al.*, 2006). This underscores the importance of identifying and characterizing additional D_1-like receptor signaling pathways that can be used to explore a structural basis for functional selectivity.

Long-term trafficking of the D_1 receptor

Chapter 6 provides the foundation for future studies to examine the mechanism of long-term receptor trafficking. These studies will test our hypothesis that ligand-receptor interactions distal to the binding pocket are responsible for the prolonged interaction of A77636 with the D_1 receptor, leading to differences in long-term receptor trafficking. However, it is important to consider alternative hypotheses. A recent study of the β_1-adrenergic receptor demonstrated that the pathway selected for receptor internalization is primarily dictated by the kinase that phosphorylates the receptor (Rapacciuolo *et al.*, 2003). The authors found that PKA-mediated phosphorylation directs internalization via the caveolae pathway, whereas GRK-mediated phosphorylation is through clathrin-coated pits. Studies indicate a role for both PKA- and GRK-mediated phosphorylation in D_1 receptor desensitization (Bates *et al.*, 1991; Black *et al.*, 1994; Tiberi *et al.*, 1996; Zhou and Fishman, 1991; Zhou and Fishman, 1991). Agonists could stabilize a

conformational state, or activate a specific G protein heterotrimer, that favors one form of receptor phosphorylation over another thus leading to differences in receptor trafficking.

It is clear that β-arrestin plays an essential role in the desensitization and endocytosis of GPCRs, however, recent studies have shown that β-arrestin can also function as a signaling intermediate in GPCR signal transduction to MAP kinase pathways independent of G proteins (Lefkowitz and Shenoy, 2005; Shenoy and Lefkowitz, 2003). An interesting future direction is to explore whether the D_1 receptor activates MAP-kinase signaling pathways via β-arrestin scaffolding. It is intriguing to hypothesize that upon endocytosis A77636-occupied D_1 receptors might activate MAP-kinase pathways.

APPENDIX:
SUMMARY OF BIOLOGICAL METHODS USED IN THIS WORK.

CELL CULTURE AND TRANSFECTION

HEK293 cells were grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. For transient expression, HEK293 cells were transfected according to the Lipofectamine protocol (GibcoBRL -Life Technologies) using 0.5 μg (hD_1) or 0.75 μg (hD_5) pcDNA3.1 constructs per 100mm culture dish containing HEK cells at approximately 80% confluency. The transfected cells were incubated with DNA/liposomes in serum free media for ~6 hours at which time Fetal Bovine Serum was added to 20%. The cells were incubated for an additional 12 hours followed by aspiration of the transfection media and addition of fresh DMEM-H containing 10% FBS. Cells were harvested 24-36 hours later for use in binding and functional assays.

MEMBRANE PREPARATION

Plates of HEK293 cells transfected with D1-like receptor DNA were harvested ~48 hours post transfection. Each 100mm dish of HEK293 cells was washed 1X with 4 mL of ice cold PBS. Cells were then lysed at 4^oC for 10 minutes using 3 mL of hypo-osmotic buffer (10mM Hepes, pH 7.4 with KOH). Cells were scraped, transferred to a centrifuge tube and spun at 28,000 x g for 20 minutes. The pellet was transferred to a Wheaton Glass/Teflon homogenizer in 10mM Hepes buffer and homogenized 4-5 times. The homogenate was then spun at 28,000 x g for 20 minutes. This process was repeated for a total of 3 times. After the third spin membranes were homogenized in storage buffer (20mM Hepes, 250mM sucrose, pH 7.4 with KOH), aliquoted at 1 mL per

microcentrifuge tube and flash frozen in 100% ethanol-dry ice mixture. Membranes were stored at -80^{o}C until use in binding and functional assays.

GENERATION OF MUTANT RECEPTORS

The human D_1 and D_5 receptor were cloned from a human cDNA library. The primers were complementary to the 5' and 3' ends and also contained an EcoRI (5') and XhoI (3') site for ligation into the expression vector pcDNA3 containing a HA tag. PCR amplification was performed in a volume of 100 μL containing 0.16 ng DNA, 0.3 μM of each primer, 0.2 mM dNTPs, 10 μL 10x reaction buffer, and 1 μL of Pfu Turbo enzyme. The PCR construct was digested with EcoRI and XhoI, gel-purified and ligated into HA-pcDNA3. The sequences of the human D_1 and D_5 receptors were verified and large quantities were generated by growth in LB overnight. This cDNA was then used as the template for the generation of the mutants. The various mutants were generated using the Quickchange kit from Stratagene (La Jolla, CA). Primers were designed according to the kit's specifications and purchased from Invitrogen (Carlsbad, CA). PCR was performed according to the manufacturer's instructions. Following PCR, the mutated receptor cDNAs were transformed in XL-1 Blue cells (Stratagene, La Jolla, CA). The mutant receptors were sequenced to verify the particular mutation and then large cultures of the mutated receptors were grown in LB broth overnight. Larger quantities of mutant cDNA were purified using the Sigma GenElute Maxiprep Kit (Sigma).

SATURATION ASSAYS USING [^{3}H]SCH23390

Saturation binding experiments were performed to determine receptor expression level and KD of SCH23390 for wild-type and mutant receptors. Membrane homogenates

from HA-hD1 HEK cells were prepared as previously described (Lewis *et al.*, 1999). For saturation binding experiments, HEK293 cell membranes were incubated for 15 minutes at 37 °C with 8-10 concentrations of [^{3}H]SCH23390 (Wyrick and Mailman, 1985) in binding buffer (50mM HEPES, 4mM $MgCl_2$, pH 7.4 with KOH). Nonspecific binding was determined using 1 μM cold SCH23390. Protein concentrations were determined using the Bradford protein assay kit.

COMPETITION ASSAYS USING [^{3}H]SCH23390

Competition binding experiments were conducted to assess the affinity ($K_{0.5}$) of each test compound at wild-type and mutant receptors. HEK293 cell membranes expressing wild-type or mutant receptors were incubated with a single concentration of [^{3}H]SCH23390 and 7-12 concentrations of test ligand in buffer (50mM HEPES, 4 mM $MgCl_2$, 0.01% ascorbic acid, pH 7.4 with KOH). Total binding was defined as the amount of [^{3}H]SCH23390 bound in the absence of a competing ligand. Non-specific binding was determined by binding in the presence of 1uM cold SCH23390.

Saturation and competition binding experiments were performed in triplicate for each assay condition in 96 well plates. Reactions were terminated by filtration using a Packard 96 Filtermate Harvester (Packard BioScience Company; Meridian, Connecticut). The filter plates were allowed to dry, 35 μL of Packard MicroScint 20 scintillation cocktail was added to each well, and radioactivity was counted using a Packard TopCount NXT Microplate scintillation counter (Packard, Downers Grove, IL).

ASSESSMENT OF CELL SURFACE RECEPTOR EXPRESSION

HEK 293 cells were seeded at ca. 50,000 cells/ well in 24-well cell culture plates. 48 hr later, cells were fixed with 4% PFA (30 min), rinsed with HBSS, and treated with blocking solution (50mM Hepes, 10%FBS, DMEM-H) for 30 min. Cells were then incubated for 1 hr with an anti-HA primary antibody (BabCO HA.11) diluted at 1:1000 (in blocking solution). Cells were washed with HBSS and subsequently incubated for 2 hr with a [^{125}I]-rabbit-anti-mouse (New England Nuclear) secondary antibody diluted at 1:500 (in blocking solution). Cells were washed with HBSS, solubilized overnight with 1M NaOH, transferred to 12x75 glass tubes and counted.

ANALYSIS OF BINDING AND FUNCTIONAL DATA

Saturation binding data were analyzed by nonlinear regression using a one-site binding rectangular hyperbola model using Prism Ver. 4 (GraphPad, Inc, San Diego CA.). Competition binding data and cAMP accumulation data were analyzed by nonlinear regression using either a variable slope or fixed slope (n_H=1) dose-response fit using Prism for the best fit of points. Functional data were expressed relative to the percentage of the stimulation produced by 1 mM dopamine at each receptor type unless otherwise noted. Affinity data were fit first to a sigmoidal model of variable slope. An ANOVA with a post-hoc Tukey test was used to compare means for all internalization and recovery experiments. Significance was set at $p<0.05$. All statistical testing was done using Prism 4.0.

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