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Delineating the Molecular Basis of Subtype-specific Ligand Binding, G Protein Coupling and Signalling Properties of D1 and D5 Dopaminergic Receptors

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Delineating the Molecular Basis of Subtype-Specific Ligand Binding, G Protein Coupling and Signaling Properties of D1 and D5 Dopaminergic Receptors

by

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Abstract

Dopamine mediates its effects through the interaction with five distinct receptors that make up the D1-like (D1 and D5) and D2-like (D2, D3, and D4) families. Dopamine receptors are members of the heptahelical G protein-coupled receptor (GPCR) family. D1-like and D2-like receptors couple to the activation and inhibition of adenylyl cyclase, respectively. Dysfunction in dopaminergic signaling has been shown to contribute, among others to the etiology of Parkinson's disease, schizophrenia, and hypertension. The high degree of structural identity between D1 and D5 receptors has hampered the development of subtype-selective drugs. Despite the structural similarities, D1 and D5 receptor subtypes exhibit distinct ligand binding and G protein coupling properties. The objective of this thesis is to delineate the structural determinants involved in the distinct ligand binding and G protein coupling properties of D1 and D5 receptors Using chimeric and mutagenesis studies I demonstrate that differences in the primary sequence within the terminal receptor locus (a region encompassing TM6, third extracellular loop (EL3), TM7, and the cytoplasmic tail) are responsible for the functional differences of D1 and D5 receptors. I describe the EL3 domain as a key determinant in the binding of antipsychotic drugs (inverse agonists) and the agonist-mediated maximal activation of adenylyl cyclase. This study highlights a novel domain (EL3) regulating binding of inverse agonists at GPCRs. Furthermore, I describe a molecular interplay between TM6 and EL3 which mediates the subtype-specific phenotypes and activation of D1 and D5 receptors. In addition, I demonstrate that in spite of structural and functional

similarities, D1-like receptors undergo a different regulatory pathway upon agonist stimulation. Specifically I demonstrate that the D5 receptor can undergo phosphorylation-independent desensitization and endocytosis. Overall, the work described in this thesis provides insight into the molecular basis of D1-like receptor signaling.

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List of Abbreviations

AC	Adenylyl Cyclase
AMP	3',5'-Adenosine Monophosphate
ANOVA	Analysis of Variance
B_{MAX}	Maximal Binding Capacity
BSA	Bovine Serum Albumin
BUT	(+)-Butaclamol
CA	[^{3}H]cAMP Accumulated
CAM	Constitutively Active Mutant
cAMP	Cyclic AMP
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
CT	Cytoplasmic Tail
DA	Dopamine
DNA	Deoxyribonucleic Acid
DOPA	Dihydroxyphenylalanine
EC_{50}	Half-maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
EL	Extracellular Loop
FBS	Fetal Bovine Serum
FLU	Z-Flupentixol
FSK	Forskolin
GABA	γ-aminobutyric Acid

G_i	Inhibitory G Protein
G_S	Stimulatory G Protein
G protein	GTP-binding Protein
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GPCR	G Protein Coupled Receptor
GRK	G Protein Coupled Receptor Kinase
GTP	Guanine Triphosphate
HBS	HEPES-buffered Saline
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	1-methyl-3-isobutylxanthine
IL	Intracellular Loop
K_D	Equilibrium Dissociation Constant
K_I	Equilibrium Inhibition Constant
MEM	Minimal Essential Medium
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide-Sensitive Factor
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography

PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phopholipase C
PMSF	Phenylmethylsulfonyl Fluoride
RGS	Regulator of G Protein Signaling
RIPA	Radioimmunoprecipitation Assay
SCH	SCH23390
SCH23390	R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5- tetrahydro-1H-3-benzazepine
SDS	Sodium Dodecyl Sulphate
SNX	Sorting Nexin
TM	Transmembrane
TRL	Terminal Receptor Locus
TU	Total Uptake

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BACKGROUND

1. G Protein-Coupled Receptors (GPCRs)

Cells exist in a complex environment comprised of a multitude of signals generated by physiological stimuli and processes. Some of these physiological inputs include sensory and chemical stimuli such as odor, taste, light, hormones, and neurotransmitters (Strader et al., 1994; Hamm, 1998; Lefkowitz, 2000; Ferguson, 2001). Dysfunction in response to these signals results in development of diseases (Marinissen and Gutkind, 2001). Signaling molecules which exert action in response to these stimuli are targets for >60% of currently available drugs (Leurs et al., 1998; Marinissen and Gutkind, 2001). Targets of these drugs are members of a superfamily of receptors that facilitate the transduction of these complex signals across the cellular membrane into second messengers that are interpreted as meaningful signals by the cell (Ferguson, 2001; Marinissen and Gutkind, 2001). One class of receptor capable of making sense of this complex cellular environment are better known as heptahelical G protein-coupled receptors (GPCRs) (Vaughan, 1998; Lefkowitz, 2000).

The early 1980s gave rise to a new area of scientific studies with the cloning of bovine rhodopsin, a GPCR capable of sensing light (Nathans and Hogness, 1983; Ji et al., 1998; Vaughan, 1998). This was followed shortly by the cloning of the hormone sensing β-adrenergic receptor (Dixon et al., 1986; Ji et al., 1998; Vaughan, 1998). Since then more than 1000 proteins with a heptahelical structure have been reported and these proteins comprise more than 1% of the human genome (Ji et al., 1998; Vaughan, 1998; Marinissen and Gutkind, 2001; Venter et al., 2001). All GPCRs exhibit a common heptahelical topography and

significant structural homology, reflecting their common mechanism of action (Strader et al., 1994; Lefkowitz, 2000). The general structure of all GPCRs is an extracellular N-terminus, seven transmembrane (TM) domains, three extracellular (EL) and three intracellular (IL) loops, an intracellular C-terminus (CT), and a fourth IL formed when a cysteine in the CT is palmitoylated (Ji et al., 1998). Among the GPCR family, the N- and C-terminal regions along with the EL and IL regions are very divergent among GPCRs. In contrast, the TM regions which are composed of 20-27 amino acids are highly conserved. The TM domains have an α-helical structure and form a seven α-helical TM core believed to be involved in ligand binding (Dohlman et al., 1991; Gether and Kobilka, 1998; Ji et al., 1998). A high resolution crystal structure of a rhodopsin receptor has provided evidence indicating that the fourth IL forms an eighth helix, which runs perpendicular to the plasma membrane (Palczewski et al., 2000). The N-terminus and EL domains have also been implicated in binding ligands (Ho et al., 1999; Ulfers et al., 2002; Wheatley et al., 2003). In contrast, the domains IL2, IL3, and CT have been shown to be critical for interaction with heterotrimeric guanine nucleotide-binding proteins (G proteins) (Wess, 1997; Gether and Kobilka, 1998). GPCRs owe their name to their interaction with these heterotrimeric G proteins.

2. G Proteins: From Structure to Function

Heterotrimeric G proteins are signal transducers that translate ligand binding to GPCRs into an intracellular response, by linking GPCRs to effectors (Hamm, 1998). G proteins consist of three subunits, α, β, and γ (Simon et al.,

1991; Neer, 1995). Currently 16 α, 5 β, and 14 γ subunit genes have been identified (Neves et al., 2002; Preininger and Hamm, 2004). G protein classes are defined by the primary sequence of their α subunits. There are four distinct classes of G proteins, G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ which transduce a large number of extracellular signals. Before any of the four classes of G proteins can exert their intracellular actions, the G protein must first be activated by a physical interaction with a ligand bound (activated) GPCR. G proteins exist in two states, a guanine diphosphate (GDP) bound inactive state and a guanine triphosphate (GTP) bound active state (Hamm, 1998; Preininger and Hamm, 2004).

Activated GPCRs act as guanine nucleotide exchange factors (GEFs) for their coupled G proteins (Preininger and Hamm, 2004). The G_α subunit bound to an activated GPCR undergoes a conformational change which releases a GDP, the rate limiting step in G protein activation. The concentration of intracellular GTP is much higher than that of GDP, thus the release of GDP leaves the G_α subunit in a transient high-affinity empty state that quickly binds GTP (Neer, 1995; Preininger and Hamm, 2004). GTP-bound G_α subunit dissociates from the $G_{\beta\gamma}$ dimer, and both subunits can now interact with multiple downstream effectors.

The $G_{\alpha s}$ pathway was first to be described and helped to elucidate concepts such as second messengers, protein phosphorylation and signal transduction (Neves et al., 2002). The $G_{\alpha s}$ pathway is coupled to the activation of the effector adenylyl cyclase (AC) which is responsible for generating intracellular cAMP. In contrast the $G_{\alpha i}$ pathway is coupled to the inhibition of adenylyl cyclase. The $G_{\alpha q}$ pathway leads to the stimulation of PLC-β which

produces intracellular messengers, inositol triphosphate and diacylglycerol. Reports suggest that $G_{\alpha 12}$ can stimulate phospholipase D, c-SRC, and PKC, but the mechanisms of these pathways remain unidentified. $G_{\alpha 13}$ has been reported to activate Rho and regulate the activity of the Na^+-H^+ exchanger (Hamm, 1998; Neves et al., 2002).

Upon dissociation of G_α-GTP from the $G_{\beta\gamma}$ dimer, free $G_{\beta\gamma}$ dimer elicits effects on numerous proteins. $G_{\beta\gamma}$ mediates effects on some second messenger enzymes; it is a positive modulator of $G_{\alpha s}$-activated ACII, ACIV, and ACVII but a negative modulator of ACI (Hamm, 1998). $G_{\beta\gamma}$ directly activates G protein-responsive K^+, Ca^{2+}, and Na^+ channels and has been reported to activate the MAP kinase pathway. Moreover, $G_{\beta\gamma}$ acts as a guanine nucleotide dissociation inhibitor (GDI) which inhibits GDP release from G_α and by this means inhibits signaling (Preininger and Hamm, 2004).

Termination of signaling by G_α-GTP involves the hydrolysis of GTP to GDP. Upon GTP cleavage, the G_α and $G_{\beta\gamma}$ subunits can re-associate and become inactive (Simon et al., 1991; Neer, 1995). The G_α subunits have an intrinsic GTPase activity but hydrolysis rates vary widely among G proteins. Regulator of G protein signaling (RGS) proteins act as GTPase activating proteins (GAPs) capable of promoting GTP hydrolysis and inactivating the signaling pathway (Preininger and Hamm, 2004). Inactivation results in the reconstitution of the heterotrimeric $\alpha\beta\gamma$ subunit, which can then again undergo the signaling process mediated by the GEF activity of activated GPCRs.

3. GPCR Activation and Regulation

GPCR activation is a complex process, which still remains to be fully unraveled. Nevertheless, the last few years have yielded an incredible amount of information about this process. At the core of GPCR activation is a change in conformation resulting from the binding of extracellular ligands. The conformational change of a GPCR is the initiation step that leads to coupling to and activation of G proteins which in turn elicits an intracellular response. Upon signaling the receptor is inactivated by a series of steps involving receptor phosphorylation, desensitization, and endocytosis. In the following section I will highlight the recent breakthroughs in understanding these processes and discuss the many proteins involved.

3.1 Model of GPCR Activation

The first model addressing receptor function was proposed by Clark in 1937 and describes the interaction between ligand (L) and receptor (R) through the formation of a ligand-receptor complex (LR) (Weiss et al., 1996). With the observation that receptors can bind and activate G proteins it became evident that a more complex model of receptor function was needed. De Lean et al. (1980) proposed the ternary complex model which described the receptor as containing two binding sites, one for ligand and one for the G protein (G) [L + R $\leftrightarrow$ LR + G $\leftrightarrow$ LRG] (De Lean et al., 1980; Costa et al., 1992).

Further experimental observations suggested that the model of GPCR activation should be based on the concept that activation of GPCRs depends on a conformational change. The idea that activation involves a receptor conformational change stems from studies showing that binding of acetylcholine to nicotinic acetylcholine receptors results in a conformational change observed as channel opening (Del Castillo and Katz, 1957). These observations gave rise to the two-state model which describes the existence of two receptor states in equilibrium. The two-state model describes an inactive R state and an active R* state existing in equilibrium [R $\leftrightarrow$ R*]. Interaction with a ligand displaces the equilibrium toward one state or the other (Birnbaumer et al., 1980; Leff, 1995). A ligand that has higher affinity for the R* state is defined as an agonist while a ligand with higher affinity for the R state is defined as an inverse agonist (Kenakin, 1995a). Ligands that do not shift the equilibrium and have equal preference for R and R* states are said to be antagonists (Gether and Kobilka, 1998).

Identification of constitutively active mutant (CAM) receptors that display varying degrees of receptor activation in the absence of agonist has given rise to the extended ternary complex model (Kjelsberg et al., 1992; Samama et al., 1993; Kenakin, 1995b). The extended ternary complex model predicts the spontaneous formation of an active R* state receptor in the absence of agonist whereby R* can then interact with and activate a G protein. The extended ternary complex model describes constitutively active receptors as those with a rightward equilibrium shift, resulting from spontaneous complex formation between receptor and G

protein in the absence of an agonist (Lefkowitz et al., 1993; Samama et al., 1993). Experimental observations have demonstrated that both receptors in an inactive R state and active R* state are able to bind G proteins. These observations have given rise to the cubic ternary complex model which accounts for the receptor having two distinct binding sites (ligand binding and G protein binding), receptors existing in two states, and considering all possible two-way and three-way interactions between receptor, ligand, and G protein (Weiss et al., 1996).

Recently it has become evident that even a cubic ternary complex model may not be sufficient to describe the complexity of GPCR activation. Several groups have demonstrated that a receptor can and undergo a transition to multiple active conformations, such as R′ or R″, which are described as intermediates between the inactive R state and active R* state (Swaminath et al., 2004). These studies demonstrate that intermediate receptor states can activate specific signaling pathways. Indeed, it was demonstrated that although inverse agonist act in opposition to agonists by suppressing constitutive activity and shifting the equilibrium towards the inactive R state, inverse agonists could also initiate unique signal transduction cascades (Azzi et al., 2003; Gbahou et al., 2003). Furthermore, a multiple active conformation model may explain how a partial agonist may stabilize the receptor in one of these intermediate states, thus increasing the chance of spontaneous transition to R* (Gether and Kobilka, 1998). In light of these observations and classification of ligands with varying degree of activity it is of even more significant therapeutic relevance to understand the specific binding determinants of ligands.

3.2 Ligand Binding Determinants

The basis of GPCR signaling rests in its ability to convey stimuli from the extracellular environment into an intracellular response. The challenge in the field of GPCRs has been in determining which domains are conserved among GPCRs and which domains represent GPCR specific functions. Many approaches have been used to compare and contrast the role of these conserved and divergent domains; some of these approaches include substitution mutants, deletion mutants, and chimeric (hybrid) receptors (Dohlman et al., 1991). Recently, Palczewski et al. published the first known high resolution crystal structure of a GPCR (rhodopsin) and it has allowed us to examine the implications and put in context prior mutagenesis studies (Palczewski et al., 2000; Huang et al., 2001; Menon et al., 2001).

Due to the diversity of GPCRs and their ligands (ranging from small molecules to large glycoproteins), the physical determinants required for binding ligands varies greatly (Strader et al., 1994). Although GPCRs share a general heptahelical structure, their extracellular and intracellular loops are very divergent. Nevertheless, there are some shared features within a GPCR family (i.e. Biogenic Amine Receptors) that allows us to draw conclusions from mutagenesis studies as to their ligand binding requirements. Chimeric receptor studies which replace a particular transmembrane domain occasionally affect the binding of either agonists, antagonists or inverse agonists, suggesting that these classes of ligands have distinct binding determinants (Marullo et al., 1990; Scheer

et al., 1996; Morin et al., 1998; Befort et al., 1999; Iwasiow et al., 1999; Tumova et al., 2003).

Numerous mutations throughout all seven TM domains have been shown to influence the binding of agonists, a testament to the diversity of ligands and GPCRs. However, a closer examination of all these mutagenesis studies reveals the conservation of domains crucial for the binding of agonists within a particular family of receptors. At the biogenic amine GPCRs (adrenergic, dopaminergic, muscarinic, serotonergic, histaminergic) TM domains 3, 5, and 6 play an important role in the binding of agonists. Residues conserved among these receptors form the transmembrane binding pocket. A conserved aspartic acid (Asp or D) at the extracellular half of the TM3 is believed to act as a counterion for the amine group of ligands (Dohlman et al., 1991; Strader et al., 1994; Shi and Javitch, 2002). The TM5 and TM6 domains are believed to dock and stabilize the catechol ring of ligands (Strader et al., 1994; Ji et al., 1998; Chen et al., 1999). However, not all of the same domains are crucial for the binding of antagonists. Although the Asp in TM3 still plays a crucial role in binding of antagonists, it is TM7 and to some extent TM6 that stabilize the binding of these ligands (Strader et al., 1994; Waugh et al., 2001; Shi and Javitch, 2002). This model may describe the binding affinity of ligands at its receptor but it does not address receptor selectivity of ligands (Huang et al., 2001; Shi and Javitch, 2002).

The selectivity of ligands such as acetylcholine for muscarinic receptors or opiates for opioid receptors may depend on the extracellular domains (Fernandez and Puett, 1996; Varga et al., 1996; Colson et al., 1998; Huang et al., 1999;

Spalding and Burstein, 2001). At opioid receptors the EL3 has been implicated as a signal initiation platform which guides ligands into the transmembrane binding pocket (Dietrich et al., 1998; Decaillot et al., 2003). The role of EL domains in biogenic amine GPCRs has not been well studied. Nevertheless, a role of EL3 in mediating the formation of β_2-adrenergic receptor active complex has been reported (Zhao et al., 1998).

3.3 GPCR Activation and G Protein Coupling

The transition between inactive R state and active R* state requires a release of intramolecular constraints resulting in conformational rearrangement of TM domains which leads to the exposure of cytoplasmic G protein binding determinants. The energy required to break the intramolecular constraints is achieved by the binding of agonists to its binding pocket (Karnik et al., 2003). Release of these constraints results in a physical movement of the cytoplasmic end of TM6 away from TM3 (Gether and Kobilka, 1998; Palczewski et al., 2000). The specific molecular determinants involved in the release of intramolecular constraints are not fully known and may be receptor specific. However, some features shared among many GPCRs have been identified as essential in mediating this process. One such feature is the DRY motif (Baldwin, 1993). The DRY motif is a stretch of three amino acids (aspartic acid = Asp = D; arginine = Arg = R; tyrosine = tyr = Y) located at the cytoplasmic end of TM3. Protonation of the Asp causes a shift of Arg out of a conserved polar transmembrane pocket (Scheer et al., 1996). A shift of Arg out of the polar transmembrane pocket results

in cytoplasmic exposure of IL2 and IL3 sequences (Gether and Kobilka, 1998; Rasmussen et al., 1999). Sequences within IL2, IL3 and CT have been shown to be determinants of G protein binding and specificity (Baldwin, 1993; Karnik et al., 2003).

3.4 Regulation of Activated GPCRs and Downstream Signaling Pathways

GPCR activation not only leads to signaling through G protein-dependent effector systems, but also is a molecular mechanism that initiates feedback regulation of GPCR coupling, endocytosis, and signaling via G protein-independent pathways (McDonald et al., 2000; Ferguson, 2001; Marinissen and Gutkind, 2001; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001; Tsao et al., 2001; Luttrell, 2002; Luttrell and Lefkowitz, 2002).

Intracellular regulation of GPCR coupling involves a process known as desensitization. Desensitization is described as a process responsible for termination or attenuation of receptor signaling in the face of acute and chronic stimulation (Lefkowitz, 1998; Ferguson, 2001). Receptors are desensitized by means of phosphorylation and binding of regulatory molecules, i.e. arrestins.

Phosphorylation of receptor intracellular determinants alters GPCR conformation and thus impedes G protein coupling, resulting in desensitization of signaling by extracellular stimuli (Lefkowitz, 1998). Desensitization is an important factor that limits the efficacy and duration of action of many therapeutics (Dohlman et al., 1991). GPCRs can undergo two types of

phosphorylation mechanisms leading to either heterologous (agonist-independent) or homologous (agonist-dependent) desensitization (Pitcher et al., 1998). Heterologous desensitization is a consequence of receptor phosphorylation by second messenger kinases, PKA and PKC. Increased activity of PKA is mediated by G_s-coupled receptors while PKC activity is elevated by G_q-coupled receptors. Nevertheless, receptor phosphorylation by PKA or PKC and in turn heterologous desensitization is the consequence of any stimulant that elevates cAMP (in the case of PKA) or diacylglycerol (in the case of PKC) and not necessarily in response to agonist stimulation. In contrast, homologous desensitization is mediated by GPCR kinases (GRKs) in response to agonist stimulation and an agonist-induced conformational change of the receptor (Ferguson, 2001). There are seven members of the GRKs family, all of which share a common structure and function. GRKs have an amino-terminal domain responsible for binding GPCRs, regulator of G-protein signaling (RGS) box, a central catalytic domain and a variable C-terminal region responsible for membrane targeting (Premont et al., 1995). The RGS box has been proposed as a potential mechanism by which GRKs may regulate GPCR signaling in a phosphorylation-independent manner (Willets et al., 2003). GPCR phosphorylation by GRKs increases the binding affinity of arrestin for GPCRs by 10-30 fold. Binding of arrestin leads to steric interference of G protein coupling and results in an attenuation of G protein-dependent GPCR signaling. Moreover, binding of arrestin targets GPCRs for endocytosis and thus terminates G protein dependent signaling (Ferguson, 2001; Pao and Benovic, 2002). An emerging concept of GPCR regulation is

phosphorylation-independent desensitization (Pao and Benovic, 2002). Catalytically inactive GRKs have been shown to bind GPCRs, serving as arrestin binding determinants and resulting in desensitization. Evidence from several GPCR models suggests that arrestins can bind agonist-activated but non-phosphorylated GPCRs and uncouple them from G protein (Gurevich et al., 1995).

Arrestins not only serve to sterically inhibit coupling with G proteins but also serve both as adaptors and scaffolds, targeting GPCRs for endocytosis and mediating G protein-independent signaling (Lefkowitz, 1998; Pierce and Lefkowitz, 2001). Arrestin functions as an adaptor in clathrin-coated vesicle mediated endocytosis of GPCRs. Arrestin targets the receptors to clathrin-coated pits through an interaction with clathrin and adaptor protein 2 (AP-2) (Goodman et al., 1997; Laporte et al., 1999; Laporte et al., 2000). Clathrin-coated pits containing the GPCR-arrestin complex are pinched off from the plasma membrane by a GTPase protein known as dynamin. In contrast, some GPCRs have been demonstrated not to undergo endocytosis through clathrin-coated pits. These GPCRs are internalized in a caveolin-dependent pathway. Nevertheless, experimental data suggests that the predominant pathway proceeds via clathrin-coated pits (Ferguson, 2001).

Besides termination of G protein signaling, endocytosis leads to GPCR resensitization or downregulation and to intracellular signaling through the MAP kinase pathway (Ferguson, 2001; Claing et al., 2002). Receptor resensitization is an important short-term (minutes) process leading to the recycling of GPCRs to

the plasma membrane where they can participate in further signaling (Dohlman et al., 1991; Luttrell and Lefkowitz, 2002). Endosomes have a low pH environment and are enriched in membrane-associated phosphatases. Within these endosomes, some GPCRs have been shown to be dephosphorylated and returned to the plasma membrane in their native, inactive state (Lefkowitz, 1998; Ferguson, 2001). This resensitization process prevents irreversible GPCR desensitization which would leave a cell unable to respond to external stimuli. In contrast, receptor downregulation is a long-term (hours) event usually in response to chronic GPCR stimulation (Tsao et al., 2001). Downregulation leads to a decrease in the number of plasma membrane GPCRs and thus an attenuation of the signal (Dohlman et al., 1991; Lefkowitz, 1998). Receptors that proceed by this pathway are either retained within the endosomes or are targeted to lysosomes for degradation (Ferguson, 2001).

Another aspect of GPCR endocytosis is the ability to mediate G protein-independent intracellular signaling. Numerous GPCRs have been shown to be able to activate the MAP kinase pathway (Daaka et al., 1998; Hall and Lefkowitz, 2002). GPCRs regulate the MAP kinase pathway through a cross-talk with classical receptor tyrosine kinases (e.g epidermal growth factor receptors), PKA and PKC as well as independently via the formation of receptor/arrestin complexes (Luttrell, 2003). Arrestin dominant negative mutants block receptor endocytosis and activation of MAP kinase (Lefkowitz, 1998). Arrestin acts as a scaffold protein which interacts with the GPCR and components (Erks, Jnks) of the MAP kinase pathway (McDonald et al., 2000). As a scaffolding protein,

arrestin brings into proximity sequentially acting kinases. The consequence of MAP kinase activation is the ability of GPCRs to regulate mitogenic effects (Lefkowitz, 1998).

4. Constitutively Active GPCRs and Diseases

Defective regulation of GPCRs has been shown to be connected with numerous human diseases. At the core of most GPCR linked diseases are naturally occurring mutations/polymorphisms that result in either loss or gain of function (Spiegel and Weinstein, 2004). Mutations leading to loss of GPCR function can result from impaired ligand binding, diminished GPCR constitutive activity, or defective trafficking of receptors to the plasma membrane, either of which results in a defective signaling pathway (Green et al., 1993; Morello et al., 2000). In contrast, mutations leading to gain of function result in increased constitutive activity of GPCRs. Constitutively active GPCRs have been shown to be tumorigenic and cause syndromes of hyperfunction (Arvanitakis et al., 1998). GPCR-mediated signaling has been implicated in the regulation of cell proliferation and differentiation (Luttrell, 2002). Gain of function mutations that lead to a defective regulation of cell cycle implicate GPCRs as oncogenes (Dohlman et al., 1991). Furthermore, viruses encoding for constitutively active GPCRs have been shown to be disease causing. The constitutively active Kaposi sarcoma-associated herpes virus GPCR is linked with the development of Kaposi's sarcoma and primary effusion lymphoma (Seifert and Wenzel-Seifert, 2002). A defining feature of cells expressing constitutively active mutant (CAM) receptors is an increase in agonist-independent activity. Such GPCR activating

mutations lead to numerous examples of human disease. An activating mutation in the luteinizing hormone results in precocious puberty, mutations in rhodopsin lead to congenital night blindness or retinitis pigmentosa, and activating mutations of the TSH receptor cause hyperthyroidism (Robinson et al., 1992; Shenker et al., 1993; Rao et al., 1994; Van Sande et al., 1995; Spiegel, 1996). Since CAMs lead to a shift of equilibrium towards R* state in the absence of agonist, therapeutic treatment of the resulting diseases may be achieved by altering the equilibrium. Inverse agonists are described as shifting the activation equilibrium towards the inactive R state and thus could be useful for the treatment of disease caused by CAM GPCRs (Seifert and Wenzel-Seifert, 2002). Furthermore, in excess of 60 naturally occurring GPCRs exhibiting constitutive activity have been described that allow for a tonic level of stimulation in vivo (Seifert and Wenzel-Seifert, 2002; Teitler et al., 2002). One such receptor is a member of the dopamine D1-like receptor family. The dopamine D5 receptor has been shown to exhibit constitutive activity, a feature that distinguishes it from the cognate D1 subtype (Tiberi and Caron, 1994).

5. Dopaminergic System

Dopamine is a simple chemical derived from the amino acid tyrosine. The biosynthesis of dopamine is a two step process, first tyrosine is converted to dihydroxyphenylalanine (DOPA) by the rate limiting enzyme tyrosine hydroxylase and secondly DOPA is decarboxylated by aromatic amino acid decarboxylase. Dopamine belongs to a family of compounds known as

catecholamines. Dopamine can be enzymatically converted to the catecholamines norepinephrine and epinephrine. The defining features of catecholamines are a catechol group (benzene ring with two adjacent hydroxyl groups) and an amine group. These defining features play a key role in the binding determinants at their cognate receptors (Dohlman et al., 1991; Ji et al., 1998).

In the 1970's dopamine was shown to exert its effects by acting on dopamine receptors and stimulating adenylyl cyclase (Kebabian and Greengard, 1971). Several years later the existence of two discrete dopamine receptor subtypes was proposed (Spano et al., 1978). Pharmacological and biochemical studies demonstrated that one dopamine receptor subtype, termed D1, was positively coupled to AC. The other dopamine receptor subtype, termed D2, was not coupled to AC (Kebabian and Calne, 1979). Subsequent studies demonstrated that in fact the D2 receptor is coupled to the inhibition of AC (De Camilli et al., 1979; Cote et al., 1982). At the same time physiological studies demonstrated the presence of dopamine in periphery systems and established the presence of peripheral dopamine receptors, termed DA1 and DA2 (Goldberg et al., 1978). The peripheral dopamine receptors were later shown to be identical to the D1 and D2 receptor subtypes found in the CNS. Growth in the areas of molecular biology and the development of gene cloning techniques resulted in the cloning of 5 distinct mammalian dopamine receptors. The gene for the D2 receptor was first to be cloned, followed shortly by the cloning of the D1 gene (Bunzow et al., 1988; Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). Low stringent hybridization using the D2 receptor as a probe identified

two related receptors, D3 and D4 which were shown to couple to the inhibition of AC (Giros et al., 1990; Van Tol et al., 1991). The D2, D3, and D4 receptors on the basis of their structural similarity and ability to inhibit AC where classified in the family of D2-like receptors. Similarly, low stringency hybridization using the D1 receptor as a probe identified the D5 (or D1B) receptor (Sunahara et al., 1991; Tiberi et al., 1991). Both D1 and D5 receptors were demonstrated to couple to the stimulation of AC and where classified as subtypes of the D1-like receptors.

Dopamine exerts its effects via D1-like and D2-like receptors in both the central nervous system (CNS) and the periphery. Dysfunction of dopaminergic system has been reported to play a key role in the etiology of disorders such as Parkinson's disease, schizophrenia, depression, Tourette syndrome, hyperprolactinemia, hyperthyroidism and hypertension (Jarvie and Caron, 1993; Missale et al., 1998).

In the CNS, dopamine acts as a neurotransmitter where it mediates a myriad of functions including locomotion, cognition, emotion, memory, and endocrine regulation. The dopaminergic system comprises three neuronal pathways: nigrostriatal, mesocorticolimbic, and tuberoinfundibular (Huang et al., 2001). The nigrostriatal pathway encompasses the substantia nigra neurons which synthesize dopamine and project to the striatum, where one of their actions is the control of locomotor activity. Degeneration of this pathway results in the etiology of Parkinson's disease. Symptoms of Parkinson's disease are described as slowness of movement, rigidity, and tremors. Dopamine receptor agonists are used as a treatment to alleviate some of the symptoms of Parkinson's disease.

Specifically, L-DOPA a precursor of dopamine which can cross the blood brain barrier has been used as a therapeutic drug of choice. However, long-term treatment with agonists leads to diminished responsiveness and in some cases psychoses. The mesocorticolimbic pathway involves the ventral tegmental area which projects to the limbic area (nucleus accumbens, amygdala, and septum). Through this pathway dopamine mediates actions on emotion and motivation. Dysfunction of this pathway gives rise to psychoses and is implicated in the etiology of schizophrenia. Antipsychotic drugs used in the treatment of schizophrenia exhibit inverse agonist properties at dopamine receptors (Levinson, 1991; Cai et al., 1999; Emsley et al., 2000; Ljubin et al., 2000; Martin et al., 2001; Seeman, 2002). Antipsychotic drugs can bind all 5 subtypes of dopamine receptors, albeit they bind D2-like receptors with higher affinity. Blockade of dopamine receptors by antipsychotic drugs can result in effects similar to those resulting from dopamine depletion in Parkinson's disease. Finally, the tuberoinfundibular pathway projects from the hypothalamus to the pituitary where it mediates endocrine function. Dysfunction of this pathway can lead to hyperprolactinemia (Missale et al., 1998; Emilien et al., 1999; Huang et al., 2001).

Within the periphery dopamine elicits effects on vascular tone, hormone secretion, gastrointestinal motility, and renal function. In the circulatory system, dopamine mediates vasodilation of blood vessels and decrease of cardiac contractility. Dopamine is produced locally in the kidney where it increases sodium and water excretion. Dopamine produces renal vasodilation, diuresis and

natriuresis. Dysfunction of the renal dopaminergic system has been shown to give rise to hypertension. Agonists of dopamine receptors are used clinically in certain pathological conditions such as heart failure (Missale et al., 1998).

6. Dopaminergic Receptor Subtypes

Analysis of the primary sequence of dopamine receptors reveals that they exhibit the structural features of members of the GPCR family (Huang et al., 2001). Sequence similarity reveals dopamine receptors are members of the rhodopsin-like GPCRs. Biochemical studies and structure analysis suggests two dopamine families, D1-like and D2-like which couple to the stimulation and inhibition of AC, respectively. The D1-like receptors are comprised of the D1 and D5 subtypes. The D2-like receptors include the D2, D3, and D4 subtypes. In comparison to D1-like receptors, D2-like receptors have a long third intracellular loop and a short C-terminus tail (Missale et al., 1998). D1-like and D2-like receptors bind the natural agonist dopamine with varying affinities. The structural differences among D1- and D2-like receptors has allowed for the development of family specific ligands. The antagonist SCH23390 has a much higher affinity for D1-like receptors while the antagonist spiperone has a significantly higher affinity for D2-like receptors (Huang et al., 2001).

6.1 Dopamine D1-like Receptors

In verterbrates the D1-like family is comprised of dopamine D1A, D1B, D1C, and D1D receptors. However, in mammals only two of the four receptors,

D1A and D1B, have been identified. The nomenclature used to describe the human D1A and D1B receptors is D1 and D5, respectively (Callier et al., 2003). These D1 and D5 receptors are encoded by two separate genes which do not contain introns (Civelli et al., 1993). The D1 receptor consists of 446 amino acids and the D5 receptor consists of 477 amino acids. Both receptors share a common topology with 82% amino acid identity within the transmembrane domains (Jarvie and Caron, 1993). Despite the similarities in their primary structure and molecular biology, D1 and D5 receptors exhibit different localization and functional properties. In comparison to D1 receptors, the D5 receptor exhibits higher agonist binding affinity and lower inverse agonist binding affinity. The D5 receptor also exhibits a higher constitutive activity and lower agonist-mediated maximal AC activity. The highly conserved structural backbone of the D1 and D5 receptors and their distinguishing pharmacological and signaling properties make the D1-like receptors an excellent model for studying the structural features responsible for functional properties of GPCRs.

The use of the D1-like specific antagonist SCH23390 has been used to map the areas of protein expression within the CNS and periphery. D1-like receptors have been observed in the forebrain areas including caudate-putamen and nucleus accumbens, also in structures of the basal ganglia pathway including the subthalamic nucleus and substantia nigra. Moreover, D1-like expression was observed in the limbic areas including the hippocampus and amygdala (Missale et al., 1998). However, lack of ligands that can discriminate between the two D1-like subtypes (D1 and D5) has made it necessary to employ other tactics in

identifying subtype specific expression patterns. Immunocytochemistry, in situ hybridization and northern blot techniques have been used to determine localization of D1-like subtypes; unfortunately the detection limit of these techniques is insufficient in identifying areas of low expression.

Although not without limitations, both D1 and D5 knockout mice have been engineered to help address receptor function in areas of overlapped expression. An inherent limitation of knockout mice is the effect of their genetic background. Transgenic mice or varying genetic backgrounds have been observed to display differing phenotypes (McNamara et al., 2003). Another limitation arises from developmental absence of a protein leading to compensatory processes (Gingrich and Hen, 2000). Moreover, methodological factors have been observed to result in inconsistent findings between different investigative groups (Waddington et al., 2001).

6.1.1 Dopamine D1 Receptor Subtype

The human D1 gene is encoded by a single exon located at position 5q35.1 of chromosome five. Distribution of D1 receptors in the rat brain has been assessed using radioligand binding, antibodies and mRNA in situ hybridization. Within the CNS, D1 expression has been reported in several specific areas including the striatum, nucleus accumbens, olfactory tubercle, substantia nigra, hypothalamus, thalamus, and cortex (Fremeau et al., 1991; Le Moine et al., 1991; Weiner et al., 1991). In the periphery, D1 receptors have been detected in the

parathyroid gland, heart, adrenal cortex, ovaries, gastrointestinal tract, and kidney (Amenta et al., 1995; Aherne et al., 1997; Vaughan et al., 2000).

D1 knockout transgenic mice have been engineered by two separate groups, and studied by numerous other laboratories. D1 knockout mice exhibited growth retardation and a reduced brain size, but no other major neurological deficits. Drago et al. reported reduced level of rearing and no significant reduction in locomotion (Drago et al., 1994). However, other groups studying the same mice but using different methodology reported increased locomotor activity (Xu et al., 1994b; Xu et al., 1994a). Moreover, other groups have reported a modest decrease in locomotor activity (Smith et al., 1998). In general these observations are in contradiction to reports that administration of D1-like antagonists to wild-type mice results in hypokinetic effects, suggesting compensatory processes in response to development in the absence of D1 receptors (Daly and Waddington, 1993; Waddington et al., 2001). Furthermore, acute administration of D1 antisense results in decreased locomotor activity. D1 knockout mice also exhibit a decrease in locomotor activity in response to amphetamine or cocaine administration (Crawford et al., 1997). Importantly, D1 knockout mice are observed to have a deficit in learning tasks and memory (Smith et al., 1998). Moreover, D1 knockout mice are reported to be hypertensive, a physiological effect attributed to deficient D1 receptor signaling in the renal proximal tubules (Albrecht et al., 1996).

6.1.2 Dopamine D5 Receptor Subtype

The human D5 gene is encoded by a single exon located at position 4q15.1-16.1 of chromosome four. The level of D5 expression in the CNS is low in comparison to that of D1 receptors. Expression of D5 receptors in the rat brain has been identified in the hippocampus, lateral mamillary nucleaus, thalamus, substantia nigra, cortex, and in the striatum at a much lower abundance in comparison to D1 receptors (Tiberi et al., 1991; Meador-Woodruff et al., 1992; Montague et al., 2001). In the periphery, D5 has been reported to be expressed in blood lymphocytes and the kidney (Amenta et al., 1995; Ricci et al., 1999).

D5 knockout mice have been reported as having normal general health and no neurological abnormalities; however, they have been described as hypertensive (Sibley, 1999; Holmes et al., 2001; Hollon et al., 2002). Hypertension in D5 knockout mice is caused by an increased sympathetic tone resulting from a CNS defect, which leads to increased blood pressure (Hollon et al., 2002). Tests evaluating locomotor activity have reported either normal or a slight increase in performance. Earlier studies using D5 antisense to inhibit D5 expression have demonstrated a modest increase in locomotor activity (Dziewczapolski et al., 1998). Administration of cocaine to wild-type mice leads to increased locomotor activity. In D5 knockout mice, locomotor activity response to cocaine administration was blunted (Elliot et al., 2003).

6.2 Dopamine D2-like Receptors

The dopamine D2-like family is comprised of three members, D2, D3 and D4 receptors. These receptors are encoded by separate genes which contain multiple introns (Missale et al., 1998). The D2-like receptors share 40% amino acid homology and couple to the inhibition of adenylyl cyclase. Despite the similarities in their primary structure and molecular biology, they exhibit differing localization and functional properties. Their importance in therapeutics has been known since the 1970s. D2-like receptors are the primary sites of action for most antipsychotic drugs. The use of the D2-like specific ligands has been used to map the areas of protein expression within the CNS and periphery. However, lack of ligands that can discriminate between the three D2-like subtypes (D2, D3 and D4) has made it necessary to employ other tactics in identifying subtype specific expression patterns. To address the issue of D2-like receptor specific function knockout mice have been engineered for D2, D3, and D4 receptors.

6.2.1 Dopamine D2 Receptor Subtype

The human D2 receptor gene is located on chromosome 11 at position 11q22-23 and contains six introns. The D2 receptor exists as two alternatively spliced isoforms (Monsma et al., 1989). The two spliced isoforms ($D2_S$ and $D2_L$) differ by a stretch of 29 amino acids in the third intracellular loop; 414 amino acids for $D2_S$ and 443 amino acids for $D2_L$ (Civelli et al., 1993). Both isoforms display similar pharmacological profiles and couple to G_i proteins through which

they inhibit AC. Moreover, both isoforms display the same distribution pattern, although the shorter variant is less abundant (Snyder et al., 1991). Functional differences among the isoforms have been identified. $D2_S$ and $D2_L$ receptors are differentially modulated by PKC (Liu et al., 1992). $D2_S$ receptors play a role in presynaptic dopamine transmission while $D2_L$ receptors participate in postsynaptic dopamine transmission (Lindgren et al., 2003).

Using a variety of approaches, D2 distribution in the CNS has been predominately reported in the striatum, olfactory tubercle, and nucleus accumbens (Bouthenet et al., 1991). D2 receptor mRNA has also been reported in the areas of amygdala, hippocampus, hypothalamus, and substantia nigra (Missale et al., 1998). In the periphery, D2 receptors have been localized to the retina, pituitary, kidney, and lymphocytes (Missale et al., 1998; Levite et al., 2001; Shin et al., 2003).

D2 knockout mice have been engineered by two groups using mice of same genetic background but a different gene deletion (Baik et al., 1995; Kelly et al., 1998). The initial study by Baik et al. (1995) reported gross neurological abnormalities, including impaired gait, impaired rotarod performance, and cataleptic-like behaviour. In contrast, the second study by Kelly et al. (1998) reported much subdued effects. They reported no abnormalities in gait and the ability to acquire normal levels of rotarod performance. They did however report modest deficiencies in locomotor activity. Other studies using further back-crossed mice constructed by Baik et al. (1995) demonstrated neither catalepsy nor impaired gait (Boulay et al., 1999a). These studies give support to the role of

genetic background of knockout mice on observed phenotype. Furthermore, Kelly et al. (1998) demonstrated that treatment of wild-type mice with antagonist (i.e. haloperidol) is characterized by hypoactivity. The activity of wild-type mice treated with haloperidol was observed lower than that of untreated knockout mice, suggesting a role of other members of the D2-like family or action of compensatory effects.

6.2.2 Dopamine D3 Receptor Subtype

The human D3 receptor gene is located on chromosome three at position 3q13.3 and contains five introns (Sokoloff et al., 1990). D3 splice variants have been reported. In rats several variants code for frameshifts or deletions resulting in non-functional D3 receptor (Giros et al., 1991). In contrast to the human genome, two functional spliced isoforms have been identified in mice (Fishburn et al., 1993). In similar fashion to D2 receptors, the mouse D3 splice variants differ by a stretch of 21 amino acids in the third intracellular loop. Both isoforms display similar pharmacological profiles and couple to G_i protein through which they inhibit AC. Moreover, both isoforms display the same distribution pattern with the long isoform being predominant (Fishburn et al., 1993).

D3 receptor distribution in the CNS shows a much lower overall abundance relative to D2 receptors. The highest amount of expression has been observed in nucleus accumbens, olfactory tubercle, and islands of Calleja (Sokoloff et al., 1990; Bouthenet et al., 1991). D3 receptors have also been detected but to a lesser extent in the areas of the striatum, substantia nigra and

hippocampus. In the periphery, D3 receptors have been localized to the kidney and lymphocytes (Levite et al., 2001; Shin et al., 2003).

Several groups have engineered D3 knockout mice, all of which exhibit no major neurobiological abnormalities. The first two groups have demonstrated increased locomotor activity which is not sustainable over an extended period of time (Steiner et al., 1997; Xu et al., 1997). However, using the same mice as Steiner et al. (1997) but backcrossed an additional generation revealed no hyperactivity (Boulay et al., 1999b). Similarly, using another line of D3 knockout mice, a study reported no increased locomotor activity (Jung et al., 1999). On the basis of pharmacological studies, D3 receptors have been implicated in an inhibitory autoreceptor role (Levant, 1997). On this basis D3 knockout mice would be expected to exhibit considerable increase in activity, which we know from the studies described above not to be the case (Waddington et al., 2001).

6.2.3 Dopamine D4 Receptor Subtype

The human D4 receptor gene is located on chromosome 11 at position 11p15.5 and contains three introns (Van Tol et al., 1991). The D4 receptor is most distantly related to the D2-like family. D4 receptors are characterized by numerous polymorphic variants resulting from insertion of a 16 amino acid repeat in the third intracellular loop. In the human population the four repeat form D4.4 is most abundant (60%). Also common are the seven repeat D4.7 (14%) and two repeat D4.2 (10%). Receptors containing upwards of 10 repeats have been identified but at much lower frequency (Van Tol et al., 1992; Seeman and Van

Tol, 1994). The pharmacological and physiological consequences of these repeats are not clear, although some studies have linked the propensity of certain repeats with disorders such as attention-deficit hyperactivity disorder (Grady et al., 2003).

The mRNA of D4 receptors have been detected in the frontal cortex, amygdala, hippocampus, and hypothalamus (Van Tol et al., 1991; O'Malley et al., 1992). Lower levels of D4 have also been reported in the basal ganglia and substantia nigra. In the periphery, D4 receptors have been identified in lymphocytes, heart, and kidney (O'Malley et al., 1992; Ricci et al., 1998; Shin et al., 2003).

D4 knockout mice develop normally and display no gross neurobiological abnormalities. Although these mice display decreased locomotor activity they are reported to have increase motor coordination as indexed by the rotarod performance test. In comparison to wild-type mice, D4 knockouts exhibit increased locomotor activity in response to non-selective dopamine receptor activation by cocaine, suggesting an inhibitory role for D4 receptors (Rubinstein et al., 1997).

7. Molecular and Biochemical Functions of D1-like Receptors

Despite the numerous studies involving dopamine D1 and D5 receptors, much work remains to be done in elucidating their specific roles. Knockout mice have shed light on the physiological role of these two closely related subtypes. However, knockout studies have also demonstrated actions of compensatory effects originating during the development process, partly in response to the

overlap in function and distribution of D1-like receptors. In recent years, with the publication of the crystal structure of rhodopsin and countless studies by researchers around the world much advancement has occurred in understanding the complex requirements for GPCR activation. In tandem these studies have demonstrated that the activation process of GPCRs involves a re-arrangement of TM domains in response to ligand binding which leads to coupling with G proteins. Nevertheless, these studies have also demonstrated that although some structural determinants (i.e. DRY motif) are highly conserved among numerous GPCR families and mediate the activation scheme, there are structural determinants specific to individual receptors which allow for the binding of select ligands and coupling to particular G proteins. Thus far the structural determinants involved in the activation of D1-like receptors have not been fully described and the subtype specific determinants discriminating between D1 and D5 receptors have not been identified. As previously described, study of the physiological role of D1 and D5 receptors has been hampered due to a lack of subtype specific drugs.

7.1 D1-like Receptors and the Etiology of Human Disease

Despite the lack of D1-like receptor subtype specific drugs, numerous studies have directly and indirectly implicated these two dopamine receptors (D1 and D5) in the etiology of human diseases such as Parkinson's, schizophrenia and hypertension. Parkinson's disease is classified as a neurodegenerative disorder resulting from the loss of dopamine producing neurons in the substantia nigra

(Huang et al., 2001). As previously discussed, the substantia nigra expresses both D1 and D5 receptors. Parkinson's disease is characterized primarily by deficiencies in locomotor activity which gives rise to symptoms including a resting tremor, bradykinesia (slowness of movement), rigidity, and postural dysfunction. Both D1 and D5 knockout mice exhibit symptoms described as deficiencies in locomotor activity. In the early stages of the disease dopamine replacement therapy using L-dopa or dopamine receptor agonists is extremely effective. However, after an extended period of treatment the efficacy decreases while side effects which include dyskinesias and psychiatric disturbances become prevalent (Moser et al., 2003). The psychiatric disturbance resulting from long term treatment with dopamine agonists bare resemblance to the positive symptoms of schizophrenia.

The etiology of schizophrenia remains unclear, but numerous hypotheses have been proposed (Duncan et al., 1999). Symptoms associated with schizophrenia are classified as either negative or positive. Negative symptoms include affective flattening and social withdrawal. Positive symptoms are characterized by delusions, hallucinations, thought disorder, and aberrant behaviors. Many antipsychotic drugs used in the treatment of these symptoms exhibit inverse agonism at dopamine receptors (Tiberi and Caron, 1994; Martin et al., 2001). Evidence suggests that schizophrenia is associated with excessive stimulation of D2 receptors in the striatum, deficient stimulation of D1 receptors in the prefrontal cortex, alterations of glutamate transmission at N-methyl-D-aspartate (NMDA) receptors, and aberrations in γ-aminobutyric acid (GABA)

receptor signaling (Laruelle et al., 2003; Wassef et al., 2003). Studies using positron emission tomography (PET) have demonstrated decreased levels of D1-like receptors in the prefrontal cortex of schizophrenic patients (Okubo et al., 1997). Decrease in D1-like receptors is thought to result in deficits of cognitive functions, such as working memory. In recent years, the glutamate and dopamine hypothesis of schizophrenia have been linked by demonstrating that D1 and NMDA receptors interact through a direct protein-protein interaction. The interaction involves the CT of D1 and results in inhibition of NMDA receptors (Lee et al., 2002). Furthermore, the D5 receptor has been shown to directly interact with $GABA_A$ receptors and inhibit GABAergic signaling (Liu et al., 2000). Deficiencies in GABAergic transmission have been postulated as contributing to both the positive and negative symptoms of schizophrenia (Lara, 2002).

The D1 and D5 knockout mice have implicated these receptors in the etiology of hypertension (Albrecht et al., 1996; Hollon et al., 2002). A defective coupling of renal D1 receptors has been proposed as a mechanism of hypertension (Hussain and Lokhandwala, 1998; Sanada et al., 1999). Dopamine is produced locally in the kidney and mediates the inhibition of the renal tubule sodium transporters, Na,K-ATPase and Na,H-exchanger (Aperia, 2000). Indeed, recent studies have suggested that essential hypertension results from a constitutive phosphorylation of the D1 receptor by GRK4, resulting in a diminished response to dopamine (Felder et al., 2002). In contrast, dysfunction in the CNS mediated by D5 receptors has been proposed as a different mechanism of hypertension. Blood

pressure has been shown to be mediated by D5 receptors through the regulation of the sympathetic tone with an involvement of adrenal catecholamines (Hollon et al., 2002).

The three examples of human disease discussed above highlight the complexity of D1-like receptor and emphasize the importance of developing subtype-specific agonists and inverse agonists for the treatment of these disorders.

7.2 Pharmacological Profile of D1-like Receptors

The evidence linking D1-like receptors to a multitude of physiological responses is overwhelming. However, despite extensive study the specific role of either D1 or D5 receptors still remains elusive and emphasizes the need for subtype-specific drugs. In light of these difficulties, an experimental approach using heterologous expression of receptors in cultured cells has been employed. One common cellular system model is that of human embryonic kidney (HEK293) cells. Numerous studies have reported that HEK293 cells are an excellent model for the study of GPCRs and more specifically D1-like receptors (Tiberi and Caron, 1994; Charpentier et al., 1996). HEK293 cells are an excellent model for numerous reasons including: they do not express any endogenous dopamine receptors, are easily transfected with high efficiency, receptors undergo post-translational modification, and these cells contain endogenous mechanisms required for signaling and trafficking by D1-like receptors (Tiberi and Caron, 1994; Charpentier et al., 1996; Ferguson et al., 1996; Zhang et al., 1997). This system allows for the easy transfection of the D1 or D5

receptor and facilitates the study of the pharmacological and signaling profile of these two receptors, despite a lack of subtype specific ligands (Tiberi and Caron, 1994).

D1 and D5 receptors differ in their ligand binding profile with the D1 receptor subtype exhibiting a lower affinity for agonists but a higher affinity for inverse agonists (Sunahara et al., 1991; Tiberi et al., 1991; Tiberi and Caron, 1994). Both receptors have been shown to couple to G_S proteins and stimulate adenylyl cyclase activity. However, a distinguishing feature of the D5 receptor is a high agonist-independent activity (constitutive activity) reminiscent of CAM GPCRs. In contrast, the D1 receptor subtype exhibits a higher agonist-mediated activity (Tiberi and Caron, 1994). On the basis of these observations and with our understanding of the ternary complex model we would propose that the D1 receptor exists in a more constrained conformation or inactive R state, while the D5 receptor exists in a less constrained (more "relaxed") conformation or active R* state.

7.3 Structure-Function Relationships of D1-like Receptors

The studies mentioned above suggest that the distinct ligand binding and G protein coupling properties of D1-like receptors may underlie their physiological functions. In response to a lack of subtype-specific ligands and absence of a dopamine receptor X-ray crystal structure, studies have resorted to using chimera and single- or double-point mutations in delineating the

determinants responsible for the discriminating pharmacological and functional properties of D1 and D5 receptor subtypes.

Mutagenesis studies have confirmed that the ligand binding determinants of D1-like receptors bare similarity to those of other GPCRs. Mutagenesis of residues in TM3 and TM5 has demonstrated them to be critical for dopamine binding at D1 receptors (Pollock et al., 1992; Tomic et al., 1993). However, these studies do not address the unique conformational states responsible for differences at the D1 and D5 receptors. Studies from numerous other GPCRs have shown that other residues in the transmembrane domain, as well as residues in extracellular and intracellular domains can influence ligand binding. In fact, studies performed on opioid receptors have suggested that the extracellular loops play an important role in directing ligands to the binding pocket (Dietrich et al., 1998; Decaillot et al., 2003). Studies on dopamine D1 receptors have demonstrated residues located in TM6 and TM7 play a role in mediating ligand binding (Kozell et al., 1994; Cho et al., 1996). Moreover, chimeric and truncation studies of the cytoplasmic tail reveal that this domain mediates structural conformations responsible for binding affinites of partial agonists at D1 and D5 receptors (Jensen et al., 1995; Sugamori et al., 1998a). In tandem, these studies demonstrate the complexity of parameters mediating receptor specific ligand binding affinities.

Studies investigating the ligand binding determinants at the D1 and D5 subtypes have correlated conformations induced by mutations and chimera with a role in mediating G protein coupling properties. Indeed, mutations in TM6 of D1 receptors not only mediate ligand binding but also elevate D1 receptor

constitutive activity, a phenotype associated with D5 receptors (Cho et al., 1996). Similarly, substitution of a single amino acid in the third intracellular loop of D1 leads to increased constitutive activity while the reciprocal mutation at the D5 receptor leads to decreased constitutive activity ("silencing" effect) (Charpentier et al., 1996). Furthermore, chimeric studies have demonstrated that IL3 is responsible for binding to G_S protein (Kozell et al., 1994). These studies support the general concept that although G protein coupling determinants are located in the intracellular domains, the overall receptor conformation mediates the G protein signaling properties of D1 and D5 receptors.

7.4 Regulation of D1-like Receptors

As previously described, ligand binding and G protein signaling is rapidly followed by regulation of the receptor, which in the classical GPCR scheme involves GPCR phosphorylation, desensitization, endocytosis, and recycling/degradation. Unlike the well established pharmacological profile of D1 and D5 receptors, a comparison of the regulatory processes of these two D1-like receptors remains to be fully appreciated. Indeed, the regulatory processes of D1 receptors have been well documented and shown to proceed through the well established GPCR scheme. D1 receptors have been shown to undergo phosphorylation by PKA and members of the GRK family (GRK 2, 3, 4, 5) (Ng et al., 1994; Zamanillo et al., 1995; Tiberi et al., 1996; Watanabe et al., 2002). Agonist stimulation leads to desensitization of D1 receptors in a phosphorylation-dependent manner (Gardner et al., 2001; Jackson et al., 2002; Lamey et al., 2002;

Kim et al., 2004). Furthermore, D1 receptors have been shown to undergo agonist-induced endocytosis (Ng et al., 1995; Ariano et al., 1997; Jackson et al., 2002). In contrast, few studies have examined the regulation of D5 receptors. Desensitization of D5 receptors has been reported but the status of receptor phosphorylation remains unclear (Jarvie et al., 1993). Fusion protein studies have suggested a lack of PKA- and PKC-mediated phosphorylation of CT (Zamanillo et al., 1995). However, no reports of GRK-dependent phosphorylation or endocytosis have been published for D5 receptors.

Functional properties of D1-like receptors can also be mediated through specific protein interactions (Bergson et al., 2003). The D1 receptor has been demonstrated to directly interact with NMDA glutamate receptors (Lee et al., 2002). Through this interaction the NMDA receptor can mediate D1 activation of adenylyl cyclase. The D1 receptor has also been shown to interact with calcyon allowing D1 cross-talk to multiple effector systems (Lezcano et al., 2000). The D5 receptor has been demonstrated to interact with $GABA_A$ receptors resulting in an inhibition of D5 signaling (Liu et al., 2000). In vitro GST-fusion protein studies have demonstrated a direct interaction between the D1 cytoplasmic tail and N-ethylmaleimide-sensitive factor (NSF), which is known to be involved in post-endocytic recycling of receptors back to the plasma membrane, and sorting nexin 1 (SNX1), known to be involved in targeting receptors to lysosomal degradation (Heydorn et al., 2004). These in vitro studies have also demonstrated the cytoplasmic tail of D5 receptors interacts with NSF and SNX1, albeit the

interaction with SNX1 is stronger than that observed for D1 receptors (Heydorn et al., 2004).

Dimerization is another mechanism that can mediate the functional properties of GPCRs. Hetero- and homodimerization of numerous GPCRs has been documented and demonstrated to mediate a diversity of effects involving ligand binding and receptor signaling (Jordan and Devi, 1999; Overton and Blumer, 2000; Rocheville et al., 2000; Pfeiffer et al., 2002). Indeed, dimerization of members of the dopamine family has been shown. Particularly the D2 receptors have been shown to dimerize (Ng et al., 1996; Zawarynski et al., 1998).

8. Objectives and Hypotheses

Despite preferential expression of specific D1-like receptor subtypes in different areas of the CNS, the basis for multiplicity of D1-like receptor subtypes remains unclear. As I have described in the above sections, a lack of subtype-specific ligands hinders the investigation of the physiological roles of D1 and D5 receptors. Numerous studies have inferred physiological actions of various GPCRs through the study of receptors expressed in cultured cells. Despite a well characterized pharmacological and signaling profile of the two D1-like receptor subtypes little work has been done to compare the regulatory processes of these two receptors. The physiological actions of D1 and D5 receptors may be differentiated on the basis of their regulatory processes. Indeed, differential regulation of these two receptors may involve, signal duration, frequency, amplitude, and unique intracellular signaling cascades.

The objectives of this PhD thesis are to delineate the molecular basis for the D1 and D5 subtype-specific ligand binding, G protein coupling, and signaling properties. Specifically, I will examine the terminal receptor locus (TRL), a domain encompassing TM6, EL3, TM7 and CT. In order to map better the determinants involved in binding of agonists and inverse agonists I will follow up this study by examining specific domains and individual amino acids within the TRL. Finally, I will focus my attention on comparing the regulation of D1 and D5 receptors in response to agonist treatment.

I hypothesize that unique receptor conformations mediated by identifiable residues located within the TRL region are responsible for the different agonist and inverse agonist binding affinities as well as the unique D1 and D5 ligand binding profiles. Secondly, I hypothesize that the multiplicity of D1-like receptor subtypes can be accounted for by the fundamental differences in the regulatory pathways controlling their responsiveness.

MATERIALS AND METHODS

1. Materials

N-[methyl-^{3}H]SCH23390 (77 – 86 Ci/mmol), [^{3}H]adenine (22 – 27 Ci/mmol), [^{14}C]cAMP (275 mCi/ml), [^{32}P]orthosphospate, [^{35}S]ATP, Sequenase version 2.0 kit, and ECL western blotting kit were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada) . Maxiprep and miniprep DNA kits, and Qiaex II beads where purchased from Qiagen Inc (Valencia, CA, USA). Agarose, phosphate buffered saline (PBS), Taq polymerase, and Kodak Biomax MR X-ray film was purchased from Life Technologies, Inc (Burlington, Ontario, Canada). Dopamine, SCH23390, Z-flupentixol (cis), E-flupentixol (trans), (+)-butaclamol, fluspirilene, thiothixene, trifluoperazine, thioridazine,1-Methyl-3-isobutylxanthine (IBMX), phenyl methyl sulfonyl fluoride (PMSF), saponin, bovine serum albumin (BSA), aprotinin, pepstatin A, benzamidine, leupeptine, and soybean trypsin inhibitor were purchased from Research Biochemicals International / Sigma (Oakville, Ontario, Canada). The Slow Fade kit and Alexa-488 (green) goat anti-rabbit antibody were purchased from Molecular Probes (Burlington, Ontario, Canada).

2. Mutagenesis of D1-like Receptors

2.1 Construction of TRL Chimeric Receptors

I took advantage of the high degree of similarity in the primary sequence of D1 and D5 receptors to swap a cassette corresponding to the terminal receptor

locus (TRL). The TRL cassette includes sequences coding for TM6, TM7, EL3 and CT (Figure 1). Using a conserved *Bcl*I restriction site located at the N-terminus of the TM6, I constructed two chimeric D1-like receptors harboring the TRL cassette of their respective cognate wild-type counterparts. The D1 chimera containing the D5 TRL and the D5 chimera containing the D1 TRL were named Chimera 1 and Chimera 2, respectively. The chimeric constructs were subcloned in pBluescript II SK+ (Stratagene) and the identity of the chimeras was confirmed by dideoxy sequencing. Expression constructs for the wild-type and chimeric D1 and D5 receptors were engineered into the expression vector pCMV5, similar to pCMV4 (Andersson et al., 1989).

2.2 Construction of EL3 and EL3/TM6 Chimeric Receptors

The chimeric receptors were constructed by gene splicing using a polymerase chain reaction (PCR)-based overlap extension approach (Horton et al., 1990). The human D1 and D5 sequences were swapped between the junctions of TM6/EL3 and EL3/TM7.

The primers used to amplify the D1-EL3D5 chimera were D1-P1(forward): 5' GAA GGC CCT CCG GCC GGC TTC CCC TGC GTC AGT GAG ACC ACC TTT GAC GTG 3'; and D1-P2(reverse): 3' AAC GGG AAG ACA TCA CCT GTG GGG TTC CCG GGA GGC CGG CCG AAG 5'. In order to engineer the chimeric receptor D1-EL3TM6D5, which involves swapping the two variant TM6 residues, a different reverse primer was designed and called D1-P2TM6(reverse): 3' TAG AAC TTG ACG TAC CAG GGA AAG ACG TCA

CCT GTG GGG CTT CCG GGA GGC 5'; the nucleotide sequence corresponding to D1 receptor are underlined.

The primers used to amplify the D5-EL3D1 chimera were D5-P1(forward): 5' GGG GAG ACG CAG CCC TTC TGC ATT GAT TCC AAC ACC TTC GAC GTC 3'; and D5-P2(reverse): 3' CAG GGA AAG ACG CCC AGA CCC CTC TGC GTC CGG AAG 5'. To engineer the chimeric receptor D1-EL3TM6D5 which involves swapping the two variant TM6 residues, a unique reverse primer was prepared and called D5-P2TM6(reverse): 3' TAG GAA TTG ACG TAA AAC GGG AAG ACA CCC AGA CCC CTC TGC GTC GGG 5'; the nucleotide sequence corresponding to D5 receptor are underlined.

A similar PCR strategy was employed for the construction of all four chimeric receptors: D1-EL3D5, D5-EL3D1, D1-EL3TM6D5, D5-EL3TM6D1. The first round of PCR using either the wild-type D1 or D5 receptor as template and its corresponding primers generated two fragments: fragment F1 was generated using a forward primer with sequence homology to the 5' end of the receptor and primer DX-P1(reverse), fragment F2 was generated using a reverse primer with sequence homology to the 3' end of the receptor and primer DX-P2 (forward); X represents either 1 or 5 depending on the chimera being generated. The result of this PCR was two fragments identical to its template receptor containing either a 5' or 3' overhang with sequence identity to its counterpart receptor EL3 domain. The conditions for this PCR step included 50 ng of template, 2 mM of dNTPs, 1.5 mM $MgCl_2$, and 50 pmol of each primer. The PCR cycle was as follows: 1x (3 min @ 94 °C → 1 min @ 42 °C → 3 min @ 72 °C) →

25x (45 sec @ 94 °C → 1 min @ 42 °C → 1 min @ 72 °C) → 1x (1 min @ 42 °C → 8 min @ 72 °C → ∞ @ 4 °C). Following the PCR extension, the PCR product was run on a 1% agarose gel. The single DNA band corresponding to the PCR product was excised and the DNA was purified using Qiaex II beads.

The second PCR step involved an overlap extension of the first two products F1 and F2, which served both as primer and template. The extracted PCR products were diluted 1:20 and 1 µl was used for the subsequent PCR step. The overhangs of F1 and F2 annealed and were extended in a single cycle; 1x (3 min @ 94 °C → 1 min @ 42 °C → 10 min @ 72 °C). Next the two flanking primers used to generate F1 and F2 were added to this PCR reaction mixture; 20x (45 sec @ 94 °C → 1 min @ 42 °C → 1 min @ 72 °C) → 1x (1 min @ 42 °C → 1 min @ 72 °C → ∞ @ 4 °C).

Subsequent to the second PCR step, the PCR product was run on a 1% agarose gel and the single DNA band corresponding the PCR product was excised and purified using Qiaex II beads. The purified PCR product was digested using restriction enzymes and once again separated by agarose gel and purified. The digested and purified PCR products were subcloned in pBluescript II SK+ (Stratagene) and the PCR extension of the chimeras was confirmed by dideoxy sequencing. Expression constructs for the wild-type and chimeric receptors were subcloned into the expression vector pCMV5.

2.3 Construction of TM6, TM7 and EL3 point mutants

The construction of point mutants was accomplished by gene splicing using a PCR-based overlap extension approach as described for the construction of EL3 chimeric receptors, using the same PCR conditions. The following double-point mutations were engineered: D1-I294M and D1-L295V, D5-M318I and D5-V319L. The following single-point mutations were engineered: D1-I294M, D1-L295V, D1-I329V, D5-M318I, D5-V319L, D5-V357I. The primers used to generate the TM6 and TM7 point mutants were engineered with silent mutations (indicated in bold) for diagnostic purposes. The mutated amino acid codons are underlined in the primer sequence.

D1-IL294/295MV (forward)
5' GTCTCCCC**GGATCC**ACAGAAGGGGACCATGCAGTTCAAGAT 3'
D1-IL294/295MV (reverse)
3' CAGAGGGG**CCTAGG**TGTCTTCCCCTGGTACGTCAAGTTCTA 5'

D1-I294M (forward)
5' GTCTCCCC**GGATCC**ACAGAAGGGCAACATGCAGTTCAAGAT 3'
D1-I294M (reverse)
3' CAGAGGGG**CCTAGG**TGTCTTCCCGTTGTACGTCAAGTTCTA 5'

D1-L295V (forward)
5' GTCTCCCC**GGATCC**ACAGAAGGGGACAATGCAGTTCAAGAT 3'
D1-L295V (reverse)
3' CAGAGGGG**CCTAGG**TGTCTTCCCCTGTTACGTCAAGTTCTA 5'

D1-I329V (forward)
5' AGGCATAAATGACGGGGTTCAAGGAT**GAATTC**GCCCACCCAA 3'
D1-I329V (reverse)
3' TCCGTATTTACTGCCCCAAGTTCCTA**CTTAAG**CGGGTGGGTT 5'

D5-MV318/319IL (forward)
5' GTGTCCA**CTACAA**AAAGGCAAAATGCAGTTAAGGATGA 3'
D5-MV318/319IL (reverse)
3' CACAGGT**GATGTT**TTTCCGTTTTACGTCAATTCCTACT 5'

D5-M318I (forward)
5' GTGTCCA**CTACAA**AAAGGGACAATGCAGTTAAGGATGA 3'
D5-M318I (reverse)
3' CACAGGT**GATGTT**TTTCCCTGTTACGTCAATTCCTACT 5'

D5-V319L (forward)
5' GTGTCCA**CTACAA**AAAGGCAACATGCAGTTAAGGATGA 3'
D5-V319L (reverse)
3' CACAGGT**GATGTT**TTTCCGTTGTACGTCAATTCCTACT 5'

D5-V357I (forward)
5' AAGGCATAGATGATGGGGTTGAGTGAG**GAATTC**GCCCAGC 3'
D5-V357I (reverse)
3' TTCCGTATCTACTACCCCAACTCACTC**CTTAAG**CGGGTCG 5'

The primers used to generate the EL3 proline point mutants were engineered to replace the desired proline with either an alanine or glycine; the resulting PCR products were verified by dideoxy sequencing. The sequence of the mutated proline is underlined. Furthermore, indicated in bold is the sequence of a silent mutation that was introduced for diagnostic purposes. At first random primers were designed to replace the desired proline with either alanine or glycine in a single PCR step, these primers are represented by X in the primer name, which indicates either alanine or glycine. The random primers contain an S in their amino acid sequence, where S corresponds to a random insertion of either G or C. In some instances a more specific primer was designed to replace proline with the desired amino acid.

D1-P296X(forward) 5'CATTTTGGSTTTCTGT**GGATCC**GGGGA3'
D1-P296X(reverse) 3'GTAAAACCSAAGACA**CCTAGG**CCCCT5'

D1-P305X(forward) 5'TTCTGT**GGATCC**GGGGAGACGCAGGSTTTCT3'
D1-P305X(reverse) 3'AAGACA**CCTAGG**CCCCTCTGCGTCCSAAGA5'

D1-P305A(forward) 5TGT**GGATCC**GGGGAGACGCAGGCTTTCTGCATT3'
D1-P305A(reverse) 3'CA**CCTAGG**CCCCTCTGCGTCCGAAAGACGTAA5'

D5-P320X (forward) 5'TGGTCGSTTT**TTGTAG**TGGACA3'
D5-P320X (reverse) 3'ACCAGCSAAA**AACATC**ACCTGT5'

D5-P320G(forward) 5'TGCATGGTCGGTTT**TTGTAG**TGGACACCCTGAA3
D5-P320G(reverse) 3'ACGTACCAGCCAAA**AACATC**ACCTGTGGGACTT5'

D5-P326X(forward) 5'CCTTT**TTGTAG**TGGACACGSTGAAGGCCCT3'
D5-P326X(reverse) 3'GGAAA**AACATC**ACCTGTGCSACTTCCGGGA5'

D5-P326A(forward) 5'TCCTTT**TTGTAG**TGGACACGCTGAAGGCCCT3'
D5-P326A(reverse) 3'AGGAAA**AACATC**ACCTGTGCGACTTCCGGGA5'

D5-P329X(forward) 5'AAGGCGSTCCG**GCTGGA**TTCCCCTG3'
D5-P329X(reverse) 3'TTCCGCSAGGC**CGACCT**AAGGGGAC5'

D5-P330X(forward) 5'AAGGCCCTGST**GCTGGA**TTCCCCTG3'
D5-P330X(reverse) 3'TTCCGGGACSA**CGACCT**AAGGGGAC5'

D5-P330A(forward) 5'AAGGCCCTGCT**GCTGGA**TTCCCCTGGGT3'
D5-P330A(reverse) 3'TTCCGGGACGA**CGACCT**AAGGGGACCCA5'

D5-P334X(forward) 5'CTCCG**GCTGGA**TTCGSTTGCGTCAGT3'
D5-P334X(reverse) 3'GAGGC**CGACCT**AAGCSAACGCAGTCA5'

3. Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) cells were from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured at 37°C and 5% CO_2 in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and gentamycin (100 mg/ml) (Life Technologies, Inc.). Cells were seeded into 100-mm dishes (2.5 x 10^6 cells/dish, 2.5 x 10^5 cells/ml). Cells were always transfected with a total of 5 μg of DNA/dish using a modified calcium phosphate precipitation procedure as described (Didsbury et al., 1991). Under sterile conditions the desired amount of DNA sufficient for transfection of two dishes (a total of 10 μg of DNA) was mixed with 100 μl of 2

M $CaCl_2$ and supplemented with sterile water to a total volume of 1 ml, to this mixture 2x HEPES buffered saline (HBS) was added drop wise. The solution was gently mixed by tapping the tube and 1 ml of solution was added drop wise to each dish. Cells were transfected with varying concentrations of DNA depending on the receptor construct used and type of experiment performed. Efficiency of transfection was estimated to be greater than 60%. For binding assays 5 μg of DNA/dish was used for transfection (giving a maximal receptor expression in HEK293 cells). For cAMP experiments the amount of DNA used in transfection was reduced to achieve expression (B_{MAX}; binding capacity, index of total receptor expression) of 1 – 3 pmol/mg protein, unless indicated otherwise. Depending on the receptor construct, the amount of DNA used ranged from 0.001 - 5 μg of DNA/dish. When less than 5 μg of DNA/dish was used empty pCMV5 vector was employed to normalize the total amount of DNA to 5 μg per dish.

4. Crude Membrane Preparation for Radioligand Assays

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 hours. Transfected HEK293 cells were then washed with cold PBS, scraped from the dish in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA) and centrifuged twice at 40 000 x g for 20 min at 4°C. The crude membrane pellet was resuspended in lysis buffer using a Brinkmann Polytron (17 000 rpm for 15 sec),

used immediately (saturation studies and cAMP experiments) or frozen in liquid nitrogen and stored at -80°C until used (competition studies).

5. Protein Assay

Two different Bio-Rad assay kits with bovine serum albumin as standard were used to measure protein concentrations in crude or solubilized membrane preparations. The Bio-Rad assay kit, for non-solubilized protein preparations (crude membrane) is based on the Bradford dye-binding procedure (Bradford, 1976). In this protocol a protein sample is mixed with the Bradford dye resulting in a colorimetric reaction, which can be quantified by a spectrometer set to a wavelength of 595 nM. For protein samples containing detergent from solubilization protocols, the Bio-Rad DC protein assay was used. The Bio-Rad DC protein assay is similar to the Lowry assay (Lowry et al., 1951). The colorimetric reaction resulting from this assay was detected by a spectrometer set to a wavelength of 650 nM.

6. Radioligand Binding Assay

A 500 µl aliquot of freshly prepared membrane (before freezing in liquid nitrogen for storage) was diluted in 2.5 ml of binding buffer (1 M Tris-HCl, pH 7.4, 0.5 M NaCl, 1M KCl, 1 M $MgCl_2$, 1.5 M $CaCl_2$, 0.5 M EDTA). Saturation binding assays were performed with 100 µl of membrane in a total volume of 500 µl using N-[methyl-^{3}H]SCH23390 as radioligand. Saturation studies were carried out using concentrations of N-[methyl-^{3}H]SCH23390 ranging from 0.01 to 15

nM. Non-specific binding was determined by the addition of 10 μM Z-flupentixol.

For competition studies frozen membranes were thawed and re-suspended in binding buffer using a Brinkman Polytron. The re-suspended membranes were incubated in a constant concentration of N-[methyl-^{3}H]SCH23390 (0.6 or 2 nM, depending on the receptor under investigation) and increasing concentrations of competing ligand. Competition studies using agonists were performed in the presence of 0.1 mM ascorbic acid.

Binding assays were incubated for 90 minutes at room temperature and terminated with rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed four times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS1701). Protein concentration was measured as described above.

7. Receptor Stability Assay

Frozen membranes were thawed and re-suspended in binding buffer using a Brinkman Polytron. Each diluted membrane was prepared in three equal aliquots and incubated in either on ice, 21 °C, or 37 °C. At time points of 0 hrs, 2 hrs, 6 hrs, 12 hrs, and 24 hrs after the start of incubation, an aliquot of 500 μl was removed to perform a binding assay using a saturating concentration of N-[methyl-^{3}H]SCH23390 (6 nM) and 10 μM Z-flupentixol to measure non-specific binding. Protein concentration was measured as described above.

8. Whole Cell cAMP Assay

8.1 Constitutive Activity and Maximal Activation of Adenylyl Cyclase

Regulation of adenylyl cyclase activity by wild-type and mutant D1 and D5 receptors was assessed in cells transfected with 5 μg DNA of receptor construct using a whole cell cAMP assay as described previously (Tiberi and Caron, 1994; Iwasiow et al., 1999; Jackson et al., 2000; Tumova et al., 2003). Following an overnight incubation with the receptor DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6 well dishes for determination of constitutive activity (agonist-independent activity) and dopamine-mediated maximal activation of adenylyl cyclase. The following day, HEK293 cells were cultured in fresh MEM containing 5% (v/v) FBS, gentamicin (100 μg/ml), and [^{3}H]adenine (1 - 2 μCi/ml) for 18–24 h at 37 °C and 5% CO2. The labeling medium was then removed and HEK293 cells incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX in the presence or absence of 10 μM dopamine for 30 min at 37 °C (in the presence of 0.1 mM ascorbic acid). All determinations were done in triplicate. At the end of the incubation period, the medium was aspirated and replaced with 1 ml of lysis solution (2.5% (v/v) perchloric acid, 1 mM cAMP, and [^{14}C]cAMP (3–5 nCi)) for 30 min at 4 °C. The lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH (neutralizing solution), and precipitates were sedimented by a low speed centrifugation (1,500 rpm) at 4 °C. The amount of intracellular [3H]cAMP was

determined from supernatants purified by sequential chromatography using Dowex (AG50WX4) and alumina columns as described previously (Johnson and Salomon, 1991). The amount of [^{3}H]cAMP (CA) over the total amount of intracellular [^{3}H]adenine (TU) was calculated to determine the relative adenylyl cyclase activity (CA/TU). Receptor expression was determined using a saturating concentration of N-[methyl-^{3}H]SCH23390.

8.2 Dopamine Dose-Response Curves

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells expressing 1 - 3 pmol/mg protein were reseeded in 12-well dishes (1 ml/well) for determination of dopamine potency (EC_{50}) and dopamine-mediated maximal activation of adenylyl cyclase (V_{MAX}). Once the labeling medium was removed, HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX in the presence or absence of dopamine for 30 min at 37 °C (in the presence of 0.1 mM ascorbic acid). All conditions were performed in triplicate using increasing concentrations of dopamine, ranging from 0.01 nM to 10 μM. At the end of the incubation period, the medium was aspirated and replaced with 1 ml of lysis solution. The lysates were then transferred to tubes containing neutralizing solution and precipitates were sedimented by a low speed centrifugation. The amount of intracellular [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex (AG50WX4) and alumina columns.

8.3 Inhibition of D1-like Receptor Constitutive Activity by Inverse Agonists

Transfected HEK293 cells were reseeded in 12 well dishes (2 ml/well) and metabolically labeled with adenine as previously described. For the inverse agonist assay, the labeling medium was removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX in the presence or absence of inverse agonist for 30 min at 37°C in the presence of 5 μM forskolin (FSK). The basal level of intracellular cAMP is relatively low thus in order to quantify accurately the decrease of basal (constitutive) activity by inverse agonists we used FSK to enhance the dynamic range of receptor constitutive activity-mediated cAMP response. Previous studies have shown that activated Gs proteins or agonist-activated Gs-coupled receptors potentiate FSK stimulated AC activity (Harry et al., 1997; Sunahara et al., 1997). Following the incubation period the medium was aspirated and each well was filled with 1 ml of lysis solution. Lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH and precipitates were sedimented by a low speed centrifugation. The amount of intracellular [^{3}H] cAMP was determined from supernatants purified by sequential chromatography using DOWEX and alumina columns.

8.4 Inverse Agonist Dose-Response Curves

Transfected HEK293 cells were reseeded in 12 well dishes (2 ml/well) and metabolically labeled with adenine as previously described. For inverse agonist

dose-response curves the labeling medium was removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX in the presence or absence of inverse agonist for 30 min at 37°C in the presence of 5 μM FSK. The concentration of inverse agonist ranged from 10^{-5} M to 10^{-11} M. Subsequent to the incubation period, the medium was aspirated and replaced with 1 ml of lysis solution. The lysates were then neutralized and the precipitates were sedimented by a low speed centrifugation. The amount of intracellular [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex (AG50WX4) and alumina columns.

8.5 Receptor Desensitization

To evaluate receptor desensitization, the ability of D1 and D5 receptors to stimulate AC activity under control and DA pretreatment conditions was assessed using whole cell cAMP assays as described previously (Jackson et al., 2002). Transfected HEK293 cells were seeded in 12-well dishes and incubated overnight as previously described. The cells were incubated in labeling medium with 0.1 mM ascorbic acid (control) or in the presence of 10 μM DA (treated) for 5 min at 37°C. Next the medium was aspirated and each well washed twice with 2 mL of PBS. Cells were then incubated in 1 mL of 20 mM HEPES-buffered MEM containing 1 mM IBMX with increasing concentrations of DA (in the presence of 0.1 mM ascorbic acid) for 10 min at 37°C. Following the incubation period, the medium was removed, and each well filled with 1 mL of lysis solution and incubated for 30 min at 4°C. The lysates were transferred to tubes containing 0.1

ml of 4.2 M KOH and the precipitates were sedimented by a low speed centrifugation. The amount of intracellular [3H]cAMP was determined from supernatants purified by sequential chromatography as previously described.

9. Immunoblotting and Immunoprecipitation

For both immunoblotting and immunoprecipitation experiments the wild-type or FLAG-tagged D1 and D5 receptor constructs were transfected in HEK293 cells using 5 μg DNA. The cells were transfected and maintained as described above. The day following transfection, the HEK293 cells were seeded and grown in six well dishes.

For immunoblotting experiments, media from the six well dishes was aspirated and the wells were washed with ice cold PBS. Next, 0.5 ml of lysis buffer containing protease inhibitors (PI) (20 μg/mL PMSF; 5 μg/ml aprotinin; 1 μg/ml pepstatin A; 10 μg/ml benzamidine, leupeptine, and soybean trypsin inhibitor) was added and HEK293 cells from two 6-wells were scraped and pooled. Scraped cells were passed six times through a syringe. The protein concentration was determined as previously described. Protein samples were diluted to desired concentration using lysis buffer + PI.

In the case of cold immunoprecipitation experiments, HEK293 cells from one 6-well were solubilized with 0.5 mL of RIPA+ (radioimmunoprecipitation assay) buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, and 10 mM disodium pyrophosphate) containing PI. Two wells were pooled and

transferred to 1.5 ml conical tubes. The cell extracts (1 ml) were solubilized for one hour at 4°C using a rotating wheel. Supernatants were clarified by centrifugation at 15000g for 15 min at 4°C and transferred to new tubes containing 50 μL of mouse monoclonal anti-FLAG affinity matrix (Sigma). Before adding the clarified supernatant to the monoclonal anti-FLAG affinity matrix two aliquots (15 μl) of the supernatant fractions were taken for protein assays as previously described. After 2 hour incubation at 4°C, anti-FLAG affinity matrix was pelleted and the supernatant discarded. The beads were then washed four times with 1 ml of ice-cold RIPA+ buffer and dried.

Protein samples were incubated in 2X SDS sample buffer (25 mM Tris-HCl (pH 6.5), 8% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol) and resolved by SDS–polyacrylamide gel electrophoresis using 10% gels. Proteins were transferred from the gel to a 0.45-μm nitrocellulose membrane (Micron Separations Inc., MA, USA). Next the nitrocellulose membrane was blocked in Blotto solution (50 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM NaCl, 5% nonfat dry milk, 0.2% Nonidet P-40, and 0.02% NaN_3) and incubated sequentially with primary rabbit polyclonal anti-D1 (D1AL24) or anti-D5 (D5JH519) antibodies and secondary rabbit HRP-linked $F(ab)_2$ fragment (1:5000) (Levey et al., 1993; Ciliax et al., 2000). The proteins immobilized on the nitrocellulose were detected by chemiluminescence using ECL reagents.

10. Whole Cell Phosphorylation Assay

Whole cell phosphorylation experiments were performed in HEK293 cells transfected with FLAG-tagged wild-type or chimeric D1 and D5 receptors using 5 μg of DNA. Following the overnight incubation with the DNA–calcium phosphate precipitate, the transfection medium was removed and replaced with fresh MEM. A day prior to the phosphorylation assay, cells were reseeded in six well dishes at a density of $1x10^6$ cells/well and grown for an additional 18 h. Media was than aspirated and replaced with 20 mM HEPES-buffered phosphate free MEM (pH 7.4) containing gentamicin (10 μg/ml) and 0.2 mCi/ml [^{32}P] orthophosphate and labeled for 90 min at 37°C. At the end of the labeling period, cells were incubated in the presence of 0.1 mM ascorbic acid (control) or 10 μM DA (treated) for 10 minutes after which the dishes were put on ice, and cells were washed three times with ice-cold PBS. Next the FLAG-tagged proteins were immunoprecipitated as described above.

Immunocomplexes were resolved by SDS–polyacrylamide gel electrophoresis using 10% gels. Gels were dried and exposed to Kodak Biomax MR Films at 80°C overnight. Receptor phosphorylation was quantified with a Typhoon PhosphorImager 8600 (Amersham Pharmacia Biotech) and values normalized for lane background and receptor expression.

11. Immunofluorescence

HEK293 cells were transiently transfected with D1 or D5 receptors and seeded on 12 mm diameter glass coverslips in a 24 well dish. Prior to use, the glass coverslips were etched using concentrated sulphuric acid for 30 minutes followed by extensive washing using distilled water. The coverslips were autoclaved before use. The receptor expressions obtained under these transfection conditions were similar (2 - 5 pmol/mg protein). Cells on coverslips were incubated with 0.1 mM ascorbic acid only (control) or in the presence of 10 μM DA (treated) for 30 min at 37°C. Following incubation the coverslips were washed with PBS at room temperature. Cells were then fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature and washed in PBS. Coverslips were incubated twice with 0.37% (w/v) glycine and 0.27% ammonium chloride in PBS for 10 min, blocked with 1% (w/v) BSA/0.4% (v/v) saponin/PBS for 30 min at room temperature, and then removed from the well and placed in a humidified dish. Next the coverslips were incubated with rabbit polyclonal anti-D1 (D1AL24) or anti-D5 (D5JH519) antibodies (1:1000) (Levey et al., 1993; Ciliax et al., 2000) in 1% (w/v) BSA/0.4% (v/v) saponin/PBS for 1 h at room temperature (100 μL per coverslip). After incubation with primary antibody cells were washed five times with 1% (w/v) BSA/0.4% (v/v) saponin/PBS for 5 min before being incubated in secondary antibody [Alexa-488 goat anti-rabbit (1:500)] in 1% (w/v) BSA/0.4% (v/v) saponin/PBS for 30 min in dark at room temperature. Following incubation in secondary antibody the coverslips were washed 3 times for 10 min in 1% (w/v) BSA/0.4% (v/v) saponin/PBS. Coverslips

were then incubated in Slow Fade Equilibrating Buffer for 5 min and excess solution was decanted prior to mounting coverslips with Slow Fade Light Antifade reagent in glycerol buffer (Molecular Probes). Confocal laser microscopy (Bio-Rad MRC-1024MP) was utilized to visualize immunofluorescence and capture images.

12. Statistical Analysis

The equilibrium dissociation constants (K_D) and inhibition constants (K_I) are reported using the geometric mean ± standard error (S.E.). All other data are reported as arithmetic means ± S.E. In brief, the geometric mean is the antilog of the arithmetic mean of the log transformed data. The standard error of the geometric mean was calculated by multiplying the value of the standard error of the averaged log transformed data by the geometric mean (De Lean et al., 1982). The homogeneity of variance was analyzed using the F_{MAX} test prior to statistical analysis. One-way ANOVA with Newman-Keuls post test and one sample t-test were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The significance was established at $p < 0.05$.

RESULTS

PART I: STRUCTURE-FUNCTION RELATIONSHIPS OF D1-LIKE RECEPTORS

1. Pharmacological and Functional Differences between the D1-like Receptors are Delineated by the Terminal Receptor Locus

1.1 Introduction

The classical paradigm for GPCR activation is described by the binding of an agonist to an inactive receptor state (R). This process leads to the formation of an active receptor state (R*) which interacts with G proteins to initiate a variety of intracellular signaling events (Lefkowitz et al., 1993; Samama et al., 1993; Leff, 1995; Iwasiow et al., 1999). In the absence of agonist, GPCRs are predominantly maintained in an inactive R state by intramolecular constraints that prohibit interaction with G proteins. These intramolecular constraints are released upon agonist binding or by mutations. As previously described, numerous mutations within GPCRs can result in receptors displaying increased levels of agonist-independent (constitutive) activity (Cotecchia et al., 1990; Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993). Constitutively active GPCRs have a greater propensity to adopt an R* state in the absence of agonists (Hasegawa et al., 1996). For instance, the dopaminergic D1 receptor subtypes have been demonstrated to display different levels of agonist-independent activity (Tiberi and Caron, 1994; Charpentier et al., 1996; Cardinaud et al., 1997).

As previously stated, both D1 and D5 receptors couple to the activation of adenylyl cyclase, however, the D5 receptor distinguishes itself from the D1 subtype by a higher agonist-independent activity, an increased affinity and potency for agonists as well as a lower affinity for inverse agonists (Tiberi and

Caron, 1994). These functional characteristics of the D5 receptor are highly reminiscent of those reported for constitutively active mutant GPCRs (Lefkowitz et al., 1993). The molecular basis underlying the differences in the ligand binding and activation properties between the D1 and D5 receptor are poorly understood.

Studies have described that replacement of a variant amino acid found in the carboxyl end of the third cytoplasmic loop of the D1 subtype (F294) by the one found in the D5 receptor (I288) can induce partially constitutive activation to the D1 receptor. In an opposite fashion, a mutant D5 receptor harboring the variant D1 amino acid exhibits a decreased level or silencing of constitutive activity (Charpentier et al., 1996). These mutant receptors display no modification in their ability to interact with inverse agonists. These results suggest that the carboxyl end of the third cytoplasmic loop plays a role in constraining the D1 and D5 receptors into their inactive and active allosteric states, respectively. However, the results indicate that the molecular properties of these two D1 receptor subtypes can only be explained partially by receptor conformations induced by amino acid sequences at the carboxyl end of the third cytoplasmic loop. Therefore, it is likely that other structural determinants within these receptors exist to define the intramolecular interactions responsible for the distinct features of the D1 and D5 receptors. Studies have shown that mutations occurring in transmembrane regions, extracellular loops, or the cytoplasmic tail of GPCRs can lead to constitutive activation (Parker and Ross, 1991; Lefkowitz et al., 1993; Hasegawa et al., 1996; Nanevicz et al., 1996; Scheer et al., 1996, 1997).

In the present study, I use a chimeric receptor approach (Figure 1) to delineate further the structural determinants that underlie the molecular properties of the human D1 and D5 receptors. I hypothesize that the terminal receptor locus (TRL), which includes the transmembrane region (TM) 6 and 7, the third extracellular loop (EL3), and the cytoplasmic tail (CT), is an important structural domain regulating the activation process of D1 and D5 receptors.

1.2 Results

1.2.1 Comparison of Wild-type and TRL Chimeric Receptors

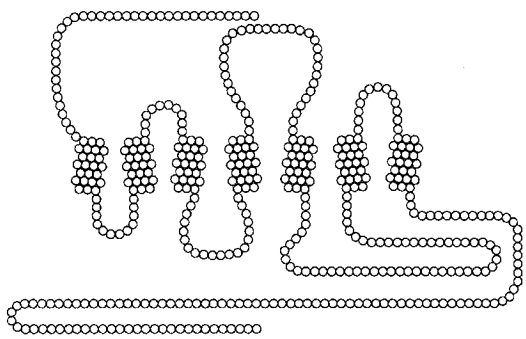
Schematic representation of wild-type and chimeric receptors is illustrated in Figure 1. The TRL sequence alignment demonstrates the high degree of identity shared between the amino acids in TM6 and TM7 of D1 and D5 receptors. Also shown are the divergent primary sequences of EL3 and cytoplasmic tail of D1 and D5 receptors.

1.2.2 TRL Chimera Delineate a Structural Domain Underlying the Dopamine Affinity for the D1-like Receptor Subtypes

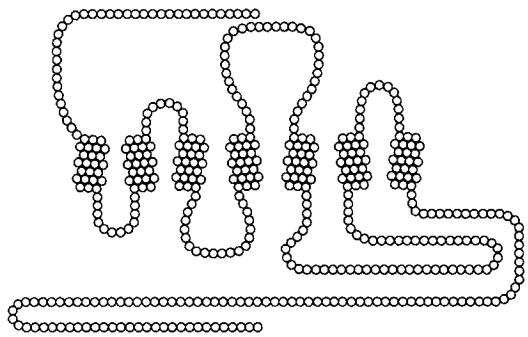
The binding affinities (K_D values) of the radioligand N-[methyl-^{3}H]SCH23390 for wild-type and TRL chimeric human D1-like receptors obtained using saturation studies are summarized in Table 1. Results indicate that the chimeric receptors retain their ability to bind N-[methyl-^{3}H]SCH23390 with high affinity. In addition, no statistical differences between the binding capacities of

Figure 1 - Schematic representation of the wild-type and TRL chimeric D1 and D5 receptors.

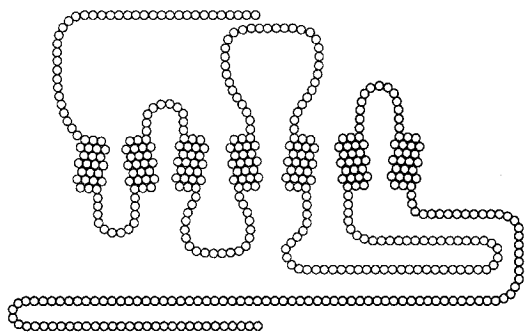
Putative topology of the wild-type D1 (red circles) and D5 receptor (blue circles), chimera 1 and chimera 2 is represented. Alignment of the primary structure corresponding to the TRL cassette derived from the human D1 and D5 receptors is shown. The TM regions are delimited with a thick line above the amino acid sequence. Identical amino acids found between the two TRL sequences are indicated with an asterisk.



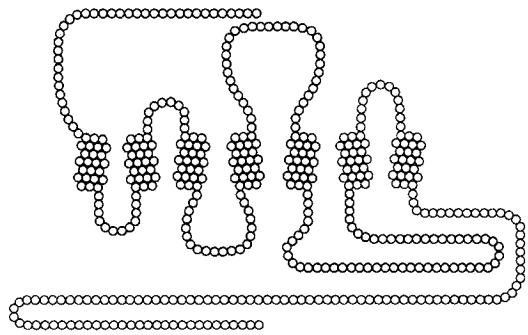
hD1



hD5



Chimera 1

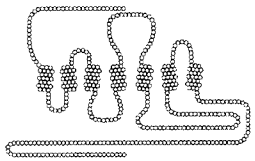
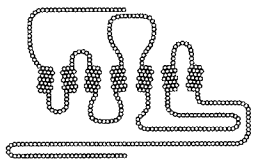
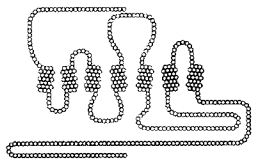
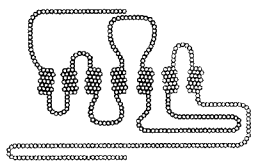


Chimera 2

```
                TM6                                EL3
hD1   VIMGVFVCCWLPFFILNCILPFC-GSGETQP--F-CIDSNT
hD5   VIMGVFVCCWLPFFILNCMVPFCSGHPEGPPAGFPCVSETT
      ******************  *** *  *  *  * *    *

                TM7
hD1   FDVFVWFGWANSSLNPIIYAFNADFRKAFSTLLGCYRLCPA
hD5   FDVFVWFGWANSSLNPVIYAFNADFQKVFAQLLGCSHFCSR
      **************** ******** * *  ****   *

hD1   TNNAIETVSINNNGAAM---FSSHHEPRGS-ISKECNLVYL
hD5   T--PVETVNISNELISYNQDIVFHKEIAAAYIHMMPNAVTP
      *    *** * *           * *     *    * *

hD1   IPHAVGSSED-LKKEEAAGIARPLEKLSPALSVILDYDTD-
hD5   GNREVDNDEEEGPFDRMFQIYQTSPDGDPVAESVWELDCEG
          *   *          *        *        *

hD1   -VSLEKIQPITQNGQHPT 171
hD5   EISLDKITPFTPNGFH-- 178
        ** ** * * ** *
```

Table 1 - Equilibrium constants and maximal binding capacity (B_{MAX}) values for wild-type and TRL chimeric D1-like receptors.

K_D, K_I, and B_{MAX} values are expressed as geometric and arithmetic means, respectively. Means are from six to eight experiments done in duplicate determinations. [3H]-SCH, N-[methyl-^{3}H]SCH23390; DA, dopamine; FLU, Z-flupentixol; BUT, (+)-butaclamol; SCH, SCH23390.

	K_D (nM)	K_I (nM)				B_{MAX} (pmol/mg prot.)
	[3H]-SCH	DA	FLU	BUT	SCH	
 **D1**	0.53 ± 0.09	8792 ± 384	13 ± 2.0	8.5 ± 0.8	0.52 ± 0.02	8.0 ± 1.3
 **D5**	0.63 ± 0.06	817 ± 48	21 ± 2.5	42 ± 1.1	0.73 ± 0.02	9.8 ± 3.1
 **CHIMERA 1**	0.30 ± 0.04	1094 ± 64	14 ± 1.8	12 ± 0.9	0.33 ± 0.01	7.6 ± 0.5
 **CHIMERA 2**	0.37 ± 0.02	6012 ± 281	20 ± 2.7	21 ± 1.5	0.34 ± 0.01	11.0 ± 1.3

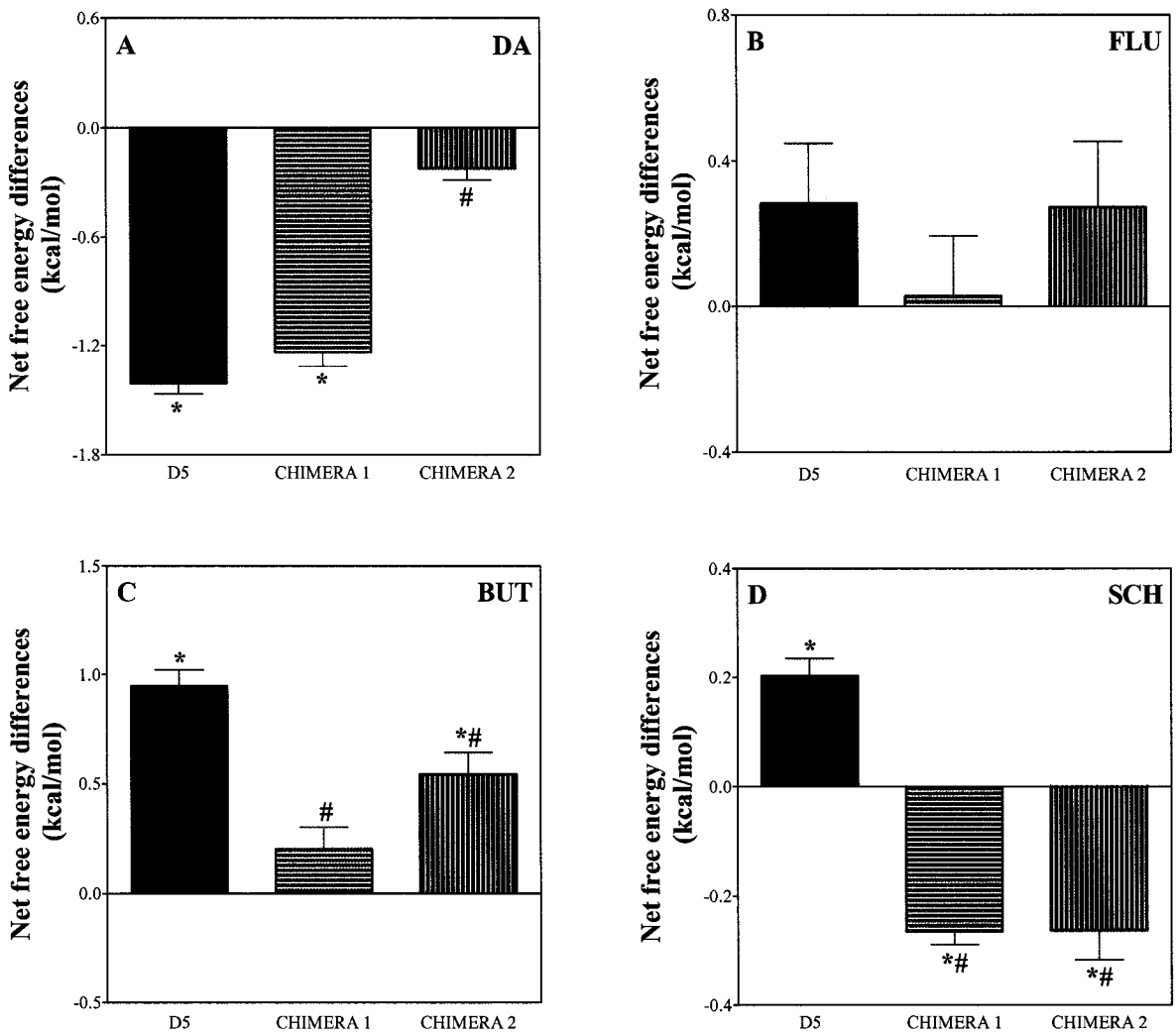
wild-type and chimeric receptors were detected (8–10 pmol/mg of protein). These results suggest that the chimeric receptors retain a proper protein folding necessary for cell expression and binding of ligands.

Competition studies were performed to determine whether the TRL contains the underlying structural requirements involved in the dopamine binding to wild-type human D1 and D5 receptors. At the wild-type D1-like receptors dopamine exhibits a higher affinity for the D5 subtype and a lower affinity for the D1 receptor (Table 1) as previously reported (Tiberi and Caron, 1994; Charpentier et al., 1996). Chimera 1 displays an affinity for dopamine which is highly reminiscent of the binding affinity observed for the D5 receptor (Table 1). In an opposite fashion, chimera 2 binds dopamine with an affinity very similar to the one measured for the D1 receptor (Table 1).

To address the issue of unique receptor conformations that preferentially bind dopamine with distinct affinity, we calculated the free binding energy using the relation $\Delta G = -RT \ln (1/K_I)$ (Catterall, 1989). As reported in Figure 2A, the calculated net free energy difference relative to the dopamine binding energy for the D1 receptor suggests that chimera 1 displays a reduction in the binding energy preference for dopamine. The binding energy for chimera 1 is statistically different from that of D1 receptor but indistinguishable from the D5 receptor. Furthermore, the increased binding energy preference of chimera 2 for dopamine is not statistically different from the D1 but is statistically different from D5 and chimera 1 receptors (Figure 2A).

Figure 2 - Free energy of binding of D1-like ligands to wildtype and TRL chimeric receptors.

For each drug tested the net free energy differences relative to hD1 was calculated using K_I values from competition studies shown in Table 1. Data are expressed as arithmetic mean ± S.E. of 7 to 10 experiments done in duplicate determinations. DA, dopamine; FLU, Z-flupentixol; BUT, (+)-butaclamol; SCH, SCH23390



* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with D5

1.2.3 The TRL Cassette Unravels Distinct Structural Requirements for the Binding of Antipsychotic Drugs

Previous studies have reported that antipsychotic drugs act as inverse agonists and bind with a lower affinity to the D5 receptor in comparison with the D1 receptor (Tiberi and Caron, 1994; Charpentier et al., 1996). We tested the binding affinity of Z-flupentixol and (+)-butaclamol, two antipsychotic drugs having a distinct chemical structure and displaying inverse agonism at the D1 and D5 receptors (Tiberi and Caron, 1994; Milligan et al., 1995). As reported before and shown in Table 1, both drugs have lower affinity for the D5 receptor in comparison to the D1 subtype (Tiberi and Caron, 1994). The binding affinities of Z-flupentixol for the chimera 1 and 2 were not statistically different from the D1 and D5 receptors, respectively (Table 1, Figure 2B). The small fold difference in the Z-flupentixol affinity (~1.5-fold) observed between the wild-type D1-like receptors remains unchanged with the exchange of the TRL cassette.

In contrast to Z-flupentixol, (+)-butaclamol displays a greater fold difference in the binding affinity (~5-fold) between the two D1-like receptor subtypes and exhibits a shift in binding affinities at the chimeric receptors (Table 1). As shown in Figure 2C, the D5 receptor has an increased binding energy preference for (+)-butaclamol in comparison with the D1 receptor. The chimera 1 binds (+)-butaclamol with an affinity that is not statistically different from the wild-type D1 receptor (Table 1, Figure 2C). However, the net binding energy preference for (+)-butaclamol at chimera 2 is decreased in comparison with D5

receptor but remains statistically different from the D1 subtype, suggesting a partial shift in affinity.

Next, I studied the binding properties of the benzazepine SCH23390 which is structurally different from both flupentixol and (+)-butaclamol. This benzazepine has been described as a classical antagonist that binds preferentially to D1-like receptors (Niznik, 1987). In the present study, SCH23390 exhibits lower affinity for the D5 receptor (Table 1) and a significant increase in the binding energy preference when compared to the D1 receptor (Figure 2D). In striking contrast to Z-flupentixol and (+)-butaclamol, SCH23390 exhibits a statistically significant increase in affinity for both chimera 1 and chimera 2 (Table 1, Figure 2D). Similar trend is also observed when using the radiolabeled benzazepine analog N-[methyl-^{3}H] SCH23390 (Table 1).

1.2.4 The TRL Cassette Is the Underlying Structural Domain of D1-like Receptor Constitutive Activation

The D5 receptor shares the functional features of constitutively activated mutant GPCRs (Tiberi and Caron, 1994; Charpentier et al., 1996). The role of the TRL cassette in the agonist-independent activation of adenylyl cyclase by wild-type and chimeric receptors was assessed using a whole cell cAMP assay. In this study, I confirm that the D5 receptor has a 3.5-fold higher agonist independent activity than the D1 receptor (Tiberi and Caron, 1994; Charpentier et al., 1996). Interestingly, chimera 1 shows an increase in its constitutive activation that is statistically different from the D1 receptor but indistinguishable from the D5

receptor (Figure 3). In contrast, chimera 2 exhibits a significant decrease of its agonist independent activity when compared with the D5 receptor (Figure 3) and is highly reminiscent of the one measured for the D1 receptor.

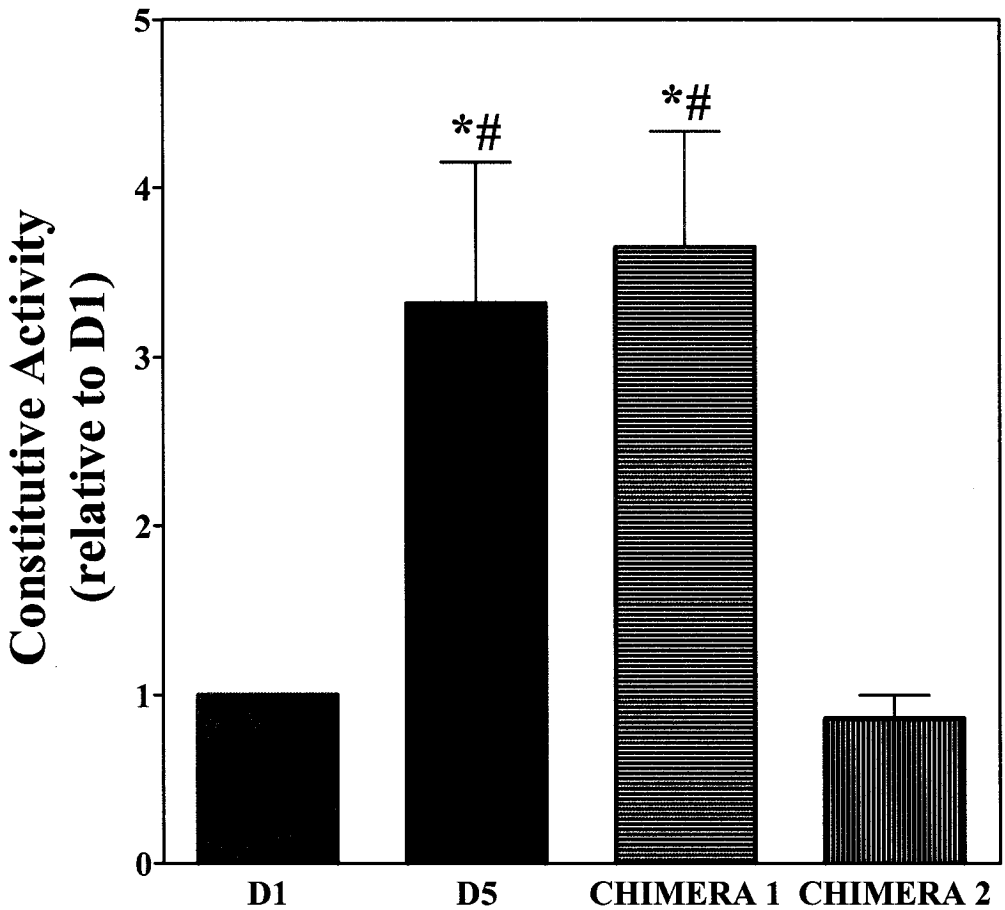
1.2.5 The TRL Cassette Is Involved in the D1 and D5 Receptor G Protein Coupling Properties

To test whether the TRL cassette delineates the structural requirements for the dopamine potency and dopamine-mediated maximal activation of adenylyl cyclase, dose-response curves were done in HEK293 cells transfected with the wild-type and chimeric receptors. As depicted in Figure 4A, the dopamine potency is about 10-fold higher at the D5 receptor in comparison with the wild-type D1, a value in agreement with previous studies (Tiberi and Caron, 1994; Charpentier et al., 1996).

Chimera 1 exhibits an increase in dopamine potency as compared with its parent D1 receptor (Figure 4A). The potency of dopamine at the chimera 1 is not statistically different from that of the D5 receptor. Alternatively, chimera 2 displays a loss of dopamine potency that is significantly different from that of D1 and D5 receptors (Figure 4A). Figure 4B shows that the maximal stimulation elicited by the D1 receptor is significantly higher than that elicited by the D5 receptors as described before (Tiberi and Caron, 1994). Chimera 1 and 2 elicited a maximal activation of adenylyl cyclase that is identical to their respective wild-type receptor counterparts (Figure 4B).

Figure 3 - Constitutive activity of wild-type and TRL chimeric D1-like receptors.

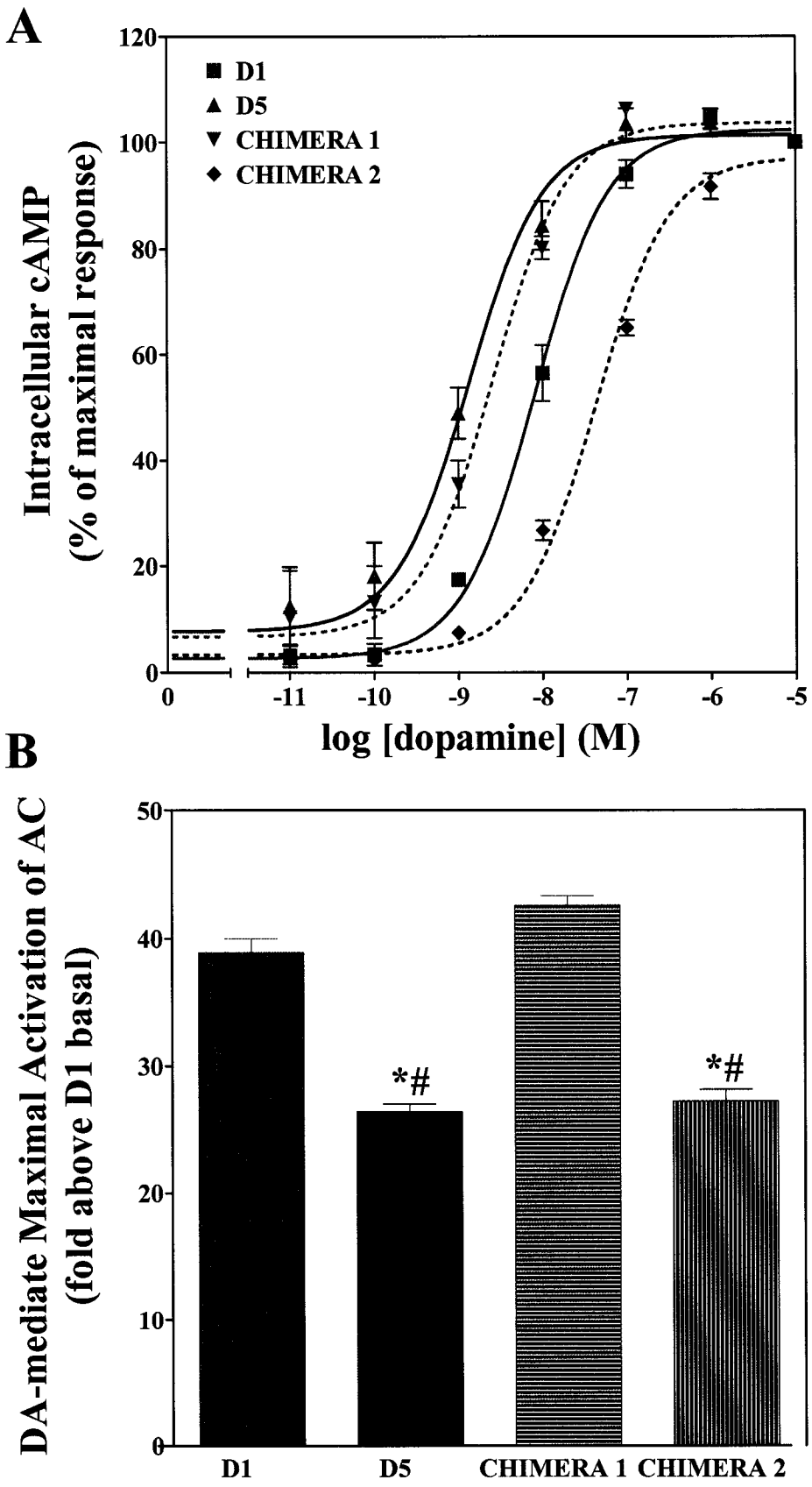
Basal levels of adenylyl cyclase activity were determined in single wells of a six-well dish using whole cell cAMP assays and calculated relative to D1 receptor. Data are expressed as arithmetic mean ± S.E. of seven experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 8.4 ± 1.9 (D1), 11.6 ± 1.8 (D5), 8.0 ± 1.1 (chimera 1) and 10.8 ± 1.8 (chimera 2).



* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with Chimera 2

Figure 4 - Dopamine-mediated activation of adenylyl cyclase activity by wild-type and TRL chimeric D1-like receptors.

A, dose-response curve of dopamine for adenylyl cyclase stimulation by wild-type and chimeric D1 receptors. Each point is the arithmetic mean ± S.E. of five experiments done in triplicate determinations using single wells from a 12-well dish. For the determination of EC_{50} values and maximal stimulation, each point was first expressed as fold relative to D1 basal activity and nonlinear regression of the data was performed using Sigmoidal dose-response (variable slope) using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. For the graphical representation, curve points are depicted as percentage of maximal response obtained with the respective wild-type or chimeric receptor after subtracting the basal value. The EC_{50} values are as follows (in nM): 9.3 ± 1.6 (D1), 1.1 ± 0.3 (D5), 2.5 ± 0.4 (chimera 1), and 41 ± 9.5 (chimera 2). The receptor expression in pmol/mg of membrane protein expressed as the arithmetic mean ± S.E. was 3.4 ± 0.9 (D1), 2.7 ± 0.7 (D5), 2.2 ± 0.5 (chimera 1), and 1.8 ± 0.5 (chimera 2). B, maximal activation of adenylyl cyclase in HEK293 transfected with wild-type and chimeric D1 receptors.



* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with Chimera 1

1.3 Conclusions

In the present study, I have used a chimeric approach to explore the structural determinants involved in the activation properties of the D1-like receptors. Previously, this approach has been useful in helping to delineate specific residues or domains underlying the binding and coupling functions of GPCRs (Kobilka et al., 1988; Suryanarayana et al., 1992; Leeb et al., 1997). Analysis of the primary structure of the D1-like receptors reveals an overall degree of identity of 48% between the D1-TRL and D5-TRL cassette (Figure 1). I demonstrate that despite this low degree of identity, the chimeric receptors retain their ability to express at high levels in HEK293 cells, as indicated by binding of the benzazepine analog N-[methyl-^{3}H] SCH23390. These results suggest that the primary sequence of the TRL does not generate structural incompatibilities which would disrupt receptor folding and maturation.

Results obtained with the benzazepine SCH23390 support a role for the TRL in regulating receptor conformation. Originally, the benzazepine SCH23390 had been described as a high affinity antagonist that binds selectively to D1-like receptors (Niznik, 1987). However, recent studies have demonstrated that benzazepine analogs behave as a partial agonist at D1-like receptors, a phenomenon not only observed in HEK293 cells (Tiberi and Caron, 1994) but also in COS-7 and Sf9 cells (Sugamori et al., 1998b; Sugamori et al., 1998a). These results are supported by *in vivo* studies showing that benzazepine-like compounds can induce behavioral responses in a D1-like agonist fashion (Collins et al., 1991). Both chimeras exhibit a statistically significant decrease in the free

binding energy when compared with their wild-type receptor counterparts (Figure 2D). The reduced binding energies translate into a higher affinity of chimera 1 and 2 for SCH23390 (Table 1). The results suggest a role for TRL in regulating specific interactions with other receptor regions to constrain the D1 and D5 receptor into a suboptimal binding state for benzazepines.

Characterization of the TRL chimeric D1 and D5 receptors has unveiled the molecular complexity underlying ligand binding at D1-like receptors. The data suggests that the TRL region releases conformational constraints involved in binding of the partial agonist SCH23390. I also report that the TRL region contains structural determinants responsible for the agonist binding profile of D1 and D5 receptors. Moreover, structural determinants within the TRL play at least in part an important role in mediating inverse agonist binding.

Binding of the inverse agonist (+)-butaclamol at D1 receptor subtypes is explained, at least partially, by TRL-induced intramolecular interactions. Chimera 2 supports a role for the D1-TRL in conferring to the wild-type D1 receptor its ability to bind (+)-butaclamol with a higher affinity. Interplay of domains within the TRL or determinants located outside the TRL may also play an important role in coordinating the conformation(s) that underlie the binding of (+)-butaclamol. In contrast, the binding affinity of the inverse agonist Z-flupentixol at the chimeric receptors remains unchanged. As reported previously, Z-flupentixol displays a lower affinity at D5 and D5-TRL receptors in comparison with the D1 and D1-TRL receptors (Tiberi and Caron, 1994). These findings suggest that Z-flupentixol binding may require conserved residues located in the TRL of both

D1-like receptor subtypes, may involve interplay of residues within the TRL, or also involve interactions with different residues existing outside the TRL boundaries. These observations give emphasis to the complex molecular pharmacology of inverse agonists. In fact, the pharmacological differences observed between these two antipsychotic drugs may suggest that different molecular mechanisms exist to induce the same physiological effect, i.e. inverse agonism.

The TRL domain is fundamental in the dopamine-independent and -dependent activation process of the human D1 and D5 receptors. I demonstrate that the TRL is involved in regulating the intramolecular interactions that underlie the distinct binding and G protein-mediated activation of AC at the D1-like receptors. The ligand binding and G protein coupling data obtained with chimera 2 suggest that the intramolecular interactions induced by the D1-TRL maintain predominantly the chimeric receptor in a constrained conformation (R state) as indicated by a decreased binding affinity and potency for dopamine, and a lower agonist-independent activity. Interestingly, the molecular properties of chimera 2 are mostly undistinguishable from those of the wild-type D1 receptor. In striking contrast, the intramolecular interactions induced by the D5-TRL enable chimera 1 to adopt a less constrained (more "relaxed") conformation (R* state) as measured by an increased dopamine binding affinity and potency, and a higher agonist-independent activity. In fact, chimera 1 exhibits dopamine binding affinity and constitutive activation properties that are indistinguishable from the wild-type D5 receptor. Most importantly, the D5-TRL contains the structural determinants that

confer the functional features of constitutively active GPCRs (Lefkowitz et al., 1993; Tiberi and Caron, 1994). Overall, these results suggest that the TRL cassette may underlie the spatial relationships specific to D1 and D5 receptors, notably those underlying the molecular properties of constitutive activation and agonist potency but not those involved in the maximal activation of adenylyl cyclase.

These results suggest the existence of different active D1-like receptor conformations evoked in the presence or absence of dopamine. The conformations responsible for agonist binding and constitutive activity are evoked by the TRL; however, the TRL does not evoke conformations responsible for inverse agonist binding or agonist-mediated maximal activation of AC at D1-like receptors. These observations describe structural determinants presumably located in the TRL region that act to define spatial relationships underlying functional properties of the D1 and D5 receptors. These studies also imply structural determinants found either within or outside the TRL region act to exert potential "antagonistic" or "counteracting" effect in the formation of active and inactive states of D1 and D5 receptors. The identification of these residues is of importance since they may underlie the molecular basis for the differential dopamine-mediated maximal activation of adenylyl cyclase and the inverse agonist binding profile of D1-like receptors.

A close examination of the primary structure of the TRL cassettes indicates a low degree of identity (38%) within the EL3 region (Figure 1). Therefore, I speculate that the EL3 region could be involved in the differential

activation processes of the D1 and D5 receptors. Alternatively, the small differences found in the primary structure of the TM6 and TM7 of D1-like receptors may alter the orientation of the TM domains and underlie the molecular basis for ligand-dependent and independent receptor conformations. Moreover, residues present in the cytoplasmic tail may also play an important role in the regulation of active and inactive conformations of the D1 and D5 receptors.

This study has delineated an important receptor domain encompassing the structural determinants involved in regulating the activation process of the human D1-like receptors. Specifically, the TRL region was shown to regulate agonist binding, agonist-independent activity, and dopamine potency. However, the inverse agonist binding and agonist-mediated maximal activation of adenylyl cyclase properties of D1-like receptors were not affected in a similar fashion, suggesting that more discrete domain within the TRL or other domains outside of the TRL region may be involved.

2. Role of EL3 in Regulating Inverse Agonist Binding and Agonist-Mediated Maximal Activation of AC

2.1 Introduction

In the following study, I examine the role of the EL3 as a structural domain responsible for differences in functional properties between D1 and D5 receptors. The previous study demonstrated that the TRL region, which encompasses the EL3 domain, is responsible for agonist binding, agonist-independent activity (constitutive activity) and dopamine potency at D1-like receptors (Iwasiow et al., 1999). However, it remained unclear if the entire TRL domain was required to observe these changes or whether more discrete domains/residues within the TRL were responsible for these effects. Based on these results, I propose that domains within the TRL may have antagonistic actions explaining why we did not observe a swap in inverse agonist binding affinity and dopamine-mediated maximal activation of AC between the two D1-like receptors. These antagonistic actions could be a consequence of residues within and/or outside of the TRL domain. Examination of the TRL domain (Figure 1) shows that the D1-like receptors are highly conserved in TM6 and TM7 but diverse in the EL3 (38% identity) and CT (31% identity). I concluded that these two diverse regions may be valid candidates for explaining the effects observed using the TRL chimera.

A recent study from our lab (in which I was involved) identified the cytoplasmic tail (CT) as an important determinant of agonist-independent activity

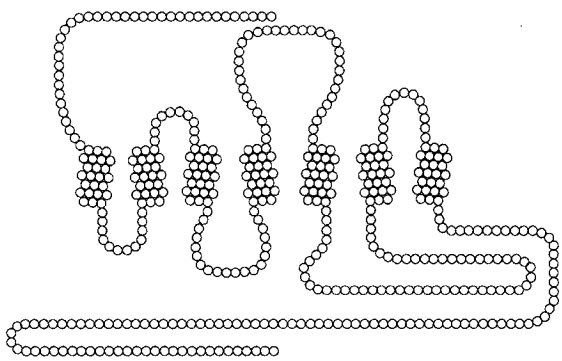
and dopamine affinity. This study demonstrated that the CT of D1-like receptors which is unlikely to play a direct role in the docking of extracellular ligands regulates receptor conformation responsible for the binding of dopamine (Jackson et al., 2000). However, this region displayed little effect on the binding affinity of inverse agonists, suggesting that inverse agonist binding is not dependent on the CT-induced conformational changes of the receptor and thus must be modulated by other residues outside of this cytoplasmic region. Furthermore, in agreement with the TRL study, the CT chimera failed to elicit a switch in the agonist-mediated maximal activation of AC at D1-like receptors (Jackson et al., 2000).

Extracellular domains of peptidergic receptors have been documented to play a key role in their function and chimeric studies of both the mu- and delta-opiod receptors have identified the third extracellular loop as a key determinant in ligand binding (Varga et al., 1996; Dietrich et al., 1998). In support of this a recent study has shown that the EL3 region plays a functional role in the regulation of ligand binding and G protein activation by the β2-adrenergic receptor (Zhao et al., 1998). There is evidence suggesting that extracellular domains can mediate, through conformational changes, the intracellular coupling of receptors to G protein (Fernandez and Puett, 1996; Zhao et al., 1998). However, it remains unclear whether this region is involved in regulating binding properties of different classes of pharmacological ligands, i.e. agonists, antagonists, and inverse agonists.

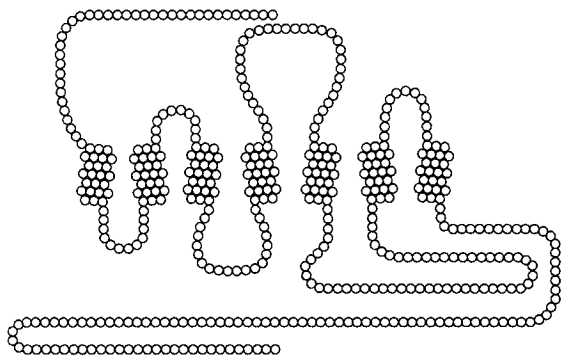
To address the issue of a potential role of extracellular domains in mediating ligand binding and G protein coupling properties, I have engineered

Figure 5 - Schematic representation of the wild-type and EL3 chimeric D1 and D5 receptors.

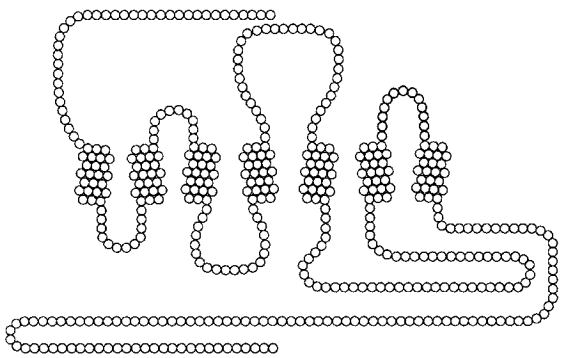
Putative topology of the wild-type D1 (red circles) and D5 receptor (blue circles), D1-EL3D5 and D5-EL3D1 is represented. Alignment of the primary structure corresponding to the EL3 region of the human D1 and D5 receptors is shown. Identical amino acids found between the wild-type receptor sequences are indicated with an asterisk.



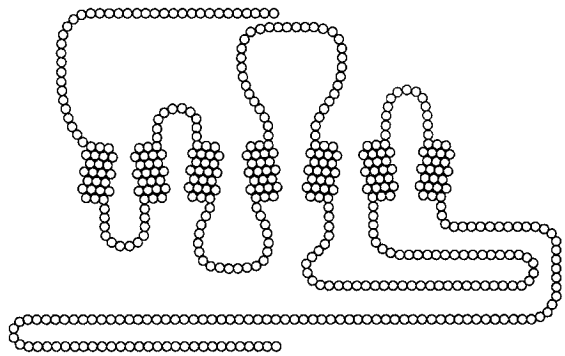
hD1



hD5



hD1-EL3D5

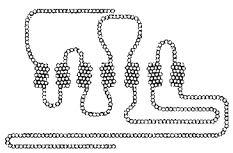
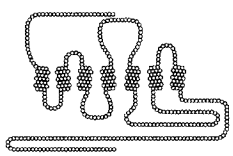
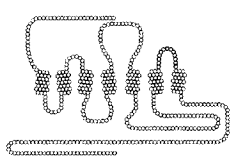
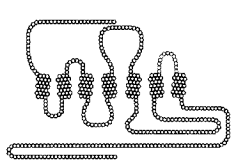


hD1-EL3D5

	TM6	EL3	TM7
hD1	FILNCMVPFC-GSGETQP--F-CIDSNTFDVFVW		
hD5	FILNCILPFCSGHPEGPPAGFPCVSETTFDVFVW		
	********** * * * * * *******		

Table 2 - Equilibrium constants and maximal binding capacity (B_{MAX}) values for wild-type and EL3 chimeric D1-like receptors.

K_D, K_I, and B_{MAX} values are expressed as geometric and arithmetic means, respectively. Means are from three to seven experiments done in duplicate determinations. [3H]-SCH, N-[methyl-^{3}H]SCH23390; DA, dopamine.

	K_D (nM) [3H]-SCH23390	K_I (nM) Dopamine	B_{MAX} (pmol/mg prot.)
 **D1**	0.48 ± 0.02	6897 ± 391	14.0 ± 2.1
 **D5**	0.62 ± 0.03	668 * ± 22.2	18.4 ± 1.5
 **D1-EL3D5**	0.41 ± 0.02	4315 *# ± 151	49.6 *# ± 5.4
 **D5-EL3D1**	**0.53** ± 0.02	1147 *#Ψ ± 35.1	6.7 *#Ψ ± 1.8

* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with D5
Ψ $p < 0.05$ when compared with D1-EL3D5

two chimeric D1 and D5 receptors, which carry a more discrete domain of the TRL cassette, namely the EL3 region. I hypothesize that the low degree of identity (38%) found between the EL3 of the D1 and D5 receptors, as shown in Figure 5, may suggest an important structural role for this region in the inverse agonist binding and agonist-mediated maximal activation of AC at D1-like receptors.

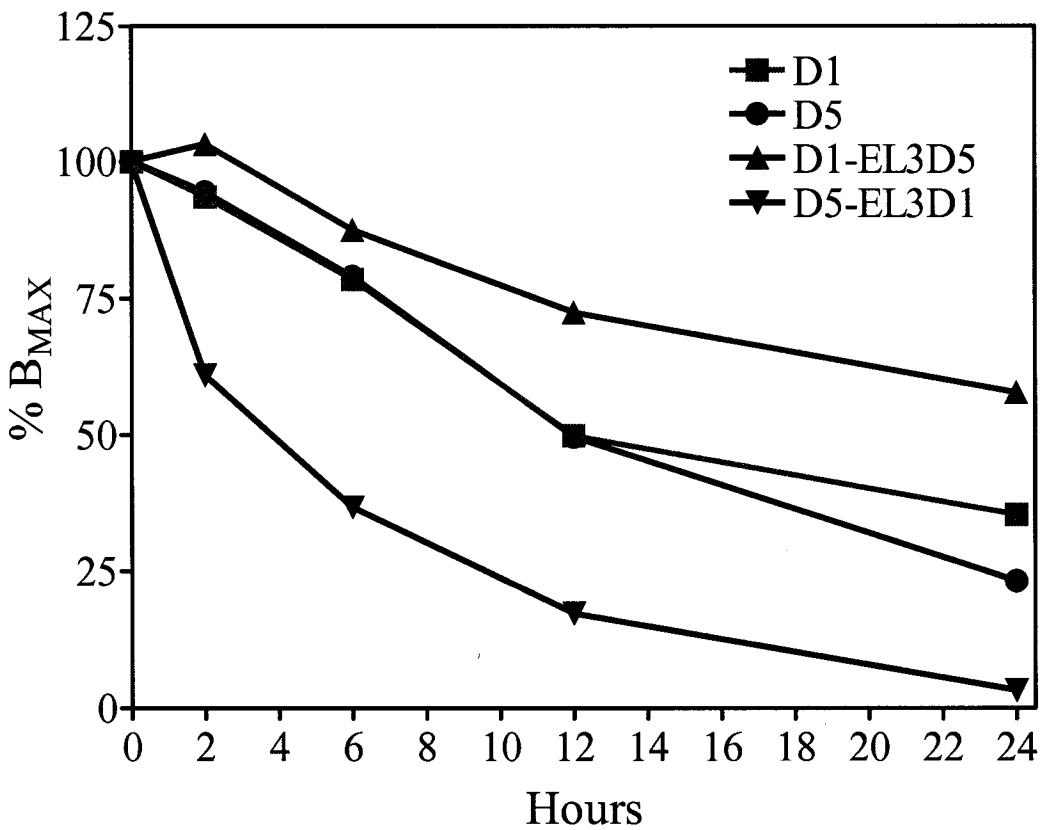
2.2 Results

2.2.1 The EL3 Region Modulates Receptor Expression, Dopamine Affinity and Agonist-Independent Activity of D1-like Receptors

D1-EL3D5 and D5-EL3D1 chimeric receptors were constructed using a polymerase chain reaction-based overlap extension method (Figure 5, Table 2). The binding affinity of N-[methyl-^{3}H]SCH23390 remains unchanged for the chimeric receptors. These results suggest that the chimeric receptors retain a proper protein folding necessary for cell expression and binding of ligands. In HEK293 cells, these chimeric receptors display high levels of expression albeit varying amounts. The D1-EL3D5 chimera expresses at B_{MAX} values 3-fold higher than the wild-type D1 receptor. In contrast, the D5-EL3D1 chimera expresses at B_{MAX} values 3-fold lower than wild-type D5 receptor (Table 2). By incubating prepared membranes at 37°C over a 24-hour period I demonstrate the varying degree of thermal stability of these wild-type and chimeric receptors (Figure 6). I show that D1-EL3D5, which expresses at higher levels, retains its ability to bind

Figure 6 - Thermal stability of wild-type and EL3 chimeric D1-like receptors.

HEK293 cell membranes expressing the D1, D5, D1-EL3D5, and D5-EL3D1 were incubated at 37 ^{o}C in the presence of protease inhibitors for the indicated time (0 – 24 hours). Residual N-[methyl-^{3}H]SCH23390 binding was determined using a saturating concentration of the radioligand, and is expressed as a percentage of control binding at t = 0.


125
100
75
50
25
0
% B_{MAX}
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours
D1
D5
D1-EL3D5
D5-EL3D1

N-[methyl-^{3}H]SCH23390 with a B_{MAX} level higher than wild-type receptors. In contrast, D5-EL3D1 exhibits almost a full loss in its ability to bind N-[methyl-^{3}H]SCH23390, suggesting that it is unstable and more prone to unfolding and/or degradation. Similar finding were recapitulated using membranes incubated at 21°C (data not shown).

Although the dopamine affinity at these chimeric receptors is not swapped as seen with either the TRL or cytoplasmic tail chimera, radioligand binding studies indicate that dopamine affinity for the D1-EL3D5 is increased when compared with the wild-type D1 receptor. In contrast, D5-EL3D1 exhibits a loss of dopamine affinity (Table 2). The modulation of dopamine affinity translates to an effect on dopamine potency (as indexed using EC_{50} values) in cells expressing these chimeric receptors. As previously described, dopamine has a ~10 fold lower potency at the D1 receptor in comparison with the D5 subtype (Figure 7) (Tiberi and Caron, 1994; Jackson et al., 2000). In comparison with its wild-type cognate D1 receptor the D1-EL3D5 chimera displays a gain in dopamine potency. In contrast, the D5-EL3D1 chimera displays a loss of dopamine potency when compared to its wild-type cognate D5 receptor (Figure 7).

The agonist-independent activity (constitutive activity) of the D1-EL3D5 and D5-EL3D1 chimera receptors was studied in intact HEK293 cells expressing similar levels of receptors. The D5-EL3D1 mutant receptor displays a loss of agonist-independent activity in comparison with the wild-type D5 receptor (Figure 8A). In contrast, the D1-EL3D5 chimeric receptor exhibits a gain of constitutive activation as compared with the wild-type D1 receptor.

Figure 7 - Dose-response curves of DA for AC stimulation by wild-type and EL3 chimeric D1-like receptors.

HEK 293 cells were transfected with wild-type or chimeric D1-like receptors. The B_{MAX} values in pmol/mg membrane protein (expressed as the arithmetic mean ± S.E.) were 3.1 ± 0.6 (D1), 2.9 ± 0.3 (D5), 4.7 ± 0.2 (D1-EL3D5), and 2.8 ± 0.3 (D5-EL3D1). Intracellular cAMP levels were measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA as described in the Methods section and plotted as a function of log of DA concentrations. Each point is the arithmetic mean ± S.E. of five experiments done in triplicate determinations and expressed as percentage of maximal response. Nonlinear regression of the data was performed using Sigmoidal dose-response (variable slope) with constrained hill slope factor using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

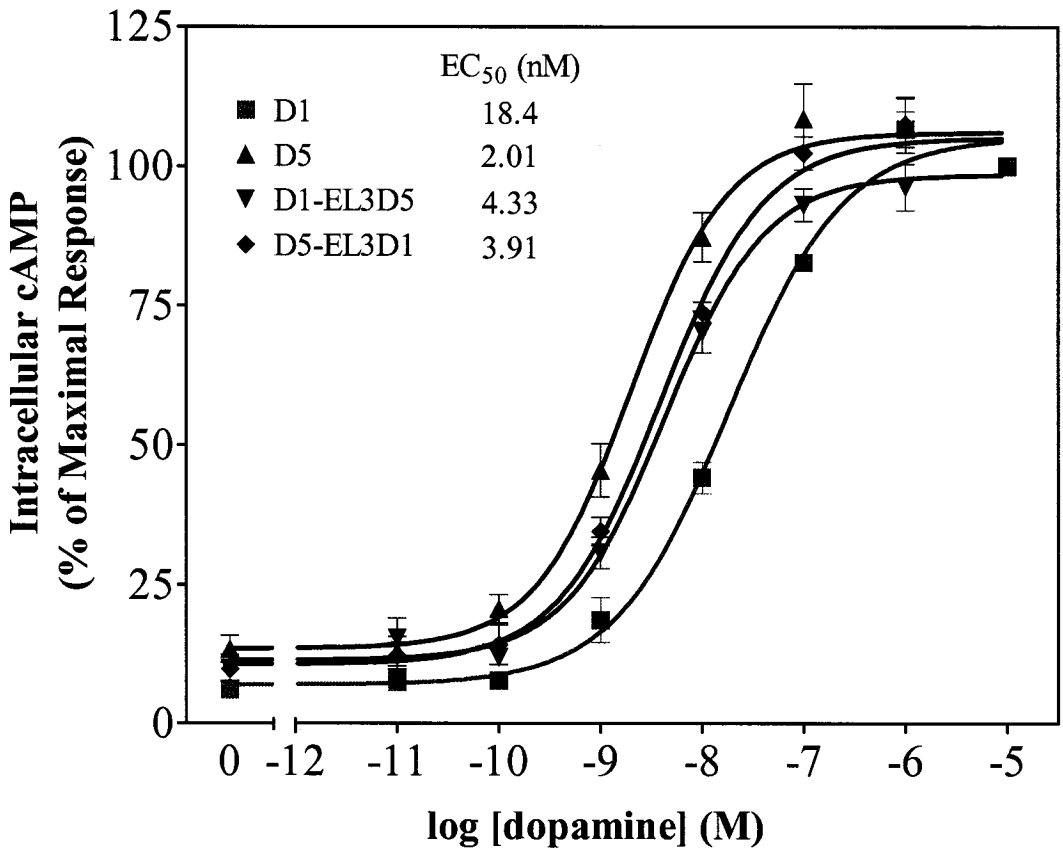
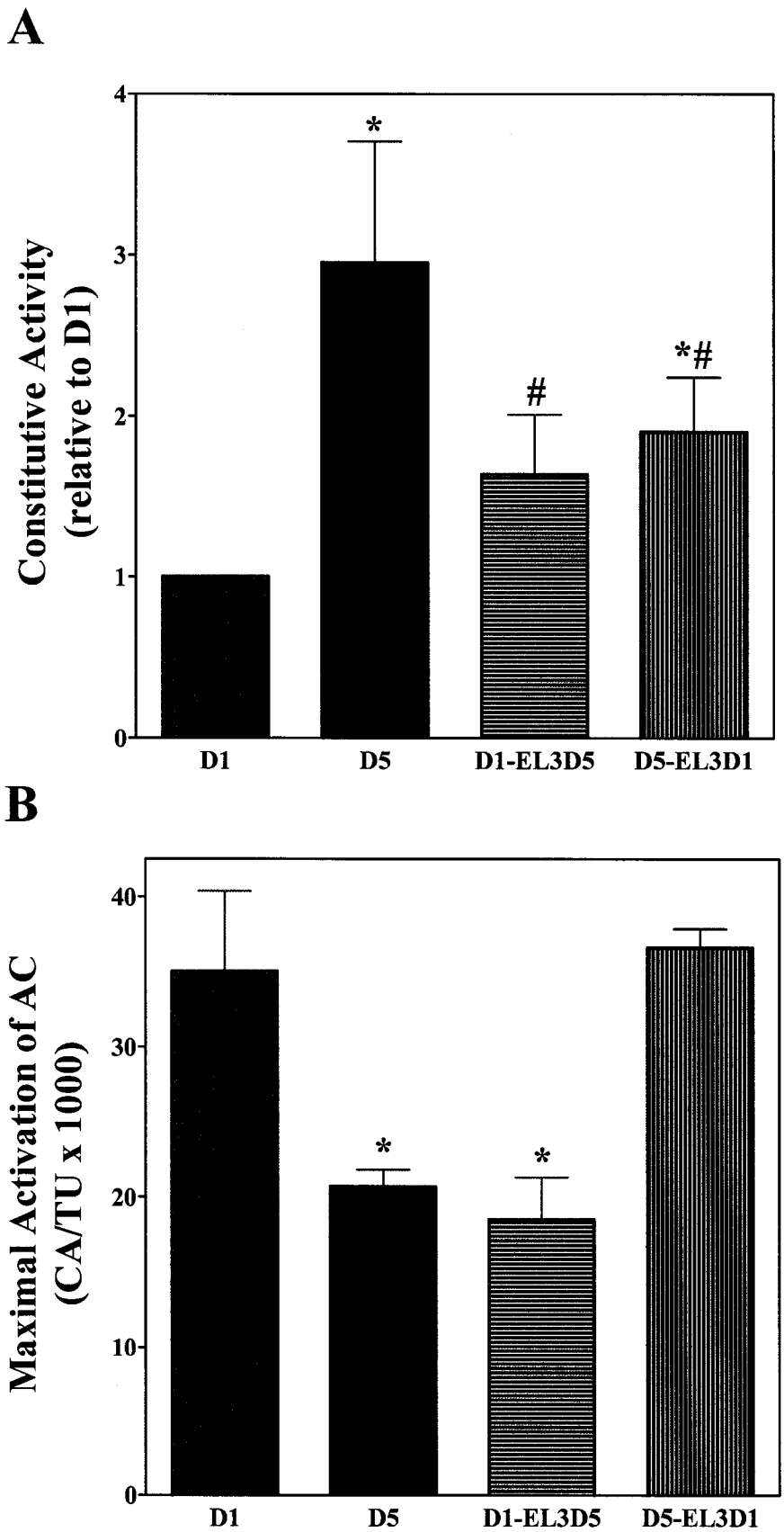

125
100
75
50
25
0
Intracellular cAMP
(% of Maximal Response)
EC50 (nM)
D1 18.4
D5 2.01
D1-EL3D5 4.33
D5-EL3D1 3.91
0 -12 -11 -10 -9 -8 -7 -6 -5
log [dopamine] (M)

Figure 8 - Agonist-independent and dopamine-mediated maximal activation of adenylyl cyclase at wild-type D1 and EL3 chimeric receptors.

A. Basal levels of adenylyl cyclase activity were determined in single wells of a 6-well dish using whole cell cAMP assays and calculated relative to D1 receptor. Data are expressed as arithmetic mean ± S.E. of seven experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 7.7 ± 1.4 (D1), 7.8 ± 0.7 (D5), 8.6 ± 3.4 (D1-EL3D5), and 5.7 ± 0.4 (D5-EL3D1). B. Maximal activation of adenylyl cyclase was determined in single wells of a 6-well dish using a final concentration of 10 μM dopamine and whole cell cAMP assays. Data are expressed as arithmetic mean ± S.E. of four to seven experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 5.3 ± 1.3 (D1), 6.1 ± 1.1 (D5), 4.4 ± 1.1 (D1-EL3D5), and 4.8 ± 0.3 (D5-EL3D1).



* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with D5

2.2.2 The EL3 Region of the D1 and D5 Receptors Contributes to the D1-like Phenotypic Expression of Dopamine-mediated Maximal Activation of AC

HEK293 cells expressing similar receptor levels of wild-type or chimeric receptors were stimulated with 10 μM dopamine, a concentration that produces a maximal activation of adenylyl cyclase. The wild-type D1 receptor elicits a significantly higher maximal activation of adenylyl cyclase than the wild-type D5 receptor (Figure 8B) (Tiberi and Caron, 1994; Jackson et al., 2000). Stimulation of HEK293 cells expressing the D1-EL3D5 chimeric receptor with 10 μM dopamine leads to a maximal activation of adenylyl cyclase that is indistinguishable from that elicited by the wild-type D5 receptor (Figure 8B). Meanwhile, stimulation of the D5-EL3D1 mutant receptor with 10 μM dopamine leads to a maximal activation of adenylyl cyclase that is indistinguishable from the maximal activation elicited by the wild-type D1 receptor (Figure 8B).

2.2.3 The EL3 Chimeric Receptors Delineate a Structural Domain Responsible for D1-like Subtype Specific Inverse Agonist Affinity

Competition studies were performed to determine whether the EL3 domain contains the underlying structural requirements involved in the binding of inverse agonists (antipsychotics) to wild-type human D1 and D5 receptors. I evaluated four different chemical classes of inverse agonists: 1) pentacyclic dibenzepine ((+)-butaclamol) 2) diphenylbutylpiperidine (fluspirilene) 3) thioxanthene (Z-flupentixol, E-flupentixol, thiothixene) 4) phenothiazine

(trifluoperazine, thioridazine) (Figure 9). As previously described, I show that the inverse agonist Z-flupentixol exhibits a higher affinity at the D1 subtype in comparison with the D5 receptor (Table 3) (Jackson et al., 2000). The D1-EL3D5 chimera displays an affinity for Z-flupentixol that is highly reminiscent of the binding affinity observed at the wild-type D5 receptor. In an opposite fashion, the D5-EL3D1 chimera binds Z-flupentixol with an affinity indistinguishable to the one measured at the wild-type D1 receptor. Similarly to Z-flupentixol, I report that the exchanged EL3 leads to a swap in the binding affinity of all inverse agonists tested at the D1-like dopaminergic receptors (Table 3).

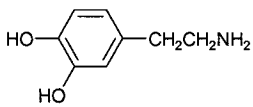
2.2.4 Regulation of FSK-Stimulated Adenylyl Cyclase Activity by D1-like Receptors and Antipsychotic Drugs

The antipsychotic-mediated inverse agonism was assessed in HEK293 cells expressing wild-type or chimeric receptors using whole cell cAMP assays. A potential limitation of these studies is the low basal intracellular cAMP levels in D1 and D5 transfected cells. The low basal intracellular cAMP levels renders difficult the analysis of dose-response curves of inverse agonists for receptor constitutive activity inhibition. To determine more accurately the inverse agonism properties of antipsychotic drugs I took advantage of the use of forskolin (FSK), a direct activator of adenylyl cyclase (Seamon and Daly, 1981). Indeed, previous studies have shown that activated Gs proteins potentiate FSK stimulated AC activity (Harry et al., 1997; Sunahara et al., 1997). I then reasoned that the degree of constitutive activity elicited in HEK293 cells transfected with Gs-coupled

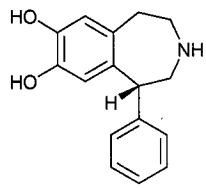
Figure 9 - Structural formula of D1-like ligands.

In HEK293 cells the following drugs are described as either agonists, partial agonists, or inverse agonists. Tested D1-like agonists agonists are dopamine, SKF-38393, apomorphine, and fenoldopam. SCH23390 behaves as a D1-like partial agonist. Tested D1-like inverse agonists are (+)-butaclamol, Z-flupentixol, E-flupentixol, thiothixene, trifluoperazine, thioridazine, and fluspirilene.

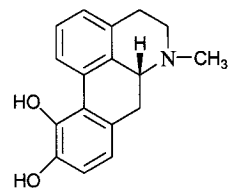
Dopamine



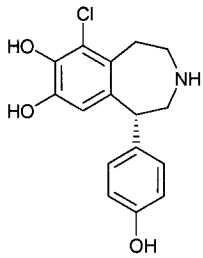
SKF-38393



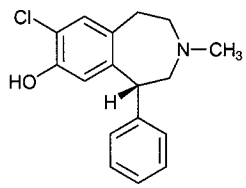
Apo-Morphine



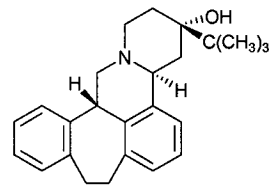
Fenoldopam



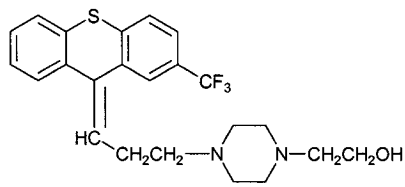
SCH-23390



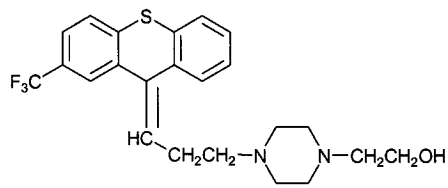
(+) Butaclamol



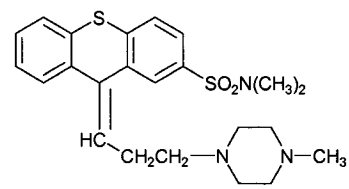
Z-Flupentixol



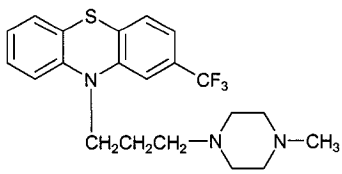
E-Flupentixol



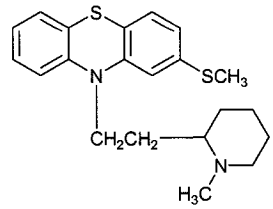
Thiothixene



Trifluoperazine



Thioridazine



Fluspirilene

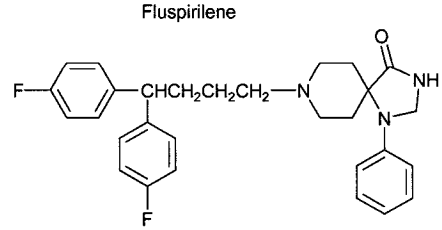
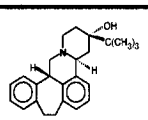
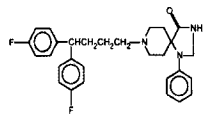
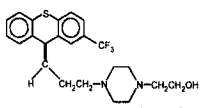
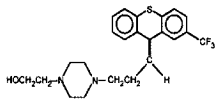
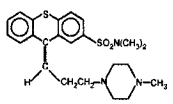
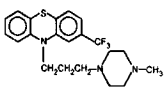
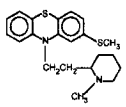


Table 3 – Equilibrium Inhibition constant (K_I) for inverse agonists at wild-type and EL3 chimeric D1-like receptors.

K_I values (nM) are expressed as geometric means and are from three to six experiments done in duplicate determinations. N-[methyl-^{3}H]SCH23390 was used as a radiotracer.

	D1	D5	D1-EL3D5	D5-EL3D1
 (+) Butaclamol	0.68 ± 0.09	15.0 * ± 0.32	3.45 *# ± 0.30	1.90 *#Ψ ± 0.18
 Fluspirilene	496 ± 75.2	1209* ± 79.6	992 * ± 51.2	465 #Ψ ± 3.19
 Z-Flupentixol	2.47 ± 0.18	4.45 * ± 0.25	5.81 * ± 0.14	2.09 #Ψ ± 0.11
 E-Flupentixol	326 ± 6.51	553 * ± 26.8	863 *# ± 17.1	251 *#Ψ ± 2.06
 Thiothixene	53.9 ± 5.97	281 * ± 16.7	152 *# ± 7.40	76.8 #Ψ ± 6.25
 Trifluoperazine	34.3 ± 4.00	41.8 ± 2.01	77.3 *# ± 0.56	16.7 *#Ψ ± 1.53
 Thioridazine	64.6 ± 2.31	200 * ± 25.7	232 * ± 15.4	68.9 #Ψ ± 2.44

* $p < 0.05$ when compared with D1

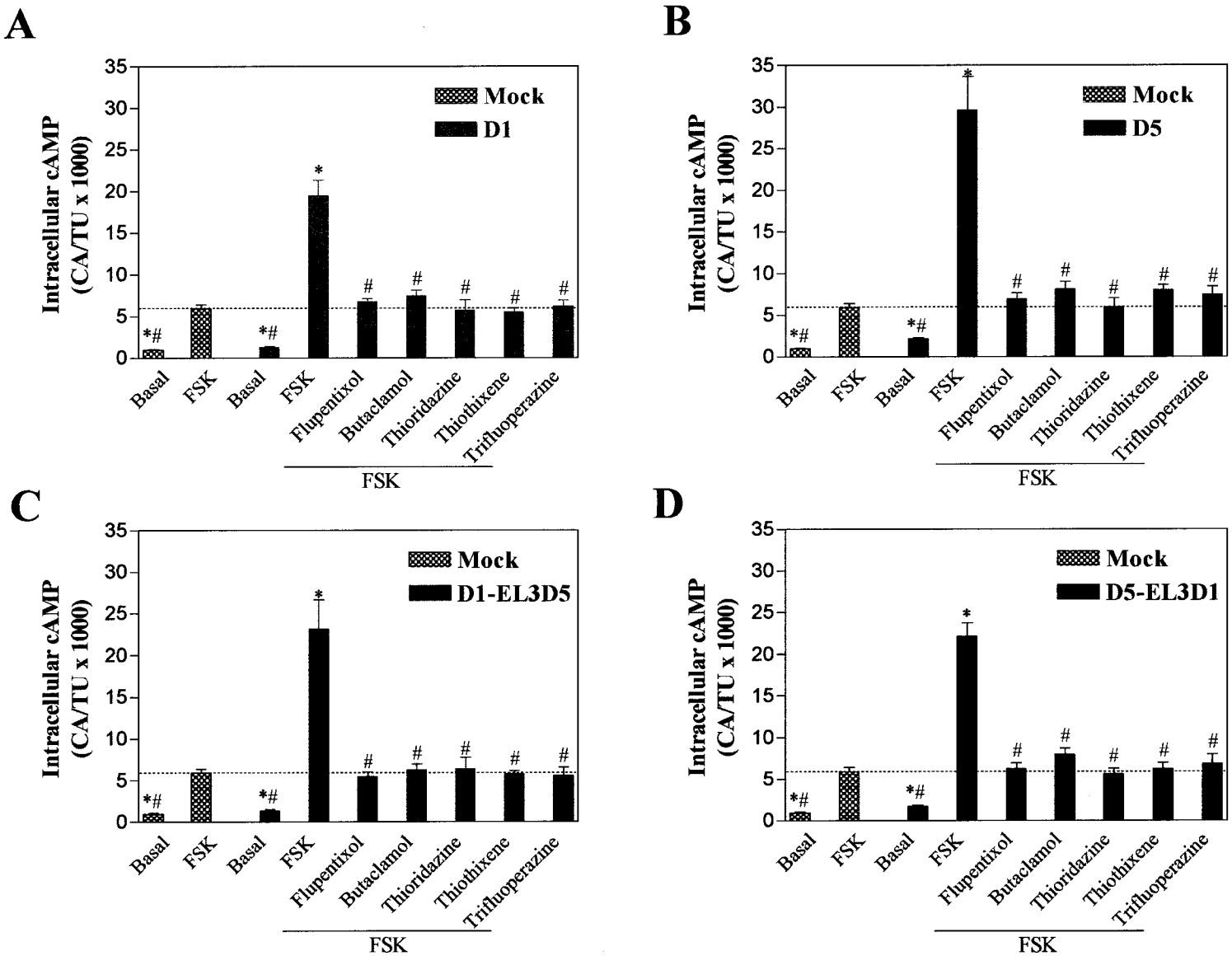
\# $p < 0.05$ when compared with D5

Ψ $p < 0.05$ when compared with D1-EL3D5

receptors would increase the extent of FSK-induced stimulation of adenylyl cyclase. Indeed, my results show that in cells expressing similar levels of D1 or D5 receptors FSK-induced stimulation of adenylyl cyclase is significantly increased in comparison with mock transfected cells. The degree of FSK-induced stimulation of AC is linearly proportional with the expression level and agonist-independent activity of Gs-coupled receptors (data not shown). In agreement with a higher agonist-independent activity of D5 receptors, my results demonstrate that in the absence of dopamine, FSK-stimulated HEK293 cells expressing D5 receptors exhibit a greater activation of AC in comparison with cells expressing D1 receptors (Figures 10A and 10B). Interestingly, potentiation of FSK-induced stimulation of adenylyl cyclase activity has been observed in the absence of agonist stimulation with other G_S-linked GPCRs, particularly the histamine H_2 and thyrotopin stimulating hormone (TSH) receptors expressed in HEK293, Sf9, or COS-7 cells (Alewijnse et al., 1997). I then took advantage of wild-type or chimeric receptor transfected HEK293 cells treated with FSK to test a series of antipsychotic drugs for their inverse agonism properties. I demonstrated that in HEK293 cells all of the tested antipsychotic drugs display full inverse agonism at both wild-type and chimeric receptors (Figure 10A-C). Full inverse agonism was indexed as the inhibition of FSK-stimulated AC activity in receptor transfected cells to a level consistent with FSK-stimulated AC activity in mock transfected cells.

Figure 10 - Inverse agonism of antipsychotic drugs at wild-type and EL3 chimeric D1-like receptors.

HEK 293 cells were transfected with wild-type or chimeric D1-like receptors. The B_{MAX} values in pmol/mg membrane protein (expressed as the arithmetic mean ± S.E.) were 9.7 ± 1.1 (D1), 8.6 ± 1.1 (D5), 13.9 ± 1.9 (D1-EL3D5), and 4.8 ± 0.5 (D5-EL3D1). FSK-stimulated intracellular cAMP levels were measured in single wells of a 6-well dish in the absence or presence of antipsychotic drug as described in the Methods section. Each point is the arithmetic mean ± S.E. of 14 to 15 experiments done in triplicate determinations.



* $p < 0.05$ when compared with FSK-stimulated Mock

\# $p < 0.05$ when compared with FSK-stimulated R (D1, D5, D1-EL3D5, or D5-EL3D1)

2.2.5 Dose-response Curves for Inhibition of Constitutive Activity of Wild-type and Chimeric Receptors by Antipsychotic Drugs

Inverse agonist potency of the antipsychotic drugs, as indexed by the inhibitory constant (IC_{50}), was modulated by the exchange of EL3 domain of D1 and D5 receptors (Figure 11). In agreement with the extended ternary complex model, the inverse agonists (+)-butaclamol and thiothixene exhibited higher potency at the D1 receptor, consistent with my results showing that these inverse agonists displayed increased affinity at the D1 receptor in comparison with the D5 receptor (Table 3, Table 4 and Figure 11). In contrast, the inverse agonists Z-flupentixol and trifluoperazine which also displayed a higher affinity at the D1 receptor did not discriminate between the D1 and D5 receptors on the basis of potency (Table 4 and Figure 11). Nevertheless, using chimeric receptors my results show that the D5-EL3 domain results in a decreased potency while the D1-EL3 domain results in increased potency at the D1 and D5 receptors, respectively. These results clearly demonstrate that a loss of inverse agonist binding affinity at the D1-EL3D5 chimeric receptor translates directly to a loss in potency. Conversely, a gain of inverse agonist binding affinity at the D5-EL3D1 chimeric receptor translates to a gain in potency (Table 3 and Table 4).

2.3 Conclusions

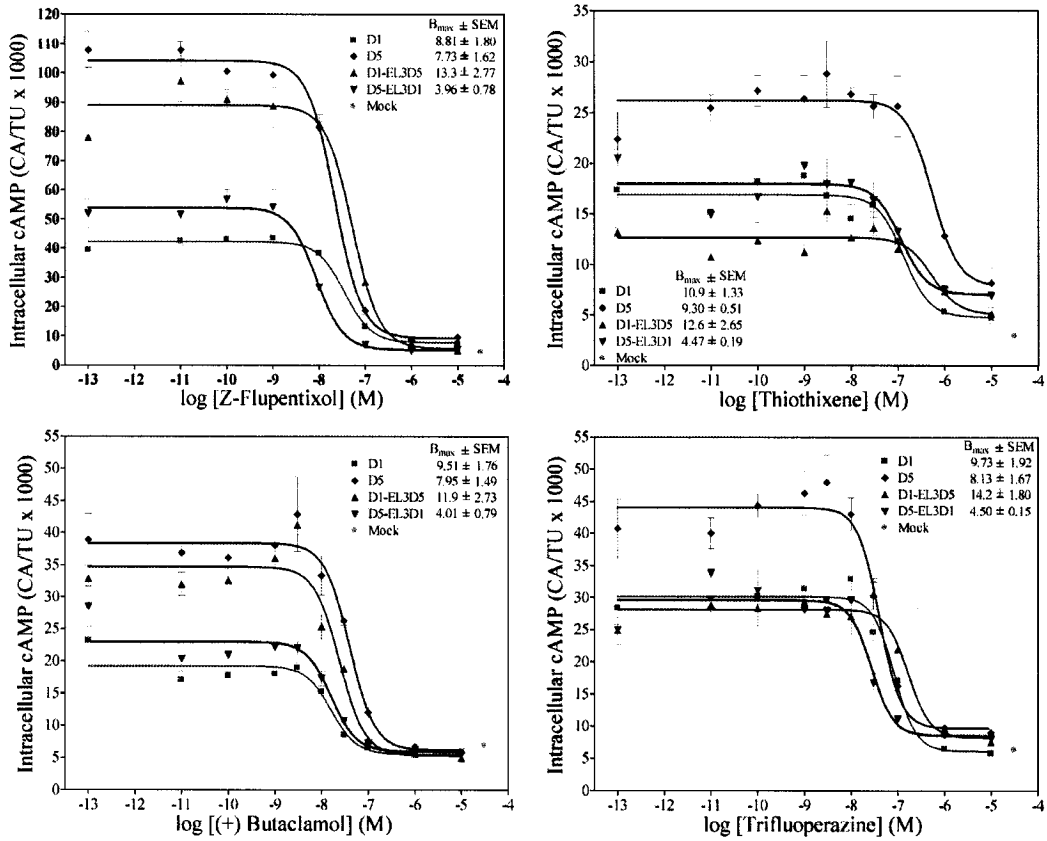
GPCR mutagenesis and modeling studies have proposed that movement of extracellular loops may constrain the accessibility to transmembrane binding sites

Figure 11 - Representative dose-response curves for antipsychotic drugs displaying inverse agonism at wild-type and EL3 chimeric D1-like receptors.

HEK 293 cells were transfected with wild-type or chimeric D1-like receptors. Intracellular cAMP levels were measured in single wells of a 12-well dish with increasing concentrations of antipsychotic drug and plotted as a function of log of [antipsychotic drug] concentrations. Representative curves are reported where each point is the arithmetic mean ± S.E. of a single experiment done in triplicate determinations. Nonlinear regression of the data was performed using Sigmoidal dose-response (variable slope) with constrained hill slope factor using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Table 4 - Inhibition constant (IC_{50}) values for antipsychotic drugs displaying inverse agonism at wild-type and EL3 chimeric D1-like receptors.

HEK 293 cells were transfected with wild-type or chimeric D1-like receptors. Intracellular cAMP levels were measured in single wells of a 12-well dish with increasing concentrations of antipsychotic drug. Values are reported as arithmetic mean ± S.E. of three to six experiments.



Inhibition constants (IC_{50}), nM

	D1	D5	D1-EL3D5	D5-EL3D1
Z-Flupentixol	50.7 ± 10.8	31.9 ± 6.70	90.3 *# ± 15.9	22.1 Ψ ± 4.60
Thiothixene	212 ± 48.4	630 * ± 25.0	740 *# ± 122	447 ± 152
(+) Butaclamol	14.4 ± 1.02	40.5 * ± 4.71	26.5 *# ± 0.95	21.1 # ± 4.39
Trifluoperazine	127 ± 13.6	73.6 ± 16.1	631 *# ± 183	61.3 Ψ ± 19.7

* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with D5
Ψ $p < 0.05$ when compared with D1-EL3D5

thus modulating ligand selectivity (Kobilka et al., 1988; Ostrowski et al., 1992; Strader et al., 1994; Mizobe et al., 1996; Colson et al., 1998; Dietrich et al., 1998; Waugh et al., 2001). Furthermore, studies have shown that extracellular domains, including EL3, can modulate receptor conformations responsible for ligand-mediated signaling and constrain the receptor into an inactive state (Fernandez and Puett, 1996; Zhao et al., 1998; Palczewski et al., 2000; Karnik et al., 2003).

My study indicates that the chimeric D1-EL3D5 and D5-EL3D1 receptors undergo proper protein folding for binding N-[methyl-3H]SCH23390 with high affinity similar to that of wild-type receptors (Table 2). The thermodynamic stability assay indicates that the EL3 domain plays a role in receptor stability (Figure 6). Specifically, I propose that although these EL3 chimeras can bind ligands and couple to G_S proteins, the EL3 domain may regulate the vulnerability of these receptors for denaturation (unfolding) or degraded. My results demonstrate that the D1-EL3D5 chimera which expresses at levels 3-fold above the wild-type receptors is more stable over a 24 hour period at 37°C. In contrast, D5-EL3D1 chimera which expresses 3-fold lower in comparison with the wild-type receptors is more prone to denaturation (unfolding) or degraded over a 24-hour period at 37°C (Figure 6). The role of degradation is however unclear. Indeed, my assay used membrane preparations in the presence of protease inhibitors. Therefore, it is more likely that the receptors were denatured (unfolded) rather than degraded.

The receptor conformations induced by the EL3 domain also exhibited effects on ligand binding and G protein coupling properties. My results indicate

that the EL3 domain plays a role in modulating the dopamine affinity of D1 and D5 receptors (Table 2). The observed partial changes in the dopamine affinity were also reflected in agonist potency (Figure 7) and agonist-independent activation of adenylyl cyclase (Figure 8A), presumably resulting from a unique inactive receptor conformation state induced by the EL3 domain. These trends are in line with our previous study using TRL chimeric receptors. However, the TRL study demonstrated a full switch in the agonist binding affinity at D1-like receptors. Taken in tandem, the TRL and EL3 studies suggest that although the EL3 domain can partially modulate these binding and G protein coupling properties, other regions within the TRL must play an important role. Indeed, our group has demonstrated that the cytoplasmic tail (CT) plays a significant role in differentiating between the D1-like subtypes (Jackson et al., 2000). A D1-CTD5 chimera displayed increased agonist binding affinity and agonist-independent adenylyl cyclase activity reminiscent of the D5 wild-type receptor. However, the D1-CTD5 chimera displayed drastically reduced expression and lower dopamine potency in comparison with the D5 receptor. A recent study by our group has described a molecular interplay between EL3 and CT (Tumova et al., 2003). This study demonstrates that the addition of variant residues of the EL3 domain of the D5 receptor into the D1-CTD5 chimera leads to a constitutively active mutant receptor displaying an increased dopamine affinity and potency, along with a rescue of B_{MAX} to levels similar to wild-type receptors (Tumova et al., 2003). These studies demonstrate that EL3 and CT can act together to induce unique agonist-bound receptor conformations.

In the present study, I demonstrate for the first time that the EL3 domain of D1-like receptors plays an important role in regulating inverse agonist binding affinity (Table 3) and dopamine-mediated maximal activation of adenylyl cyclase (Figure 8B). This is in contrast with our TRL chimeric study, which although included the EL3 region, did not exhibit any major effect on the inverse agonist binding affinity or dopamine-mediated maximal activation of adenylyl cyclase properties at the D1-like receptors.

The D1-EL3D5 chimera exhibited a dopamine-mediated maximal activation of adenylyl cyclase similar to that of the D5 receptor whereas the D5-EL3D1 chimera exhibited a dopamine-mediated maximal activation of adenylyl cyclase reminiscent of the D1 receptor (Figure 8B). I propose that the EL3 regulates the dopamine-mediated maximal activation of adenylyl cyclase by controlling intramolecular interactions of D1-like receptors involved in the agonist-bound R* state. The conformational changes induced by EL3 are also crucial in regulating the binding affinity of inverse agonists at D1-like receptors. The D1-EL3D5 and D5-EL3D1 chimeric receptors exhibited affinity for inverse agonists that was reminiscent of their counterpart D5 and D1 receptors, respectively. These results implicate the EL3 domain as a key structural determinant of D1 receptor subtypes for the binding of inverse agonists.

Examination of the binding properties of inverse agonist drugs at the D1-like receptors suggests that the EL3 domain may not necessarily contain the binding determinants. The extent of binding affinity swap was observed to be drug dependent but independent of chemical class. In fact, the results identify

three categories of inverse agonists, those that exhibit a full swap in binding affinity, those that exhibit an amplified swap, and those that exhibit a partial swap. A full swap in binding affinities was observed for the drugs fluspirilene, Z-flupentixol, and thioridazine. The drugs (+)-butaclamol and thiothixene exhibited only a partial swap in binding affinity at the EL3 chimeric receptors while the drugs E-flupentixol and trifluoperazine exhibited a more pronounced swap (Table 3).

On the basis of these results, I propose that the EL3 domain of D1 and D5 receptors modulates the structural arrangement of transmembrane domain binding determinants for inverse agonists or modulates inverse agonist accessibility to the binding pocket. In fact studies on other GPCR systems support the notion that extracellular domain mediate ligand accessibility to the binding pocket and are not involved directly with ligand docking (Colson et al., 1998; Dietrich et al., 1998; Karnik et al., 2003).

All of the antipsychotic drugs tested in this study displayed full inverse agonism at wild-type and chimeric D1-like receptors (Figure 10). In accordance with the extended ternary complex model, the binding of inverse agonists at chimeric receptors is closely related to their potency. Loss of binding affinity at the D1-EL3D5 chimera translated directly to a loss in potency. In contrast, a gain in binding affinity at the D5EL3D1 chimera resulted in a gain of potency (Table 4, Figure 11). Based on the demonstration that antipsychotic drugs exhibit inverse agonism at the D1-like receptors, I would predict that antipsychotic drugs with increased binding affinity would exhibit increased bio-efficacy. Moreover,

development of antipsychotics with increased bioavailability and half-life may contribute to the bio-efficacy of such drugs. Potentially, such inverse agonists would exhibit greater therapeutic benefits in treating diseases associated with increased constitutive activity.

Overall, I have demonstrated that the EL3 plays an important role in regulating the conformational state involved in inverse agonist binding and the dopamine-mediated maximal activation of adenylyl cyclase by the D1 and D5 receptor. Importantly, these effects were not observed with the exchange of TRL, implicating that another domain within the TRL region may be antagonizing the receptor conformations and effects elicited by EL3. In the next study, I explore the underlying role of TM6 and TM7 in regulating EL3 induced receptor conformations.

3. Conformational Interactions of TM6, EL3, and TM7 Domains

3.1 Introduction

In the previous study, I demonstrated that the EL3 domain is responsible for the inverse agonist binding affinity and dopamine-mediated maximal activation of adenylyl cyclase at the D1-like receptors. These effects were not disclosed in the first study using TRL chimera. In the present study, I investigate the role of TM6 and TM7 domains in interfering with the EL3-induced receptor conformations responsible for D1-like inverse agonist binding affinity and dopamine-mediated maximal activation of adenylyl cyclase.

Recently, a study from our group has demonstrated an interplay between EL3 and the cytoplasmic tail (Tumova et al., 2003). Previously our group has reported that D1-like chimeric cytoplasmic tail receptors exhibit almost a full switch in agonist binding affinity and a swap in agonist-independent activity, but did not result in switching agonist potency. Furthermore, I demonstrated that the EL3 domain only partially modulated these properties, suggesting that other regions within the TRL were involved. Tumova et al. (2003) showed that chimera involving both the EL3 and cytoplasmic tail of D1-like receptors demonstrated a full switch in agonist binding affinity, dopamine potency, and agonist-independent activity. However, these cytoplasmic tail/EL3 chimeric studies do not provide a clear insight into why the inverse agonist binding affinity and dopamine-mediated maximal activation of AC are switched using EL3 chimera but not disclosed using TRL chimera. Indeed, the cytoplasmic tail/EL3 chimeric

receptors recapitulated the same inverse agonist affinity observed with EL3 chimera (Tumova and Tiberi, unpublished data).

In light of the evidence obtained from previous studies, I hypothesize that variant residues within the highly conserved TM6 and/or TM7 must be involved in constraining subtype-specific conformations responsible for inverse agonist binding and dopamine-mediated maximal activation of AC. As previously described, TM domains are highly conserved within a GPCR family. Indeed, the D1 and D5 receptors only differ by two amino acids in TM6 and one amino acid in TM7 (Figure 1). In order to examine the role of these two transmembrane domains in context of the EL3 chimera I have engineered four new chimeric receptors. First I engineered chimeric receptors which swapped TM6, EL3 and TM7 between the D1 and D5 receptors; these were named D1-EL3TM6/7D5 and D5-EL3TM6/7D1. Furthermore, to discriminate between the effects of TM6 and TM7, I constructed two other chimeras where only TM6 and EL3 were swapped; these were named D1-EL3TM6D5 and D5-EL3TM6D1.

3.2 Results

3.2.1 Receptor Expression and Ligand Binding at D1-EL3TM6/7D5 and D5-EL3TM6/7D1 Chimeric Receptors

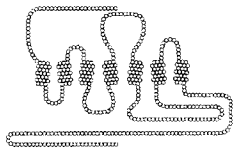
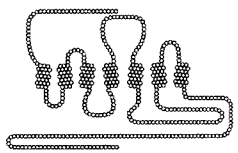
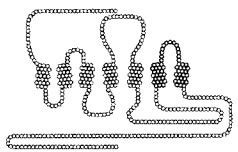
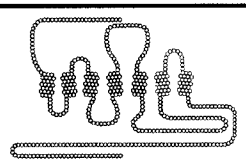
The binding affinities (K_D values) of the radioligand N-[methyl-^{3}H]SCH23390 at wild-type and D1-EL3TM6/7D5 and D5-EL3TM6/7D1 chimeric D1-like receptors obtained using saturation studies are summarized in

Table 5. Results indicate that the chimeric receptors retain their ability to bind N-[methyl-^{3}H]SCH23390 with high affinity and retain also proper protein folding necessary for membrane expression and D1-like ligand binding. In HEK293 cells, these chimeric receptors display high levels of expression albeit varying amounts, reminiscent of the EL3 chimeras. The D1-EL3TM6/7D5 chimera expresses at B_{MAX} values 3-fold higher than the wild-type receptors. In contrast, the D5-EL3TM6/7D1 chimera exhibits B_{MAX} values 3-fold lower in comparison with wild-type receptors (Table 5).

Competition studies were performed to determine whether the TM6/7 domains in conjunction with EL3 could reproduce the full swap in dopamine affinity observed using the TRL chimeras. Furthermore, competition studies were performed to determine whether the effects on inverse agonist binding seen with the EL3 chimeras could be masked by the presence of TM6/7 divergent residues. The competition studies demonstrate that the dopamine binding affinity was not swapped at D1-EL3TM6/7D5 and D5-EL3TM6/7D1 chimeric receptors (Table 5), consistent with our previous studies implicating the cytoplasmic tail as the determinant regulating agonist binding affinities of D1-like receptors. In agreement with my hypothesis, the competition studies for inverse agonists Z-flupentixol and (+)-butaclamol recapitulated the results observed with the TRL chimeras and not the results observed with the EL3 chimeras. Specifically, the binding affinity for Z-flupentixol at the D1-EL3TM6/7D5 and D5-EL3TM6/7D1 chimeric receptors was unchanged when compared with wild-type D1 and D5 receptors, respectively.

Table 5 - Equilibrium constants and maximal binding capacity (B_{MAX}) values for wild-type and EL3TM6/7 chimeric D1-like receptors.

K_D, K_I, and B_{MAX} values are expressed as geometric and arithmetic means, respectively. Means are from three to five experiments done in duplicate determinations. [3H]-SCH, N-[methyl-^{3}H]SCH23390; DA, dopamine; FLU, Z-flupentixol; BUT, (+)-butaclamol; SCH, SCH23390.

	K_D (nM)	K_I (nM)				B_{MAX} (pmol/mg prot.)
	[3H]-SCH	DA	FLU	BUT	SCH	
 **D1**	0.51 ± 0.01	4620 ± 424	9.70 ± 1.26	8.82 ± 0.56	0.54 ± 0.03	18.9 ± 2.69
 **D5**	0.75 * ± 0.05	430 * ± 25.5	18.4 * ± 0.31	43.0 * ± 2.34	0.91 * ± 0.02	23.2 ± 2.32
 **D1-EL3TM6/7D5**	0.79 * ± 0.04	4170 # ± 393	14.7 ± 0.89	14.3 *# ± 1.11	0.46 # ± 0.02	52.2 *# ± 5.87
 **D5-EL3TM6/7D1**	0.42 #Ψ ± 0.02	546 *Ψ ± 30.4	21.2 * ± 0.54	21.3 *# ± 0.68	0.66 #Ψ ± 0.02	5.46 *#Ψ ± 0.52

* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

Ψ $p < 0.05$ when compared with D1-EL3TM6/7D5

3.2.2 Characterization of D1-EL3TM6D5 and D5-EL3TM6D1 Receptor Expression and Agonist Binding Properties

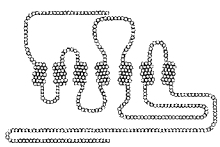
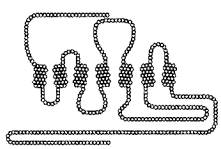
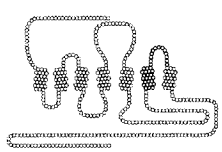
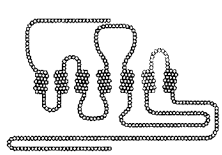
The pattern of D1-EL3TM6D5 and D5-EL3TM6D1 receptor expression and dopamine affinity is reminiscent of the EL3 chimeric receptors (Table 6). Both D1-EL3TM6D5 and D5-EL3TM6D1 bind N-[methyl-3H]SCH23390 with high affinity and express high receptor number. Nevertheless, receptor expression (B_{MAX}) of D1-EL3TM6D5 was 3-fold higher than wild-type D1 while D5-EL3TM6D1 receptor displays a B_{MAX} value that is 3-fold lower than wild-type D5 receptors. Competition studies for the agonist dopamine suggest that the EL3TM6 chimeras do not display any major change in dopamine binding affinity (Table 6). However, the small trend observed in dopamine affinity was reminiscent of the EL3 chimeric receptors. These results suggest that TM6 residues are not modulating EL3-induced receptor conformation for dopamine binding.

3.2.3 TM6 Residues Inhibit the EL3-Induced Effects on Inverse Agonist Binding Properties

Competition studies were performed to determine the role of a molecular interplay between the variant TM6 residues and EL3 in mediating the structural requirements for inverse agonist / antipsychotic binding at wild-type D1 and D5 receptors. I evaluated the same four chemical classes of inverse agonists tested in the EL3 studies (Figure 9). In striking contrast to the EL3 chimeras, the EL3TM6 chimeras do not display a swap in the inverse agonist binding affinities. The D1-EL3TM6D5 chimera displays a binding affinity for flupentixol (Z- and E-

Table 6 - Equilibrium constants and maximal binding capacity (B_{MAX}) values for wild-type and EL3TM6 chimeric D1-like receptors.

K_D, K_I, and B_{MAX} values are expressed as geometric and arithmetic means, respectively. Means are from four to eight experiments done in duplicate determinations.

	K_D (nM) [3H]-SCH23390	K_I (nM) Dopamine	B_{MAX} (pmol/mg prot.)
 **D1**	0.64 ± 0.04	6270 ± 391	14.0 ± 0.42
 **D5**	0.75 ± 0.03	570 * ± 22.2	17.5 ± 0.46
 **D1-EL3TM6D5**	0.51 *# ± 0.02	4990 # ± 151	31.8 *# ± 0.86
 **D5-EL3TM6D1**	0.40 *# ± 0.02	437 *Ψ ± 35.1	3.71 *#Ψ ± 0.22

* $p < 0.05$ when compared with D1
\# $p < 0.05$ when compared with D5
Ψ $p < 0.05$ when compared with D1-EL3TM6D5

stereoisomers), (+)-butaclamol, and fluspirilene that is indistinguishable from the binding affinity observed at wild-type D1 receptors while the D5-EL3TM6D1 chimera binds both flupentixol stereoisomers and (+)-butaclamol with a binding affinity resembling the one measured at wild-type D5 receptors (Table 7). In contrast, the binding affinity of fluspirilene at the D5-EL3TM6D1 chimera was indistinguishable from the binding affinity observed at wild-type D1 receptors, which is reminiscent of results obtained from studies using EL3 chimeric receptors. Furthermore, the inverse agonists thiothixene and thioridazine exhibited only a partial modulation of binding affinities at the D1-EL3TM6D5 and D5-EL3TM6D1 chimera (Table 7).

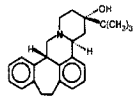
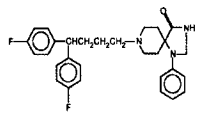
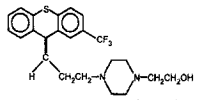
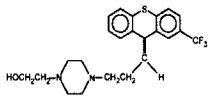
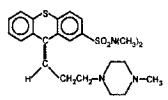
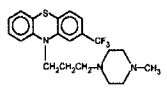
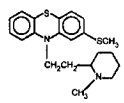
3.2.4 TM6 Residues Inhibit the Effects on Dopamine-mediated Maximal Activation of Adenylyl Cyclase Activity Mediated by EL3

The agonist-independent activity of the D1-EL3TM6D5 and D5-EL3TM6D1 chimeric receptors was studied in intact HEK293 cells expressing similar levels of receptors. The D5-EL3TM6D1 mutant receptor displays a loss of agonist-independent activity in comparison with the wild-type D5 receptor. In contrast, the D1-EL3D5 chimeric receptor exhibits a gain in the extent of constitutive activation as compared with the wild-type D1 receptor (Figure 12A). These results are consistent with those observed for the TRL and EL3 chimera, as described in our previous studies.

HEK293 cells expressing similar levels of wild-type or chimeric receptors were stimulated with 10 μM dopamine, a concentration that produces a maximal

Table 7 – Equilibrium Inhibition constant (K_I) for inverse agonists at wild-type and EL3/TM6 chimeric D1-like receptors.

K_I values (nM) are expressed as geometric means from three to nine experiments done in duplicate determinations. N-[methyl-^{3}H]SCH23390 was used as a radiotracer.

	D1	D5	D1-EL3TM6D5	D5-EL3TM6D1
 (+) Butaclamol	1.00 ± 0.14	17.8 * ± 0.07	2.51 # ± 0.07	8.18 *Ψ ± 0.16
 Fluspirilene	361 ± 21.1	1248 * ± 71.9	573 # ± 23.9	578 # ± 34.9
 Z-Flupentixol	2.70 ± 0.15	4.77 * ± 0.21	2.36 # ± 0.01	4.48 *Ψ ± 0.24
 E-Flupentixol	347 ± 8.44	572 * ± 30.5	263 # ± 11.2	582 *Ψ ± 17.7
 Thiothixene	65.4 ± 6.53	292 * ± 13.1	115 *# ± 3.67	109 *# ± 2.66
 Trifluoperazine	29.1 ± 1.23	42.7 ± 2.15	28.3 ± 1.63	26.7 ± 2.65
 Thioridazine	67.8 ± 2.30	228 * ± 14.9	129 *# ± 5.63	99.3 *# ± 2.76

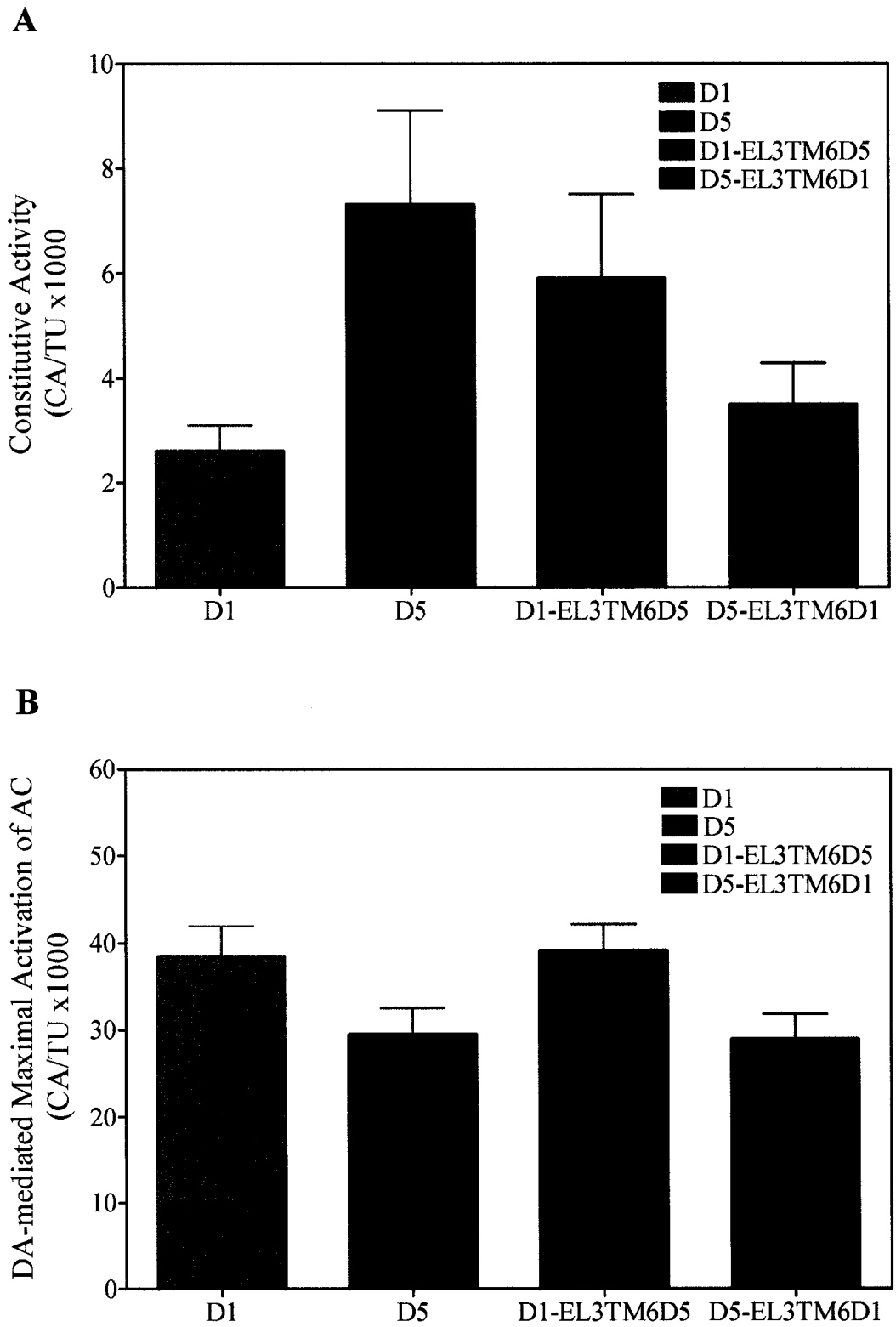
* $p < 0.05$ when compared with D1A

\# $p < 0.05$ when compared with D1B

Ψ $p < 0.05$ when compared with D1AEL3B

Figure 12 - Agonist-independent and dopamine-mediated maximal activation of adenylyl cyclase at wild-type D1 and EL3/TM6 chimeric receptors.

A. Basal levels of adenylyl cyclase activity were determined in single wells of a 6-well dish using whole cell cAMP assays and calculated relative to D1 receptor. Data are expressed as arithmetic mean ± S.E. of four to eight experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 14.9 ± 1.8 (D1), 17.9 ± 1.5 (D5), 32.1 ± 2.3 (D1-EL3TM6D5), and 3.8 ± 0.6 (D5-EL3TM6D1). B. Maximal activation of adenylyl cyclase was determined in single wells of a 6-well dish using a final concentration of 10 μM dopamine and whole cell cAMP assays. Data are expressed as arithmetic mean ± S.E. of four to seven experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein (expressed as the arithmetic mean ± S.E.) was 5.2 ± 1.2 (D1), 6.4 ± 0.8 (D5), 7.6 ± 1.6 (D1-EL3D5), and 2.7 ± 0.3 (D5-EL3D1).


A
Constitutive Activity
(CA/TU x1000
10
8
6
4
2
0
D1
D5
D1-EL3TM6D5
D5-EL3TM6D1
D1
D5
D1-EL3TM6D5
D5-EL3TM6D1
B
DA-mediated Maximal Activation of AC
(CA/TU x1000
60
50
40
30
20
10
0
D1
D5
D1-EL3TM6D5
D5-EL3TM6D1
D1
D5
D1-EL3TM6D5
D5-EL3TM6D1

activation of adenylyl cyclase. As previously reported, the wild-type D1 receptor elicits a significantly higher maximal activation of adenylyl cyclase than the wild-type D5 receptor in the presence of 10 μM dopamine (Figure 12B) (Tiberi and Caron, 1994; Jackson et al., 2000; Tumova et al., 2003). In contrast to the EL3 chimera, HEK293 cells expressing the D1-EL3TM6D5 chimeric receptor with 10 μM dopamine leads to a maximal activation of adenylyl cyclase that is indistinguishable from the maximal activation elicited by its parent wild-type D1 receptor (Figure 12B). Similarly, stimulation of the D5-EL3TM6D1 mutant receptor leads to a maximal activation of adenylyl cyclase that is indistinguishable from the maximal activation elicited by its parent wild-type D5 receptor (Figure 12B). The dopamine-mediated maximal activation of AC elicited by the EL3TM6 chimera is reminiscent of results obtained with the TRL chimera and opposite to data obtained with the EL3 chimera, suggesting that the TM6 residues have an inhibitory effect on the dopamine-mediated maximal activation of AC activity mediated by the EL3 domain.

3.3 Conclusions

This study identifies a potential molecular interplay between determinants located in TM6 and EL3. This interplay appears to be important in regulating the activation process of the D1-like receptors and demonstrates that intramolecular interactions are responsible for some of the observed differences between D1 and D5 receptor functional properties. Previously, I have demonstrated that the EL3 domain is a structural determinant responsible for discriminating inverse agonist

binding affinities and dopamine-mediated maximal activation of AC between D1 and D5 receptors. Furthermore, studies from our group have reported an interplay between the cytoplasmic tail and the EL3 in regulating the dopamine binding affinity and constitutive activity at the D1 and D5 receptors (Tumova et al., 2003).

Similarly, I report that the TM6 domain displays interfering effects on the functional properties mediated by EL3. In the present study, I describe potential structural constraints between the EL3 and TM6 domains that are implicated in regulating inverse agonist binding affinity and dopamine-mediated maximal activation of AC at the D1 and D5 receptors. Our TRL chimeric study did not demonstrate any role in mediating the inverse agonist binding affinity and dopamine-mediated maximal activation of AC at the D1-like receptors. This observation was disputed by the EL3 study, which described this receptor region as containing structural determinants for inverse agonist binding affinity and dopamine-mediated maximal activation of AC. Altogether these two studies suggested that other receptor regions within the TRL must be involved in regulating these properties and reconciling the differences observed with the TRL and EL3 chimeras.

I now confirm that the lack of observed effect on the inverse agonist affinity and dopamine-mediated maximal activation of AC using TRL chimeric receptors was a result of the TM6 induced interfering effects on the binding and activation properties regulated by EL3. In this new study, introduction of the TM6 variant residues to the D1-EL3D5 chimeric receptor (D1-EL3TM6D5) reverses the inverse agonist binding affinity to a value highly reminiscent of the D1

receptor. An opposite effect was observed for the D5-EL3D1 chimeric receptors that display higher affinity for inverse agonists while the D5-EL3TM6D1 chimeric receptors exhibit a lower affinity for inverse agonists reminiscent of the D5 receptor. These data would suggest that conformational interactions between the EL3 and TM6 domains are key structural determinants for inverse agonist binding and dopamine-mediated maximal activation of adenylyl cyclase at the D1 and D5 receptors. I propose that the two variant amino acids at the C-terminal end of TM6 may be involved in regulating the D1 and D5 specific phenotypes for inverse agonist binding and dopamine-mediated maximal activation of AC.

4. Role of Specific Amino Acids within the TM6, EL3, and TM7 Domains Delineated using Point Mutations

4.1 Introduction

Mechanisms of GPCR activation involve the release of constraining intramolecular interactions (Karnik et al., 2003). Movement of transmembrane domains has been suggested as essential for the activation of function (Gether et al., 1997; Chen et al., 1999; Sheikh et al., 1999; Ballesteros et al., 2001; Miura et al., 2003). The activation signal is believed to be generated by the extracellular loops, which in turn mediate a TM movement and elicit intracellular GPCR function. Among all studied GPCRs, 52% of inactivating mutations outside of the TM regions are located in the EL2 and EL3 domains (Karnik et al., 2003). Moreover, a consequence of GPCR activation is believed to be a tilt in the TM6 domain (Sheikh et al., 1996; Gether et al., 1997; Sheikh et al., 1999; Ballesteros et al., 2001).

Evidence from our previous chimeric receptor studies supports the notion that the differences observed between the D1-like subtypes are a result of different conformational states. These unique states of receptor activation are mediated by numerous receptor domains including TM6, TM7, EL3, and the cytoplasmic tail. Our studies have demonstrated both positive and negative interplay between these domains. However, due to the nature of chimeric receptors we do not know what specific residues may be involved in mediating these conformational differences.

In the present study, I use a single-point mutation approach to investigate the role of individual amino acids within the EL3, TM6, and TM7 that may be involved in regulating a conformational switch between inactive and active receptor states. Due to the diversity in the EL3 domain of D1-like receptors, I have selected to focus the investigation on the role of proline residues. There is a striking difference in the number and positioning of prolines in the EL3 of D1-like receptors. The EL3 of the D1 receptor contains only two proline residues while the EL3 of the D5 receptor contains five proline residues (Figure 5). Proline residues are known to introduce kinks in the secondary structure of proteins (Palczewski et al., 2000), thus I hypothesize that the receptor conformation induced by proline residues plays a role in regulating the distinguishing functional properties of D1 and D5 receptors. In order to test this hypothesis I have systematically mutated all proline located in the EL3 of D1 and D5 receptor to both alanine and glycine.

Furthermore, our EL3/TM6 study has demonstrated a negative interplay of these two domains. The amino acid sequence of TM6 displays a high degree of identity between D1 and D5 receptors. In fact, these differ only by two amino acids (Figure 1). I hypothesize that the interfering effects of TM6 on the inverse agonist affinity and dopamine-mediated maximal activation of adenylyl cyclase regulated by EL3 results from these two variant amino acids. In order to address which of these two variant amino acids plays a role in mediating the overall conformational state of the receptor, I have generated a series of point mutations

in which either both or a single amino acid has been swapped between the D1 and D5 receptors.

Moreover, mutagenesis studies have demonstrated that the TM7 domain plays a role in the binding of inverse agonists / antagonists at GPCRs (Strader et al., 1994; Waugh et al., 2001; Shi and Javitch, 2002). Thus to investigate the role of TM7 in regulating the functional properties of D1-like receptors, I generated mutant receptors in which the single variant TM7 amino acid was swapped between D1 and D5 subtypes.

4.2 Results

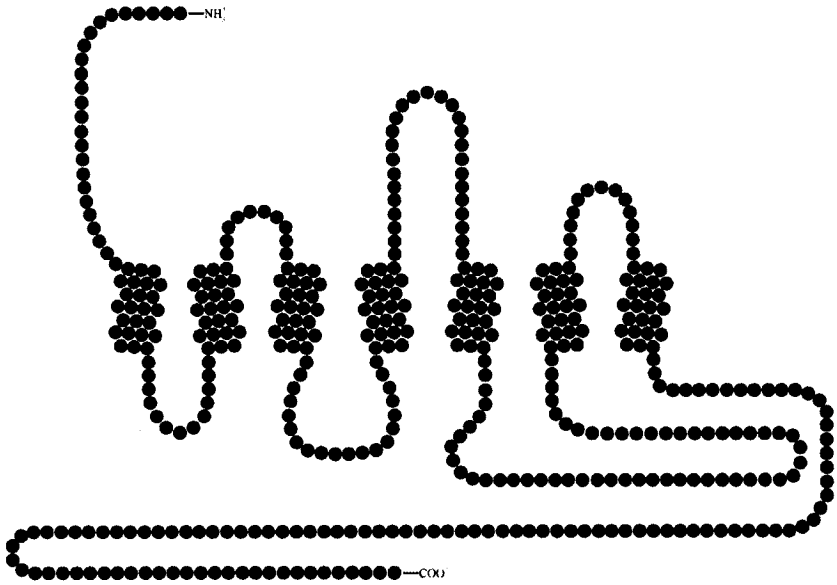
4.2.1 Comparison of Wild-type and Proline Point Mutant Receptors

The series of four D1 and eleven D5 receptor point mutants are illustrated in Figure 13, along with a sequence alignment of TM6 and EL3. Each proline was mutated to alanine and glycine. Resulting from a PCR missense mutation, cysteine at position 335 in the D5 receptor was mutated to tryptophan in the presence of the D5P330G mutation.

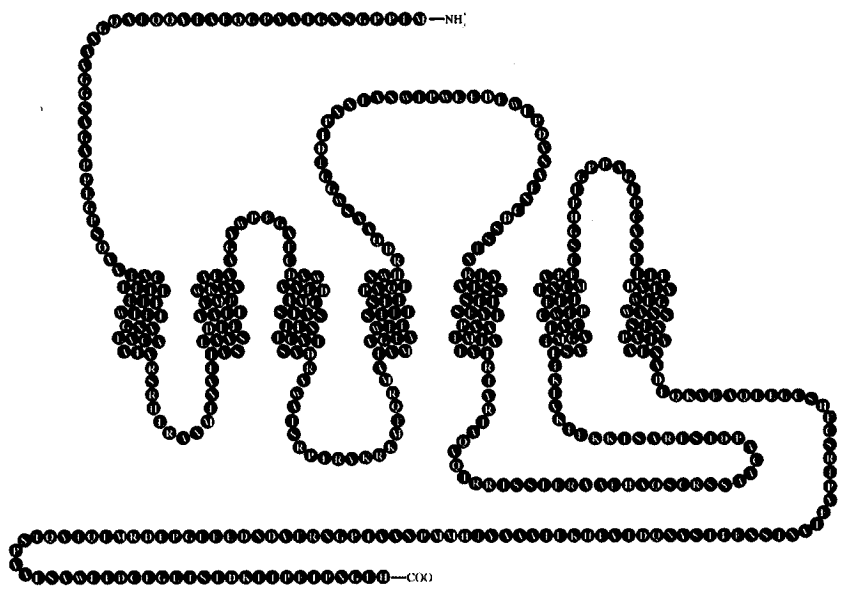
Figure 13 - Schematic representation of the wild-type and EL3 chimeric D1 and D5 receptors.

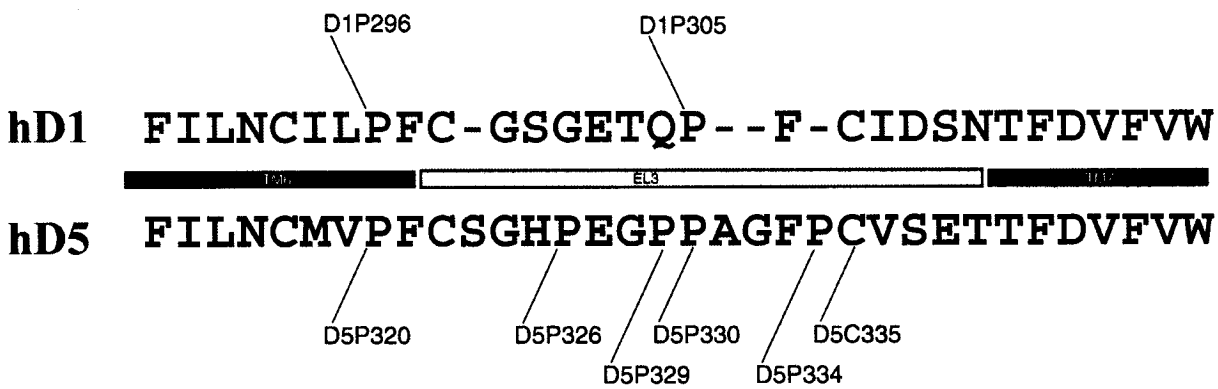
Putative topology of the wild-type D1 (red circles) and D5 (blue circles) receptors. Alignment of the primary structure corresponding to the EL3 region of the human D1 and D5 receptors is shown. Mutated amino acids are identified based on their one letter amino acid code and relative position in the receptor.

hD1


NH
COO

hD5


NH
COO


D1P296
D1P305
hD1 FILNCILPFC-GSGETQP--F-CIDSNTFDVFVW
EL3
hD5 FILNCMVPFCSGHPEGPPAGFPCVSETTFDVFVW
D5P320
D5P326
D5P329
D5P330
D5P334
D5C335

4.2.2 Mutations of Proline Residues at the Junction of TM6 and EL3 Regulate High Affinity Binding of N-[methyl-^{3}H]SCH23390 and Receptor Expression

The binding affinities (K_D values) of the radioligand N-[methyl-^{3}H]SCH23390 for wild-type and point mutant receptors obtained using saturation studies are summarized in Table 8. Results indicate that the mutant receptors retain their ability to bind N-[methyl-^{3}H]SCH23390 with high affinity. However, I observe that substitution of proline at positions D1P296 and D5P320 results in a significant loss in binding affinity. As shown on the sequence alignment (Figure 13), these are corresponding proline located at the junction of TM6 and EL3. Substitution of other proline within the EL3 had no significant effect, except for mutant D5P330G/C335W that exhibited a significant gain in binding affinity.

In addition, maximal binding capacities (B_{MAX}) of N-[methyl-^{3}H]SCH23390 for cells expressing wild-type and mutant receptors were detected in the range of 14 – 40 pmol/mg of protein, with the exception being D5P330G/C335W that exhibited a significant reduction of expression (0.58 pmol/mg of protein) (Table 8). In line with our EL3 studies, I observed that disruption of the native D1-EL3 conformation yielded a significant gain in maximal binding capacity (B_{MAX}) while disruption of the native D5-EL3 conformation resulted in a significant loss in maximal binding capacity. Particularly, there was a statistically significant gain in B_{MAX} values when P305 was substituted in the D1 receptor and there was a statistically significant loss in B_{MAX} values when P329 was substituted in the D5 receptor.

Table 8 - Equilibrium Dissociation constant (K_D) and maximal binding capacity (B_{MAX}) values for wild-type and proline point mutant D1-like receptors.

K_D and B_{MAX} values are expressed as geometric and arithmetic means, respectively. Means are from three to seven experiments done in duplicate determinations.

	^{3}H-SCH23390 (nM)	Binding Capacity (pmol/mg protein)
D1	0.55 ± 0.02	19.7 ± 1.19
D1P296A	2.02 ± 0.12 *#	23.5 ± 1.70
D1P296G	2.61 ± 0.19 *#	14.7 ± 0.75
D1P305A	0.69 ± 0.06	40.4 ± 3.32 *
D1P305G	1.06 ± 0.06	37.1 ± 1.32 *
D5	0.87 ± 0.06	23.8 ± 0.93
D5P320A	2.79 ± 0.22 *#	14.6 ± 1.29
D5P320G	1.01 ± 0.05	17.4 ± 0.22
D5P326A	1.05 ± 0.06	26.5 ± 1.20
D5P326G	0.57 ± 0.02	27.9 ± 1.76
D5P329A	0.97 ± 0.02	13.0 ± 1.05 #
D5P329G	0.43 ± 0.01	36.0 ± 3.41
D5P330A	0.89 ± 0.05	22.6 ± 1.77
D5P330G	0.69 ± 0.06	29.0 ± 1.16
D5P330G C335W	0.30 ± 0.05 #	0.58 ± 0.05 *#
D5P334A	0.81 ± 0.03	15.4 ± 0.83
D5P334G	0.47 ± 0.02	20.3 ± 0.60

* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

4.2.3 Mutations of Proline Residues at the Junction of TM6 and EL3 Regulate High Affinity Agonist Binding

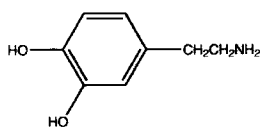
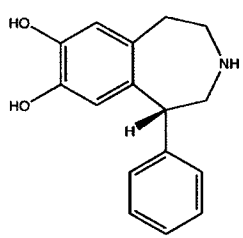
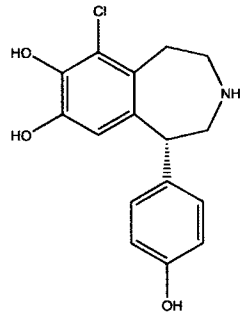
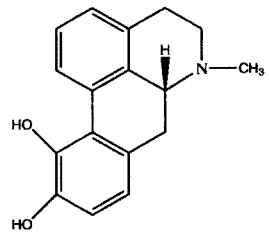
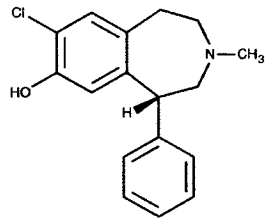
Competition studies were performed to determine the role of individual proline residues within the EL3 in mediating the structural requirements involved in the agonist (Figure 9; dopamine, SKF38393, fenoldopam, apomorphine) binding to wild-type human D1 and D5 receptors. Our studies were divided into two sets of experiments. The first set of experiments examined the role of proline at the junction of TM6/EL3 (Table 9; D1P296, D5P320). A second set of experiments examined the role of proline located within the EL3 (Table 10; D1P305, D5P326, D5P329, D5P330, D5P334).

At the wild-type D1-like receptors, the agonists dopamine and fenoldopam exhibit a significantly higher affinity for the D5 subtype in comparison with the D1 receptor (Table 9 and 10). In contrast, the agonists SKF38393 and apomorphine did not discriminate between the two D1-like receptors. For all agonists tested I report a significant decrease of binding affinity at the D1P296 and D5P320 mutant receptors. The agonist dopamine exhibited a greater decrease in binding affinity. A decrease in binding affinity was observed for both glycine and alanine substitutions; however, the degree of binding affinity decrease was greatest when the proline was substituted with an alanine (Table 9).

Substitution of proline within the EL3 of D1 and D5 receptors has a much smaller effect on the binding affinity of agonists in comparison with proline substitutions made at the junction of TM6 and EL3 (Table 9 and 10). Dopamine exhibits a significant 2-fold loss in binding affinity for the mutant receptor

Table 9 – Equilibrium Inhibition constant (K_I) for agonists at wild-type and proline point mutant D1-like receptors.

K_I values (nM) for proline mutated at the junction of TM6 and EL3 (D1P296 and D5P320) are expressed as geometric means. N-[methyl-^{3}H]SCH23390 was used as a radiotracer. Means are from three to six experiments done in duplicate determinations.

	Dopamine	SKF38393	Fenoldopam	Apomorphine	SCH23390
					
D1	3990 ± 231	120 ± 2.69	43.5 ± 2.25	432 ± 8.30	0.46 ± 0.02
D1P296A	50100 ± 2630 *#	296 ± 6.13 *#	75.2 ± 1.15 *#	1490 ± 35.5 *#	1.48 ± 0.07 *#
D1P296G	37400 ± 555 *#	465 ± 32.5 *#	101 ± 4.62 *#	1340 ± 25.4 *#	1.78 ± 0.07 *#
D5	662 ± 29.8 *	83.6 ± 3.29	21.1 ± 1.17 *	453 ± 25.4	0.90 ± 0.05 *
D5P320A	12500 ± 943 *#	513 ± 3.79 *#	63.7 ± 2.10 #	2510 ± 210 *#	2.30 ± 0.04 *#
D5P320G	3090 ± 126 #	201 ± 5.90 *#	38.8 ± 2.15 #	1160 ± 55.7 *#	1.03 ± 0.01 *

* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

Table 10 – Equilibrium Inhibition constant (K_I) for agonists at wild-type and proline point mutant D1-like receptors.

K_I values (nM) for proline mutated within the EL3 (D1P305 and D5P326, D5P329, D5P330, D5P334) are expressed as geometric means. N-[methyl-^{3}H]SCH23390 was used as a radiotracer. Means are from three to six experiments done in duplicate determinations.

	Dopamine	SKF38393	Fenoldopam	Apomorphine	SCH23390
D1	3990 ± 231	120 ± 2.69	43.5 ± 2.25	432 ± 8.30	0.46 ± 0.02
D1P305A	5260 ± 204 #	120 ± 4.85	53.7 ± 2.51 #	554 ± 17.9	0.44 ± 0.02 #
D1P305G	7800 ± 442 *#	156 ± 4.07 #	62.4 ± 1.65 #	671 ± 30.0	0.62 ± 0.02
D5	662 ± 29.8 *	83.6 ± 3.29	21.1 ± 1.17 *	453 ± 25.4	0.90 ± 0.05 *
D5P326A	677 ± 40.4 *	90.7 ± 0.60	23.5 ± 1.55 *	406 ± 44.5	0.98 ± 0.07 *
D5P326G	317 ± 15.8 *#	48.9 ± 1.87 *#	12.6 ± 1.47 *	291 ± 28.9	0.57 ± 0.03 #
D5P329A	665 ± 65.5 *	92.0 ± 5.44	16.9 ± 1.24 *	379 ± 31.8	0.83 ± 0.02 *
D5P329G	308 ± 2.80 *#	44.1 ± 1.84 *#	11.0 ± 0.78 *	309 ± 40.0	0.43 ± 0.01 #
D5P330A	640 ± 41.0 *	99.2 ± 6.02	18.8 ± 2.12 *	403 ± 34.9	1.08 ± 0.12 *
D5P330G	444 ± 3.70 *	62.7 ± 1.08 *	15.9 ± 0.59 *	412 ± 37.7	0.62 ± 0.01
D5P330G C335W	439 ± 8.81 *	N.D.	14.6 ± 1.56 *	208 ± 2.80 #	N.D.
D5P334A	643 ± 81.5 *	75.4 ± 3.45 *	16.3 ± 1.17 *	355 ± 36.6	0.78 ± 0.06 *
D5P334G	353 ± 8.12 *#	45.0 ± 3.01 *#	11.9 ± 0.81 *	261 ± 25.6	0.36 ± 0.01 #

* $p < 0.05$ when compared with D1
# $p < 0.05$ when compared with D5
N.D., Not Determined

D1P305G. The binding affinity of the other tested agonists is not significantly altered although I do report a consistent trend of decreased binding affinity and the degree of decrease is greater when D1P305 is substituted for glycine as opposed to alanine. In contrast, agonists exhibited a gain of binding affinity when proline within EL3 of the D5 receptor were substituted. Dopamine and SKF38393 exhibit a significant gain in binding affinity for D5P326G, D5P329G, and D5P334G mutant receptors. Furthermore, a similar trend although not significant is observed for agonists fenoldopam and apomorphine.

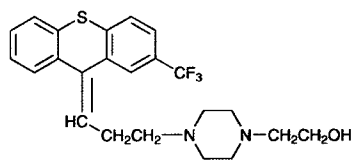
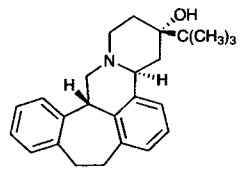
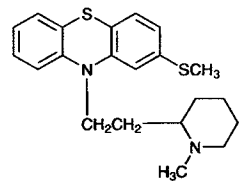
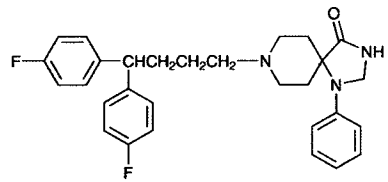
4.2.4 Proline Residues within the EL3 Domain Modulate Inverse Agonist Binding Properties at D1-like Receptors

Competition studies were performed to determine the role of proline residues within the EL3 in regulating the structural requirements involved in inverse agonist binding at wild-type D1 and D5 receptors (Figure 9; SCH23390, Z-flupentixol, (+)-butaclamol, thioridazine, fluspirilene). The studies were divided into two series of experiments, the first examining the role of proline residues at the junction of TM6/EL3 (Table 11; D1P296, D5P320), and a second set of experiments examining the role of proline residues located within the EL3 (Table 12; D1P305, D5P326, D5P329, D5P330, D5P334). At the wild-type D1-like receptors all tested inverse agonists display a significantly higher affinity for the D1 receptor in comparison with the D5 receptor (Table 11 and 12).

Substitution of D1P296 with either glycine or alanine leads to a slight increase in inverse agonist binding affinity. Similarly, substitution of D5P320

Table 11 – Equilibrium Inhibition constant (K_I) for inverse agonists at wild-type and proline point mutant D1-like receptors.

K_I values (nM) for proline mutated at the junction of TM6 and EL3 (D1P296 and D5P320) are expressed as geometric means. N-[methyl-^{3}H]SCH23390 was used as a radiotracer. Means are from three to six experiments done in duplicate determinations.

	Z-Flupentixol	(+)-Butaclamol	Thioridazine	Fluspirilene
				
D1	**4.81 ± 0.48**	**2.04 ± 0.17**	**59.9 ± 0.79**	**317 ± 9.20**
D1P296A	**4.07 ± 0.57 #**	**1.73 ± 0.16 #**	**45.1 ± 5.20 #**	**172 ± 8.00 *#**
D1P296G	**3.31 ± 0.47 #**	**1.45 ± 0.11 #**	**33.4 ± 2.29 *#**	**132 ± 20.0 *#**
D5	**13.4 ± 0.45 ***	**30.1 ± 0.82 ***	**180 ± 3.59 ***	**1060 ± 33.5 ***
D5P320A	**24.0 ± 0.13 *#**	**53.7 ± 1.20 *#**	**358 ± 7.21 *#**	**1430 ± 15.0 ***
D5P320G	**12.2 ± 0.07 ***	**17.3 ± 1.36 ***	**115 ± 5.54 ***	**587 ± 58.5 #**

* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

Table 12 – Equilibrium Inhibition constant (K_I) for inverse agonists at wild-type and proline point mutant D1-like receptors.

K_I values (nM) for proline mutated within the EL3 (D1P305 and D5P326, D5P329, D5P330, D5P334) are expressed as geometric means. N-[methyl-^{3}H]SCH23390 was used as a radiotracer. Means are from three to six experiments done in duplicate determinations.

	Z-Flupentixol	(+)-Butaclamol	Thioridazine	Fluspirilene
D1	4.81 ± 0.48	2.04 ± 0.17	59.9 ± 0.79	317 ± 9.20
D1P305A	6.61 ± 0.63 #	3.63 ± 0.28 *#	78.4 ± 2.73 #	412 ± 15.2 #
D1P305G	5.15 ± 0.54 #	2.60 ± 0.21 #	55.8 ± 0.93 #	361 ± 29.3 #
D5	13.4 ± 0.45 *	30.1 ± 0.82 *	180 ± 3.59 *	1060 ± 33.5 *
D5P326A	10.5 ± 0.44 *	31.4 ± 0.52 *	198 ± 5.24 *	998 ± 30.2 *
D5P326G	9.40 ± 0.22 *	21.5 ± 0.40 *	120 ± 1.57 *	553 ± 45.0 #
D5P329A	11.7 ± 0.45 *	27.1 ± 1.36 *	171 ± 1.13 *	947 ± 27.2 *
D5P329G	12.0 ± 0.16 *	20.3 ± 0.49 *	128 ± 5.49 *	515 ± 29.0 #
D5P330A	10.7 ± 0.55 *	30.7 ± 1.09 *	182 ± 6.60 *	913 ± 56.9 *
D5P330G	12.1 ± 0.38 *	29.1 ± 0.32 *	173 ± 4.08 *	676 ± 39.4 *
D5P334A	9.30 ± 0.33 *	20.6 ± 0.81 *	130 ± 3.13 *	774 ± 19.4 *
D5P334G	9.70 ± 0.28 *	21.3 ± 0.48 *	123 ± 6.57 *	458 ± 33.3 #

* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

with glycine results in a small increase in inverse agonist binding affinity. In contrast, substitution of D5P320 with alanine leads to a decrease in inverse binding affinity at the D1-like receptors (Table 11).

At the D1 receptor, substitution of D1P305 with either glycine or alanine leads to a decreased inverse agonist binding affinity, albeit a less pronounced effect was observed when the more flexible glycine residue was introduced. In contrast, substitution of D5P326, D5P329, D5P330, or D5P334 with either glycine or alanine leads to an increase in inverse agonist binding affinities, an observation consistent with the effects reported for the D5-EL3D1 chimera. The increase in inverse agonist binding affinities is more pronounced when the more flexible glycine residue is introduced (Table 12).

4.2.5 Proline Residues in EL3 Regulate D1-like Receptor - G Protein Coupling Properties

Substitution of either D1P296 or D5P320 at the junction of TM6 and EL3 results in a striking ~ 3-fold decrease in agonist-independent activity (Table 13). In contrast, I did not observe an effect on the agonist-independent activity of D1 or D5 receptors harboring mutated proline residues located within the EL3 region (Table 13).

To test whether the proline mutant receptors delineate the structural requirements for the dopamine potency and dopamine-mediated maximal activation of adenylyl cyclase, cAMP assays were performed using HEK293 cells transfected with wild-type or mutant receptors. I report that the dopamine-

Table 13 - Constitutive activity and dopamine-mediated maximal activation of adenylyl cyclase at wild-type D1 and proline point mutant D1-like receptors.

Basal levels of adenylyl cyclase activity were determined in single wells of a 6-well dish using whole cell cAMP assays. Maximal activation of adenylyl cyclase was determined in single wells of a 6-well dish using a final concentration of 10 μM dopamine and whole cell cAMP assays. Data are expressed as arithmetic mean ± S.E. of three experiments done in triplicate determinations.

	cAMP Assay (CA/TU x 1000)	
	Constitutive Activity	Maximal Stimulation (10μM DA)
D1	4.39 ± 1.94	49.3 ± 8.35
D1P296A	1.89 ± 0.56	48.8 ± 5.28
D1P296G	1.12 ± 0.15	54.8 ± 5.35
D1P305A	5.21 ± 1.54	45.7 ± 5.64
D1P305G	5.56 ± 3.06	47.5 ± 1.01
D5	9.66 ± 2.06	38.6 ± 7.29
D5P320A	2.57 ± 1.13	33.3 ± 7.20
D5P320G	3.49 ± 1.30	43.1 ± 2.89
D5P326A	10.1 ± 2.35	51.2 ± 11.8
D5P326G	12.3 ± 3.22	45.1 ± 4.79
D5P329A	8.82 ± 1.87	44.1 ± 5.13
D5P329G	11.7 ± 3.29	46.5 ± 7.68
D5P330A	10.0 ± 3.31	41.4 ± 4.87
D5P330G	8.71 ± 1.36	43.8 ± 3.85
D5P334A	8.93 ± 2.77	43.5 ± 5.34
D5P334G	11.1 ± 3.05	50.0 ± 6.18

mediated maximal activation of AC for all tested mutant receptors was not altered when compared to their respective wild-type receptors (Table 13). Substitution of either D1P296 or D5P320 at the junction of TM6 and EL3 leads toa decrease in dopamine potency (Figure 14). The decrease in dopamine potency is more pronounced at the D1 mutant receptor. Furthermore, I observed a greater decrease in dopamine potency when proline was substituted with a glycine as opposed to alanine (Figure 14). This observation is in contrast to the effect on dopamine binding affinity in which case an alanine for proline substitution yielded the most potent loss of binding affinity (Table 9). Moreover, substitution of glycine for proline in the EL3 domain of D1 receptors leads to a loss of dopamine potency; while at D5 receptors a similar substitution leads to a gain of dopamine potency.

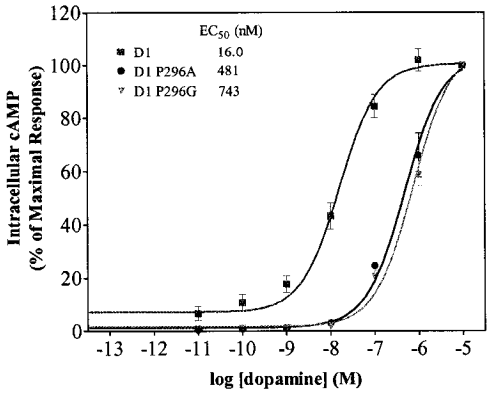
4.2.6 Variant TM6 and TM7 Amino Acids Play a Minor Role in Regulating Binding Affinities of Agonists and Inverse Agonists at D1-like Receptors

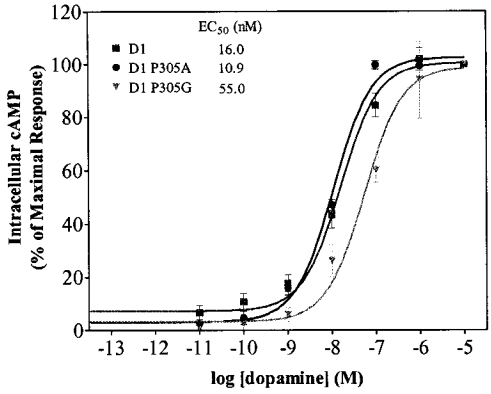
A series of single and double point mutations were engineered in TM6 and TM7 regions of D1 and D5 receptors. Specifically at either the D1 or D5 receptor, I engineered a double point mutant (D1IL294MV or D5MV318IL) and two single point mutations (D1I294M, D1L295V or D5M318I, D5V319L) in the TM6 domain and a single point mutation in TM7 (D1I329V or D5V357I) (Figure 15).

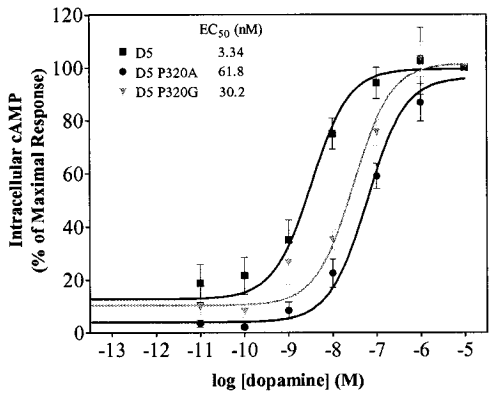
The binding affinities (K_D values) of the radioligand N-[methyl-^{3}H]SCH23390 for wild-type and TM6/TM7 mutant receptors obtained using saturation studies are summarized in Table 14. Results indicate that the mutant receptors retain their ability to bind N-[methyl-^{3}H]SCH23390 with high affinity.

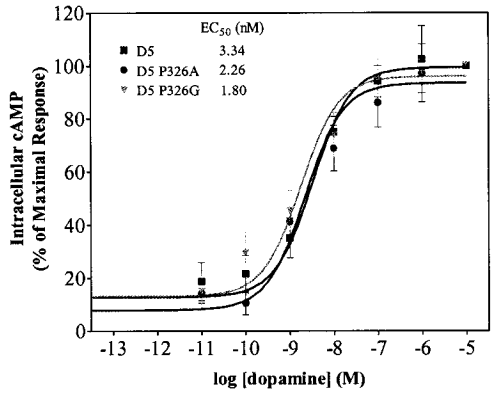
Figure 14- Dose-response curves of DA for AC stimulation by wild-type and mutant D1-like receptors.

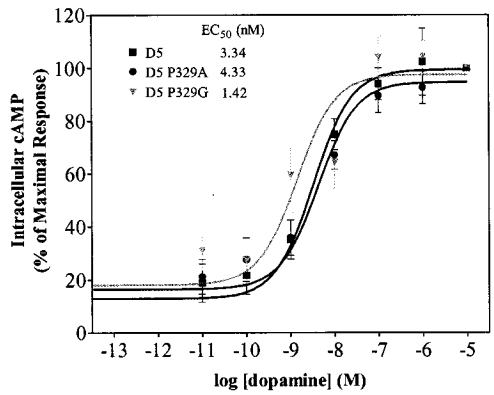
HEK 293 cells were transfected with wild-type or proline point mutant D1-like receptors. The B_{MAX} values in pmol/mg membrane protein expressed as the arithmetic mean ± S.E. are inset as a table in the lower right corner. Intracellular cAMP levels were measured in single wells of a 12-well dish in presence of increasing concentrations of DA as described in the Methods section and plotted as a function of log of DA concentrations. Each point is the arithmetic mean ± S.E. of three to eleven experiments done in triplicate determinations and expressed as percentage of maximal response. Nonlinear regression of the data was performed using Sigmoidal dose-response (variable slope) with constrained hill slope factor using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

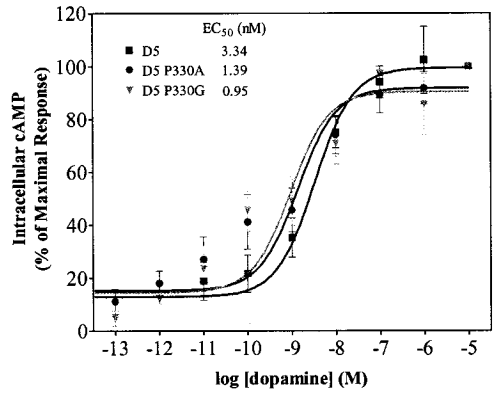


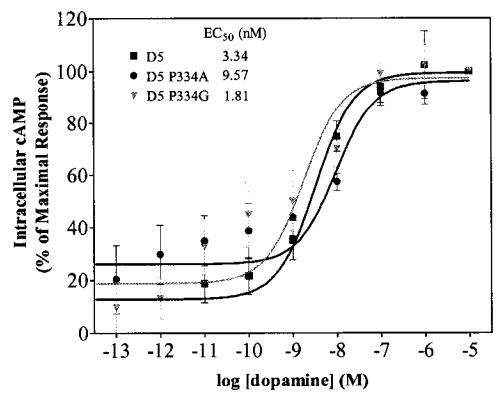








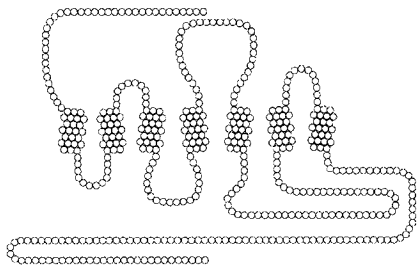




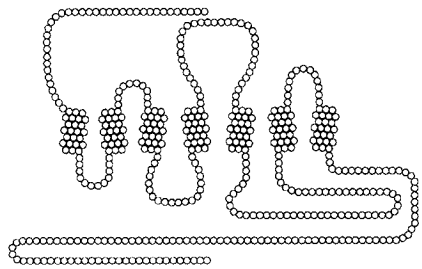
	B_{max}	SEM
D1	2.47	0.87
D5	2.07	0.96
D1-P296A	3.71	1.54
D1-P296G	1.19	0.21
D1-P305A	2.96	0.62
D1-P305G	1.44	0.05
D5-P320A	1.06	0.31
D5-P320G	1.70	0.51
D5-P326A	2.00	0.63
D5-P326G	1.77	0.67
D5-P329A	1.34	0.24
D5-P329G	2.07	0.47
D5-P330A	1.14	0.25
D5-P330G	2.73	0.73
D5-P334A	1.11	0.22
D5-P334G	3.07	0.82

Figure 15 - Schematic representation of the wild-type and TM6/TM7 mutant D1 and D5 receptors.

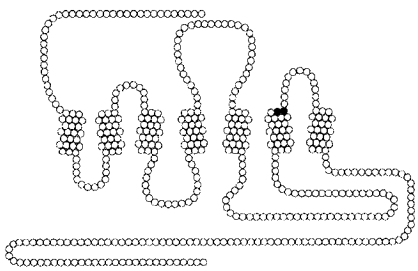
Putative topology of the wild-type D1 (red circles) and D5 (blue circles) receptors, identifying the mutated amino acids.



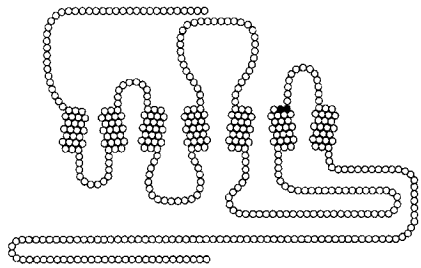
D1



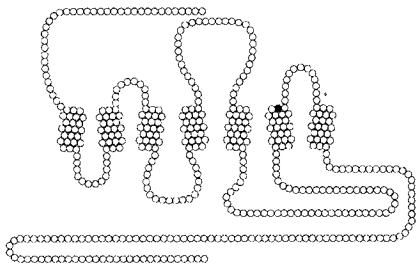
D5



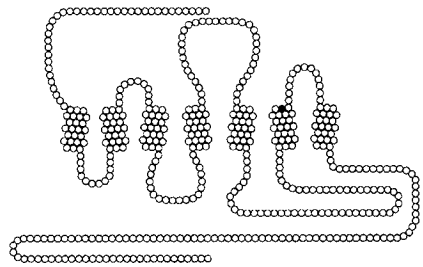
D1 IL294MV



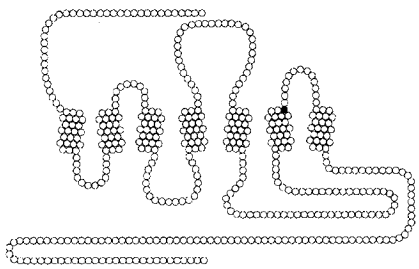
D5 MV318IL



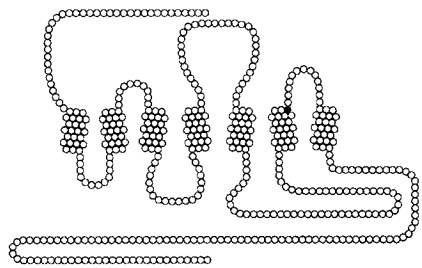
D1 I294M



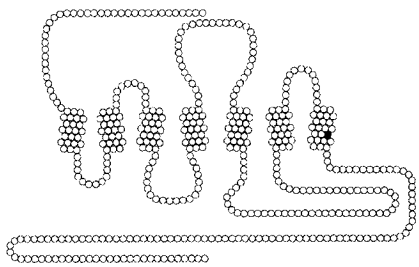
D5 M318I



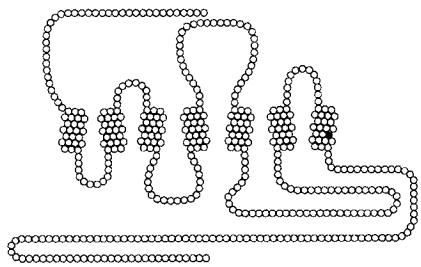
D1 L295V



D5 V319L



D1 I329V



D5 V357I

The binding capacities of wild-type and mutant receptors were detected in the range of 10 – 28 pmol/mg of protein. These results suggest that the mutantreceptors retain a proper protein folding necessary for membrane expression and binding of ligands.

Competition studies show that substitution of D1I329 and D5V357 in TM7 has no major effect on the ligand binding properties. Substitution of variant amino acids in TM6 modulates agonist but not inverse agonist / antipsychotic binding. At the D1 receptor, substitution of TM6 variant amino acids leads to a loss of binding affinity, which is more pronounced when both isoleucine and leucine or only leucine are substituted. In contrast, at the D5 receptor, only substitution of both TM6 variant amino acids methionine and valine results in a significant gain in agonist binding affinity (Table 14).

4.2.7 TM6 Variant Amino Acids Play a Limited Role in Mediating Receptor – G Protein Coupling Properties

The role of TM6 and TM7 variant amino acids in mediating the agonist-independent and dependent activation of adenylyl cyclase by wild-type and mutant receptors was assessed using a whole cell cAMP assay. I report no statistically significant effect on either the agonist-independent or dependent G protein coupling properties. For the agonist-independent activity, I observe a trend showing a gain at the D1 receptor and loss at the D5 receptor when D1L295 or D5V319 where mutated, respectively (Figure 16).

Table 14 - Equilibrium constants and maximal binding capacity (B_{MAX}) values for wild-type and TM6/TM7 mutant D1-like receptors.

K_D, K_I, and B_{MAX} values are expressed as geometric and arithmetic means, respectively. N-[methyl-^{3}H]SCH23390 was used as a radiotracer. Means are from three to eight experiments done in duplicate determinations.

	D1	D5	D1I329V	D1IL294MV	D1I294M	D1L295V	D5V357I	D5MV318IL	D5M318I	D5V319L
B_{max} (pmol/mg prot)	9.95 ± 0.58	15.6 ± 3.28	18.1 ± 1.85	22.2 ± 2.49	22.3 ± 1.76	28.7 * ± 1.87	17.2 ± 4.39	14.1 ± 3.41	15.6 ± 3.92	15.6 ± 0.56
3H-SCH23390 (nM)	0.37 ± 0.02	0.58 ± 0.01	0.36 ± 0.01	0.51 ± 0.05	0.44 ± 0.01	0.39 ± 0.01	0.54 ± 0.02	0.37 ± 0.01	0.45 ± 0.05	0.58 ± 0.03
Dopamine (nM)	4580 ± 215	476 * ± 15.0	3890 # ± 96.3	8510 *# ± 265	6810 *# ± 211	8130 *# ± 292	508 * ± 24.9	235 *# ± 10.5	347 * ± 40.8	595 * ± 47.0
Z-Flupentixol (nM)	6.92 ± 0.46	13.8* ± 0.73	5.51 # ± 0.62	4.12 # ± 0.51	7.39 ± 0.76	5.94 # ± 0.68	9.91 ± 1.10	19.9* ± 1.98	16.7* ± 1.24	19.3 * ± 0.88
(+) Butaclamol (nM)	2.18 ± 0.27	29.2* ± 0.98	2.11 # ± 0.55	1.07 # ± 0.29	1.94 # ± 0.03	1.26 # ± 0.07	23.1* ± 1.42	32.6* ± 1.18	25.7* ± 0.92	35.3 * ± 2.08
SCH23390 (nM)	0.35 ± 0.01	0.53* ± 0.01	0.36 # ± 0.01	0.41 # ± 0.01	0.32 # ± 0.01	0.30 # ± 0.01	0.54* ± 0.01	0.36# ± 0.01	0.37# ± 0.02	0.56 * ± 0.03

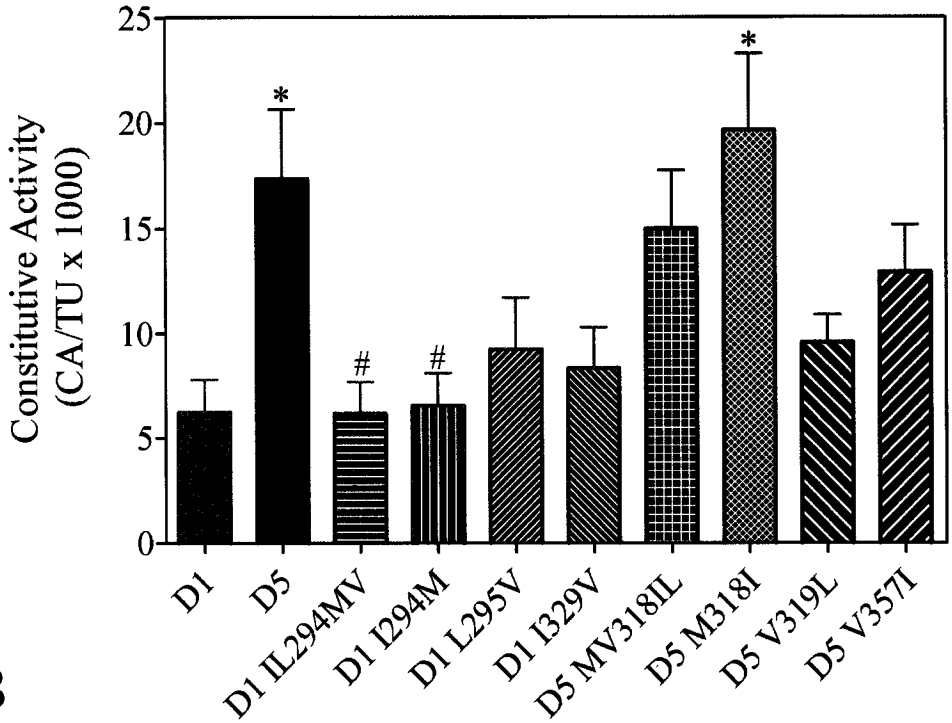
* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

Figure 16 - Agonist-independent and dopamine-mediated maximal activation of adenylyl cyclase at wild-type D1 and TM6/TM7 mutant receptors.

A. Basal levels of adenylyl cyclase activity were determined in single wells of a 6-well dish using whole cell cAMP assays. Data are expressed as arithmetic mean ± S.E. of five to six experiments done in triplicate determinations. The receptor expression in pmol/mg of membrane protein expressed as the arithmetic mean ± S.E. was 10.9 ± 1.9 (D1), 11.9 ± 0.9 (D5), 16.9 ± 2.6 (D1-IL294MV), 16.0 ± 2.5 (D1-I294M), 18.0 ± 3.5 (D1-L295V), 14.0 ± 2.1 (D1-I329V), 9.4 ± 1.5 (D5-MV318IL), 11.4 ± 2.3 (D5-M318I), 8.9 ± 1.5 (D5-V319L), 12.3 ± 1.8 (D5-V357I). B. Maximal activation of adenylyl cyclase was determined in single wells of a 6-well dish using a final concentration of 10 μM dopamine and whole cell cAMP assays. Data are expressed as arithmetic mean ± S.E. of four experiments done in triplicate determinations.

A



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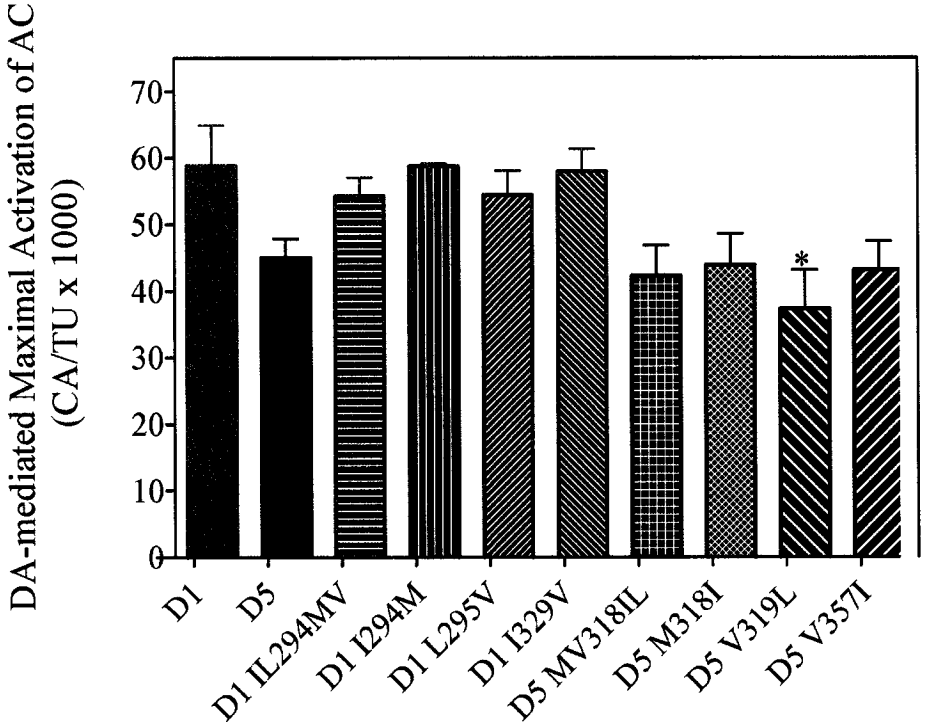


* $p < 0.05$ when compared with D1

\# $p < 0.05$ when compared with D5

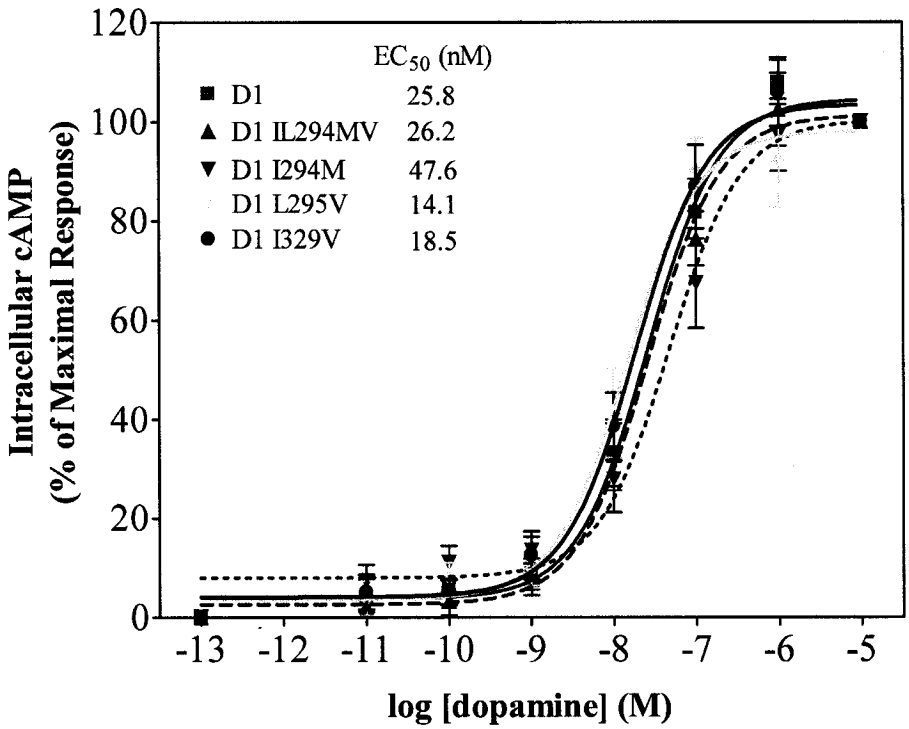
To test whether these variant amino acids participate in the structural requirements for the dopamine potency, dose-response curves were performed using HEK293 cells transfected with wild-type and mutant receptors. At the D1 receptor TM6 point mutants, I report that a double mutation of isoleucine and leucine to methionine and valine has no effect on dopamine potency. However, the single mutations have opposite effects, D1I294M and D1L295V displayed a loss and gain in dopamine potency, respectively (Figure 17). At the D5V319L mutant, I observe a loss of dopamine potency that was not recapitulated in cells expressing D5M318I or D5MV318IL mutants. Moreover, I demonstrate that TM7 mutation alter dopamine potency at D1 and D5 receptors. Interestingly, D1I329V and D5V375I display a gain and loss of dopamine potency, respectively (Figure 17).

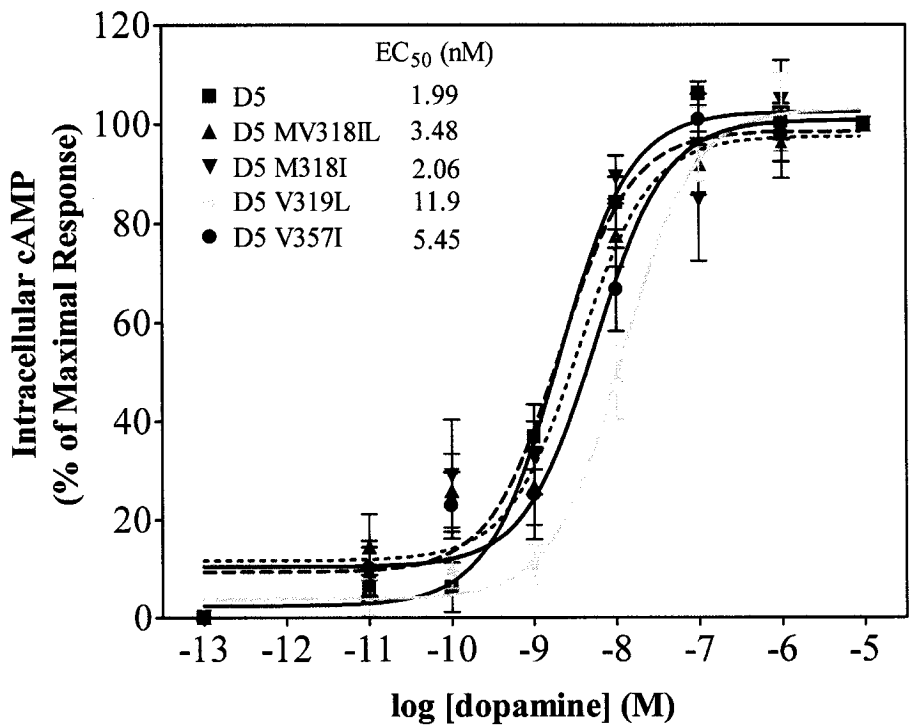
4.3 Conclusions

Using point mutations within TM6, EL3, and TM7 of D1-like receptors, this study identifies specific amino acids responsible for influencing the functional properties of the D1 and D5 receptors. Specifically, I show that specific amino acids of EL3, TM6, and TM7 regulate the conformational determinants necessary for binding of ligands (notably agonists) and coupling to G protein (notably agonist-independent activity and dopamine potency). However, this study did not identify specific amino acids contributing to structural determinants necessary for inverse agonist binding and dopamine-mediated maximal activation of AC, which I have previously shown to involve the EL3 and TM6 domains. Not

Figure 17 - Dose-response curves of DA for AC stimulation by wild-type and TM6/TM7 mutant D1-like receptors.

HEK 293 cells were transfected with wild-type or TM6/TM7 mutant D1-like receptors. The B_{MAX} values in pmol/mg membrane protein (expressed as the arithmetic mean ± S.E.) were 1.7 ± 0.4 (D1), 2.1 ± 0.3 (D5), 1.8 ± 0.5 (D1-IL294MV), 1.8 ± 0.6 (D1-I294M), 1.9 ± 0.7 (D1-L295V), 1.5 ± 0.3 (D1-I329V), 1.5 ± 0.3 (D5-MV318IL), 1.6 ± 0.3 (D5-M318I), 1.5 ± 0.2 (D5-V319L), 1.3 ± 0.2 (D5-V357I). Intracellular cAMP levels were measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA as described in the Methods section and plotted as a function of log of DA concentrations. Each point is the arithmetic mean ± S.E. of five experiments done in triplicate determinations and expressed as percentage of maximal response. Nonlinear regression of the data was performed using sigmoidal dose-response with shared hill slope factor using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.


EC50 (nM)
D1 25.8
D1 IL294MV 26.2
D1 I294M 47.6
D1 L295V 14.1
D1 I329V 18.5
Intracellular cAMP
(% of Maximal Response)
log [dopamine] (M)


EC50 (nM)
D5 1.99
D5 MV318IL 3.48
D5 M318I 2.06
D5 V319L 11.9
D5 V357I 5.45
Intracellular cAMP
(% of Maximal Response)
log [dopamine] (M)

Surprisingly, my studies highlight the fact that the structural constraints required for the D1 and D5 specific pharmacological and functional phenotypes are not mediated through a single residue but rather a combination of specific amino acids or entire receptor domains.

At the completion of my EL3 and EL3/TM6 studies, I had hypothesized that specific residues within these domains are at the root of the structural constraints responsible for the D1 or D5 specific phenotypes. However, our EL3 point mutant studies have not reproduced the effects observed using chimeras harboring the entire EL3 of D1 or D5 receptors. Nevertheless, mutation of proline residues did lead to a general trend of losing inverse agonist binding affinity at the D1 receptor and gaining inverse agonist binding affinity at the D5 receptor. This trend serves to support the idea that the overall conformation of the EL3 is important in mediating D1 and D5 specific phenotypes for inverse agonist binding, however a single proline residue is insufficient to generate this effect. Interestingly, I observed also a more prominent effect when glycine was substituted for a proline, thereby increasing flexibility in the secondary structure of the EL3. Therefore it is likely that multiple prolines need to be mutated concurrently in order to observe the effects reported using EL3 chimera. It may also be plausible that other amino acids within the EL3 may play a critical role or that spacing of the EL3 (number of amino acids) may be crucial in determining the correct structural constraints. As previously described, the activation of GPCRs involves the movement of TM6 relative to TM3. The overall conformation of the EL3 which links TM6 with TM7 may play an important role

in the activation of GPCRs upon ligand binding by mediating the movement of TM6.

PART II: REGULATION OF D1-LIKE RECEPTORS

1. D1 and D5 Receptor Regulation and Trafficking in the Absence and Presence of Agonist Stimulation

1.1 Introduction

Several studies have demonstrated that post-translational modifications of the D1 receptor are potentially contributing to the basis of CNS and peripheral disorders. However, the role of post-translational modification of the D5 receptor in the etiology of physiological disorders has not been well studied (Sanada et al., 1999; Sibley, 1999; Dumartin et al., 2000; Zizak et al., 2000; Ferguson, 2001; Zhen et al., 2001).

Studies involving other members of the GPCR family have shown that agonist-promoted phosphorylation, desensitization and endocytosis of GPCRs play an important role in the physiological response to extracellular stimuli (Felder et al., 1990; Xu et al., 1999; Angers et al., 2000; Koch et al., 2000; Miller and Lefkowitz, 2001; Pierce and Lefkowitz, 2001; Mason et al., 2002). In agreement with other GPCR systems, agonist-dependent regulation of the D1 receptor responsiveness has been well documented (Chneiweiss et al., 1990; Bates et al., 1991; Ng et al., 1994; Ng et al., 1995; Tiberi et al., 1996; Jiang and Sibley, 1999; Vickery and von Zastrow, 1999). The D1 receptor has been shown to undergo phosphorylation both by PKA and GRKs (Zamanillo et al., 1995; Tiberi et al., 1996; Lewis et al., 1998; Gardner et al., 2001; Mason et al., 2002). D1 receptors treated with agonist undergo desensitization mediated by residues within the third intracellular loop (IL3) and cytoplasmic tail (CT) (Jensen et al.,

1995; Jiang and Sibley, 1999; Jackson et al., 2002; Lamey et al., 2002). Moreover, upon agonist stimulation D1 receptors have been shown to be endocytosed (Ng et al., 1995; Vickery and von Zastrow, 1999; Jackson et al., 2002; Lamey et al., 2002). Interestingly, little evidence exists to support the classical GPCR regulation paradigms at the D5 receptor. In fact, only a few studies have demonstrated desensitization of the D5 receptor and no evidence exists for the role of D5 receptor phosphorylation or endocytosis (Jarvie et al., 1993; Le Crom et al., 2002).

In the present study, I investigate the agonist-mediated regulatory pathway of both D1 and D5 receptors and compare them in respect to their ability to be phosphorylated, desensitized, and endocytosed. I hypothesize that D1 and D5 receptors are differentially regulated following agonist stimulation.

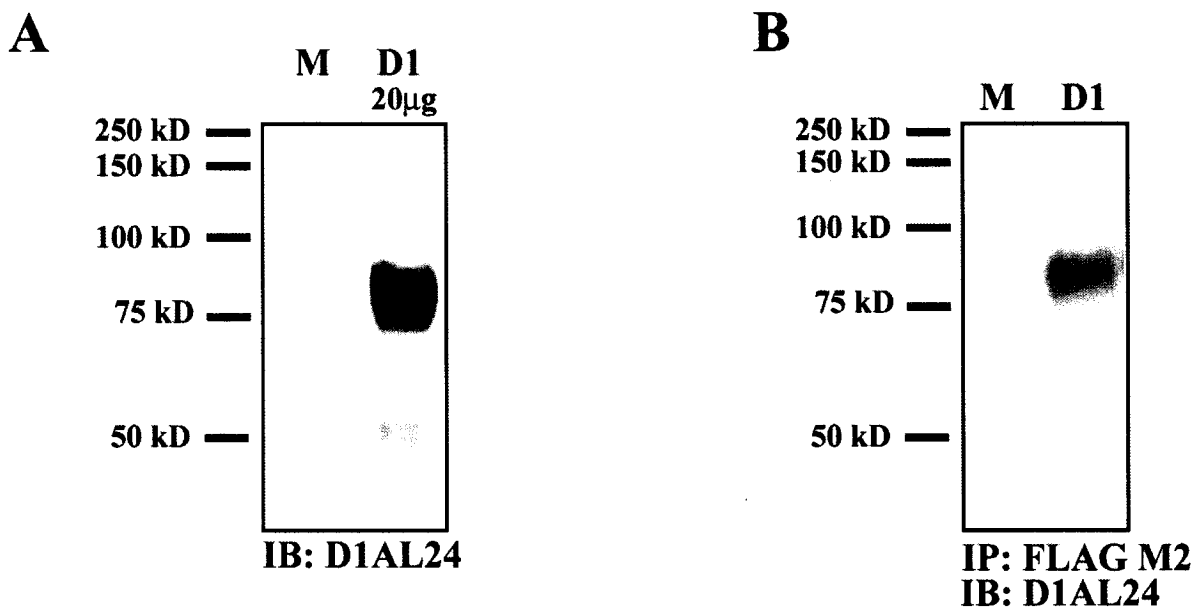
1.2 Results

1.2.1 Electrophoretic Mobility of D1 and D5 Receptors

The electrophoretic mobility of human D1 and D5 receptors in transiently transfected HEK293 cells was assessed both after preparation of crude membrane extract or membrane solubilization with RIPA buffer. Figure 18A and 18C show immunoblotting using human D1 and D5 specific antibodies, D1AL24 and D5JH519 respectively (Levey et al., 1993; Ciliax et al., 2000). As previously described, the D1 receptor runs as a band ranging from 75 – 85 kD (Tiberi et al., 1996). I also observe a minor band around 50 kD, a molecular size consistent with non-glycosylated receptor. In contrast, the D5 receptor runs as a band ranging from 120 – 140 kD. Both antibodies were specific as no band was detected in mock transfected cells. For immunoprecipitation assays, I used D1 and the D5 receptors that were re-engineered at the N-terminus with a FLAG tag (Guan et al., 1992; Einhauer and Jungbauer, 2001). Detergent solubilized samples were immunoprecipitated using an anti-FLAG M2 matrix and immunoblotted using D1 or D5 specific antibodies (Figures 18B and 18D). Similarly to untagged receptors, immunoprecipitated FLAG-tagged receptors have an electrophoretic mobility between 75 -85 kD (FLAG-D1) and 120 – 140 kD (FLAG-D5). Therefore, the FLAG tag does not seem to modify the electrophoretic mobility of either D1 or D5 receptors.

Figure 18 - Immunoprecipitation and immunoblotting of wild-type and FLAG-tagged D1-like receptors.

Immunoprecipitation and immunoblotting of wild-type and FLAG-tagged D1-like receptors expressed in HEK cells was performed as described in the Methods section. Shown is a representative example of an experiment repeated three to five times. Panels A and C are representative examples of immunoblotted wild-type D1 and D5 receptors. Panels B and D are representative examples of FLAG-tagged D1-like receptors immunoprecipitated with FLAG M2 matrix and immunoblotted with D1 or D5 specific antibodies D1AL24 and D5JH519, respectively. The symbol M represents mock samples containing HEK cells transfected with empty pCMV-5 vector.


A
M
D1
20μg
250 kD
150 kD
100 kD
75 kD
50 kD
IB: D1AL24
B
M
D1
250 kD
150 kD
100 kD
75 kD
50 kD
IP: FLAG M2
IB: D1AL24
C
M
D5
10μg 20μg
250 kD
150 kD
100 kD
75 kD
50 kD
IB: D5JH519
D
M
D5
250 kD
150 kD
100 kD
75 kD
IP: FLAG M2
IB: D5JH519

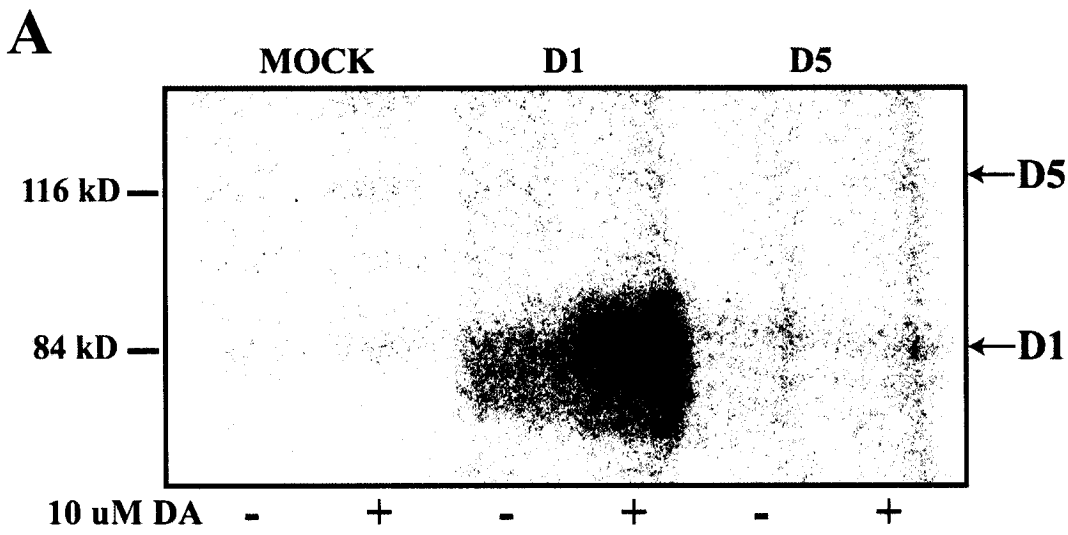
1.2.2 The D5 Receptor is Not Phosphorylated in HEK293 Cells

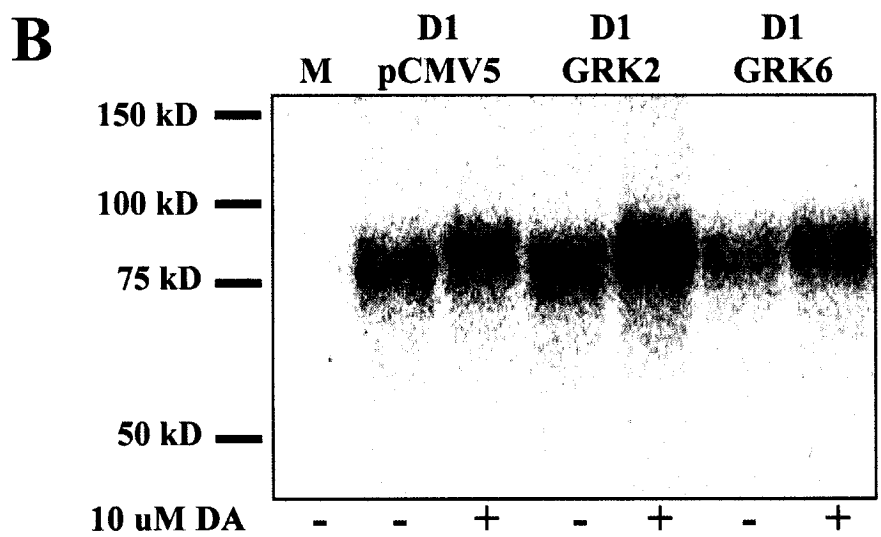
Using metabolically labeled cells expressing either FLAG-D1 or FLAG-D5 receptors, I assessed receptor phosphorylation in the absence and presence of 10 μM dopamine (agonist). A representative whole cell phosphorylation assay is shown in Figure 19A. I show that the human D1 receptor is phosphorylated under basal conditions and that the extent of phosphorylation increases upon agonist stimulation, as observed by increased band intensity and upward shift (Tiberi et al., 1996; Gardner et al., 2001). In striking contrast, I did not detect any phosphorylation of D5 receptors, in the absence or presence of agonist. The D1 receptor has been shown to be a substrate for GRKs, in particular GRK2, GRK3, GRK5, and more recently GRK4 (Tiberi et al., 1996; Watanabe et al., 2002). Figure 19B demonstrates that the human FLAG-D1 receptors are substrate for GRK2 but not GRK6 as demonstrated by the increased level of phosphorylation upon agonist stimulation in cells co-expressing FLAG-D1 receptors and GRK2. Co-expression of GRK2, GRK5, or GRK6 with the human FLAG-D5 receptor did not promote receptor phosphorylation in the presence or absence of dopamine (Figure 19C).

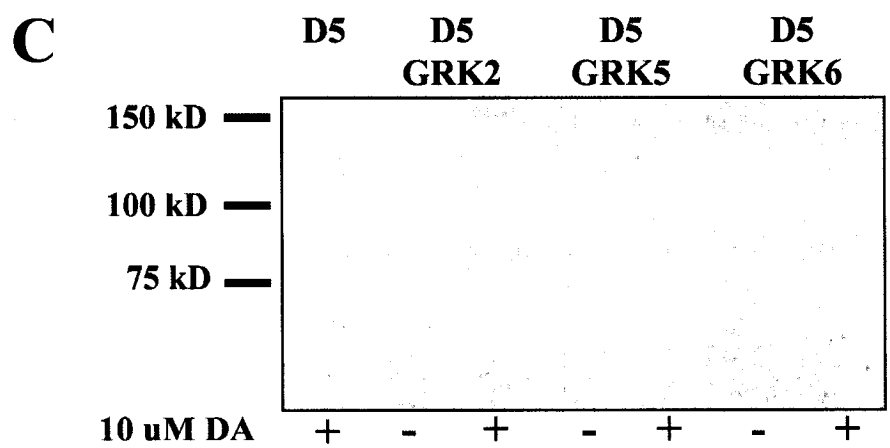
In order to investigate the role of IL3 and CT in mediating potentially the observed differences in phosphorylation, six human FLAG tagged D1/D5 chimeric receptors were engineered (Figure 20). The extent of agonist-induced phosphorylation was determined and results for the wild-type and chimeric receptors are reported in Figure 21. My data suggests that introduction of

Figure 19 - Phosphorylation of the wild-type FLAG-D1 and FLAG-D5 receptors expressed in HEK293 cells.

HEK cells transfected with empty pCMV5 vector or the FLAG-tagged D1 or D5 receptor were incubated in phosphate free media supplemented with ^{32}P for 90 minutes prior to treatement with or without 10 μM dopamine (DA) for 10 min and subjected to immunoprecipitation using anti-FLAG M2 affinity matrix as described in the Methods section. Immunocomplexes were then resolved by SDS-polyacrylamide gel electrophoresis using 10% gels. B. HEK cells transfected with empty pCMV5 vector or the FLAG-tagged D1 receptor co-transfected with either GRK2 or GRK6. Transfected cells were treated as described in (A). C. HEK cells transfected with empty pCMV5 vector or the FLAG-tagged D5 receptor co-transfected with GRK2, GRK5, or GRK6. Transfected cells were treated as described in (A).


A
MOCK
D1
D5
116 kD
84 kD
D5
D1
10 uM DA - + - + - +


B
M
D1 pCMV5
D1 GRK2
D1 GRK6
150 kD
100 kD
75 kD
50 kD
10 uM DA - - + - + - +


C
D5
D5 GRK2
D5 GRK5
D5 GRK6
150 kD
100 kD
75 kD
10 uM DA + - + - + - +

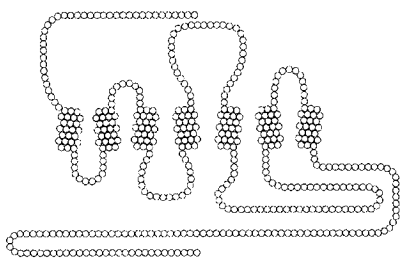
cytoplasmic D5 domains (IL3 and/or CT) into D1 receptors (Figure 20: Chimera 1, 3, 5) results in a loss of phosphorylation, however, D5 phosphorylation remains undetectable in the presence of cytoplasmic D1 domains (Figure 20: Chimera 2, 4, 6; Figure 21).

1.2.3 D5 Receptor Undergoes a More Pronounced Desensitization in Comparison with the D1 Receptor

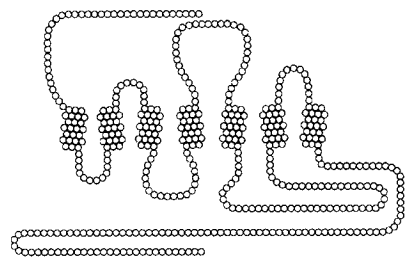
The ability of D5 receptors to undergo desensitization (in the absence of phosphorylation) in this cellular system was questioned and thus required further investigation. In contrast to the phosphorylation data, the dopamine-induced desensitization studies indicate that both D1 and D5 receptors undergo agonist-dependent desensitization (Figure 22). Interestingly, the D5 receptor undergoes a more pronounced agonist-dependent desensitization despite lack of dopamine-induced phsophorylation. Pre-treatment of HEK293 cells expressing D1 receptors with 10 μM DA for 5minutes leads to a small 1.2-fold rightward shift in EC_{50} and a 22% decrease in dopamine mediated maximal activation of adenylyl cyclase. Similar pretreatment of D5 receptors leads to a 2-fold rightward shift in EC_{50} and a 45% decrease in maximal activation of adenylyl cyclase. These results suggest strongly that the D5 receptor undergoes phosphorylation-independent desensitization in HEK293 cells.

Figure 20 - Schematic representation of the wild-type and chimeric D1-like receptors.

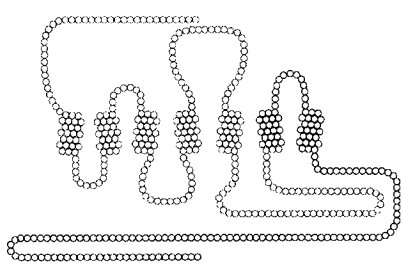
Putative topology of the wild-type D1 (red circles), chimera 1, chimera 3, chimera 5 and wild-type D5 (blue circles), chimera 2, chimera 4, chimera 6.



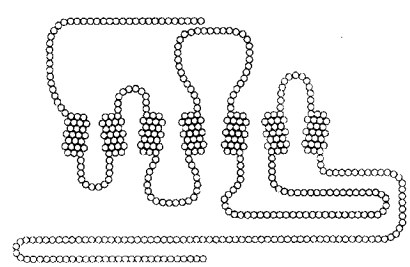
hD1



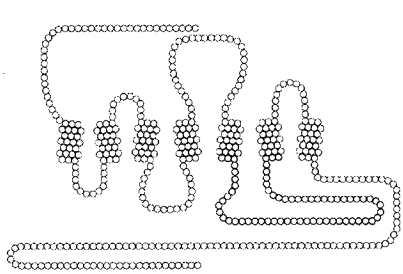
hD5



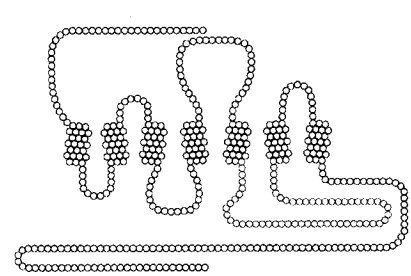
CHIMERA 1



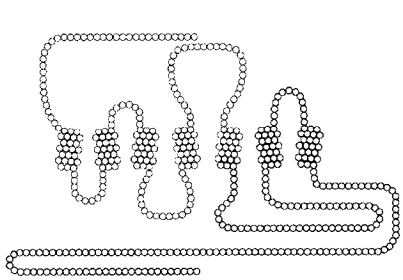
CHIMERA 2



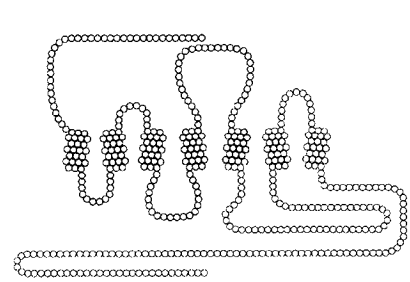
CHIMERA 3



CHIMERA 4



CHIMERA 5



CHIMERA 6

Figure 21 - Phosphorylation of the wild-type D1-like and chimeric receptors expressed in HEK293 cells.

Receptor phosphorylation for D1-like and chimeric receptors was performed as described in the Methods section. Shown are quantified basal and dopamine-stimulated phosphorylation levels of D1-like and chimeric receptors. Phosphorylation was quantified with a Typhoon PhosphorImager 8600 and normalized relative to dopamine-stimulated D1 receptor phosphorylation.

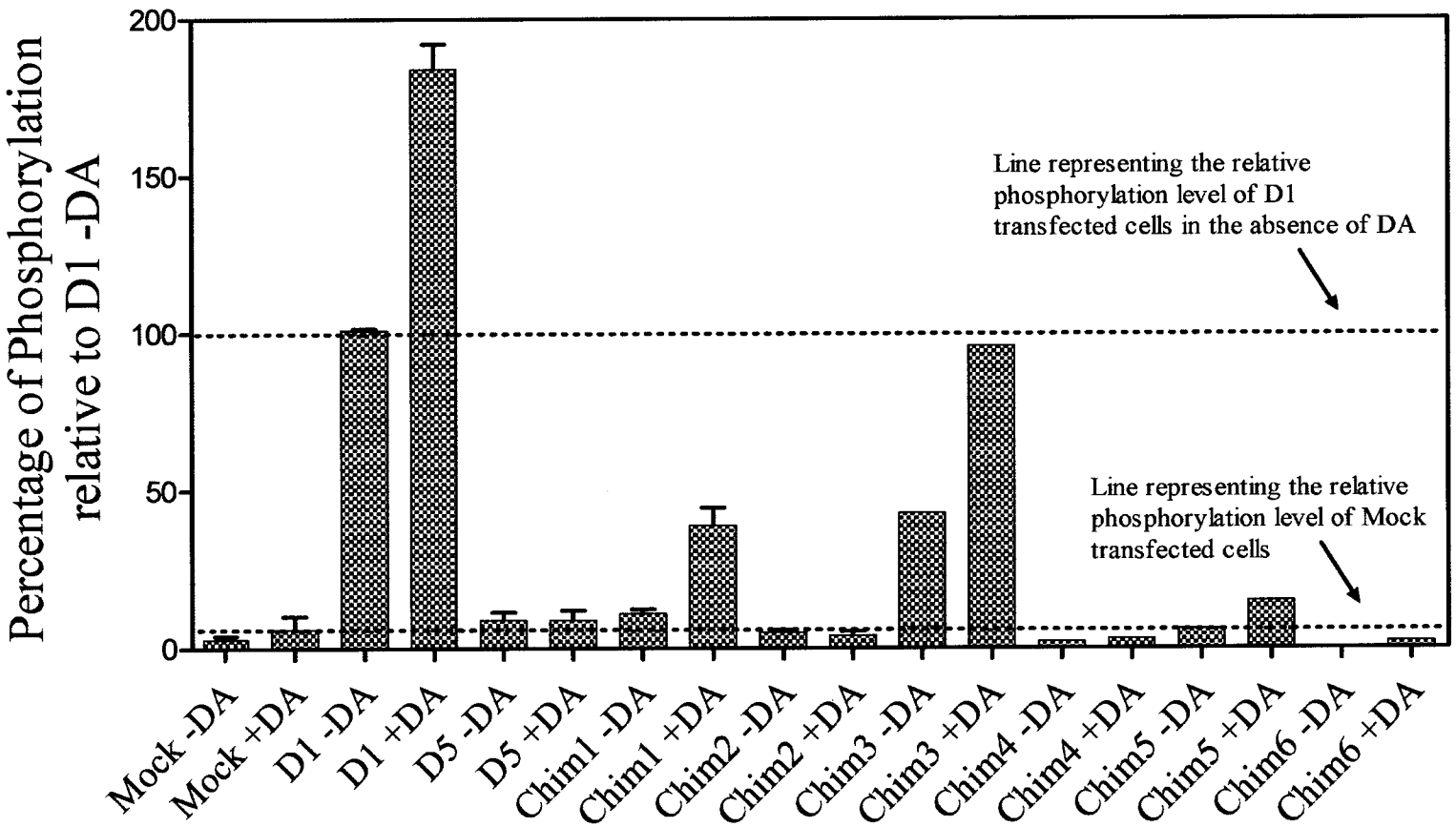
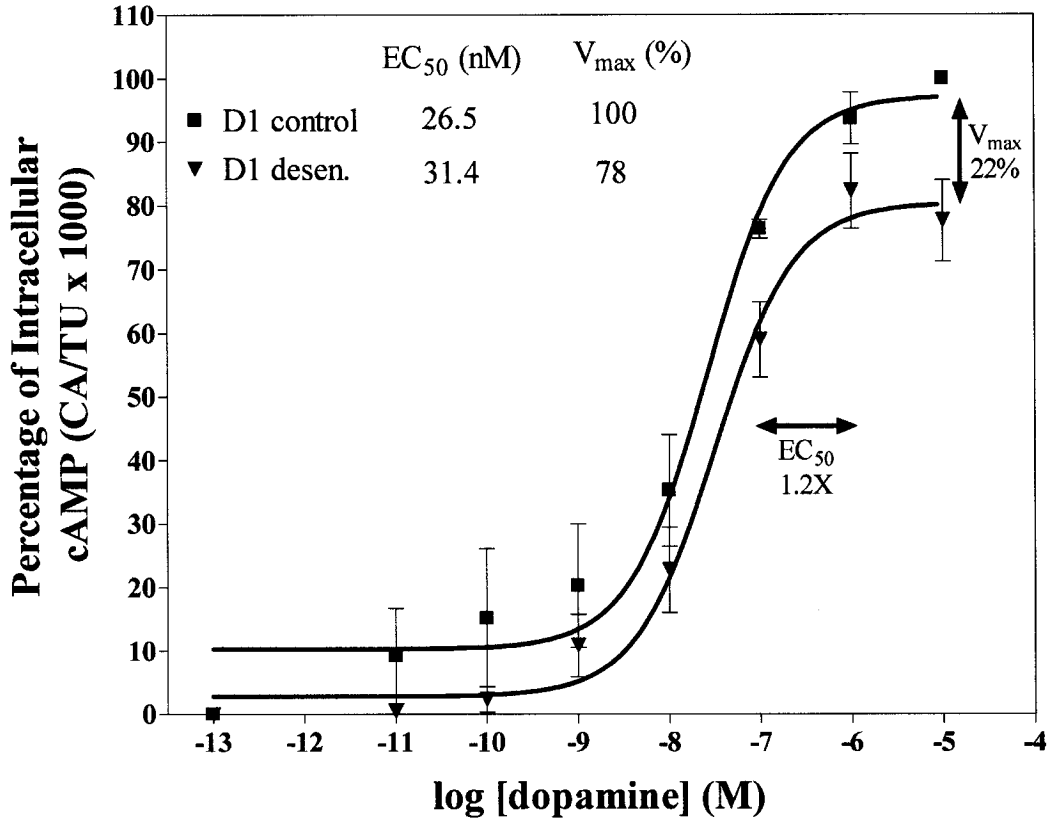
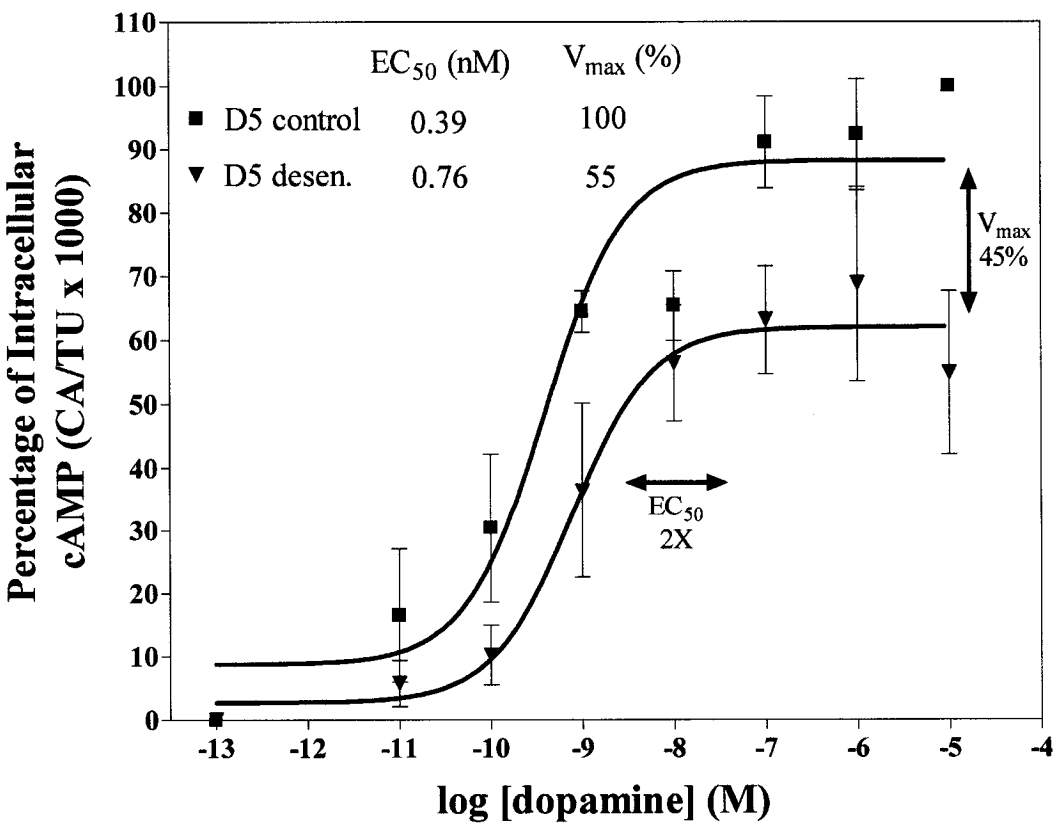

Percentage of Phosphorylation relative to D1 -DA
200
150
100
50
0
Line representing the relative phosphorylation level of D1 transfected cells in the absence of DA
Line representing the relative phosphorylation level of Mock transfected cells
Mock -DA
Mock +DA
D1 -DA
D1 +DA
D5 -DA
D5 +DA
Chim1 -DA
Chim1 +DA
Chim2 -DA
Chim2 +DA
Chim3 -DA
Chim3 +DA
Chim4 -DA
Chim4 +DA
Chim5 -DA
Chim5 +DA
Chim6 -DA
Chim6 +DA

Figure 22 - Dopamine-induced desensitization of adenylyl cyclase activity in HEK293 cells transfected with wild-type D1 and D5 receptors.

HEK cells transfected wild-type D1 and D5 receptors were seeded in 12-well dishes and labeled with [^{3}H]adenine as described in the Methods section. Cells were treated with or without 10 μM dopamine (DA) for 5 min at 37 °C. At the end of the treatment, each well was washed twice with PBS, and cells were incubated in the presence or absence of increasing concentrations of DA for 10 min at 37 °C. Results are expressed as arithmetic means ± SE of eight experiments done in triplicate determination and reported as percentage of maximal response obtained under control experimental conditions. Production of intracellular cAMP is plotted as a function of log of DA concentrations, and curves were analyzed by using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The receptor number in pmol/mg of membrane protein (expressed as the arithmetic mean ± SE) was 1.46 ± 0.19 (D1, control), 1.26 ± 0.12 (D1, treated), 0.79 ± 0.06 (D5, control), 0.76 ± 0.08 (D5, treated).


EC50 (nM)
Vmax (%)
D1 control
26.5
100
D1 desen.
31.4
78
Vmax
22%
EC50
1.2X
Percentage of Intracellular
cAMP (CA/TU x 1000)
log [dopamine] (M)


EC50 (nM)
Vmax (%)
D5 control
0.39
100
D5 desen.
0.76
55
Vmax
45%
EC50
2X
Percentage of Intracellular
cAMP (CA/TU x 1000)
log [dopamine] (M)

1.2.4 Dopamine Stimulation Leads to Dynamin-Dependent Endocytosis of D1-like Receptors

Using confocal laser microscopy and permeabilized HEK293 cells, I investigated agonist-mediated D1 and D5 receptor endocytosis. Permeabilized HEK293 cells expressing D1 or D5 receptors were labeled with primary D1 and D5 receptor specific antibodies, respectively.

Under basal conditions, HEK293 cells expressing either D1 or D5 receptors exhibit an immunostaining predominantly localized on the cell surface (Figure 23A-C and Figure 24A-C). Following a 30 minute stimulation with 10 μM DA, permeabilized HEK293 cells expressing either D1 or D5 receptors exhibit a punctuate labeling in the cytoplasm (Figure 23D-F and Figure 24D-F). Furthermore, DA stimulated cells expressing D1 receptor exhibit a partial loss of cell surface immunostaining, consistent with receptor endocytosis (Figure 23D-F). The effect is more striking in cells expressing D5 receptors, which exhibit a lack of detectable cell surface immunostaining following DA stimulation (Figure 24D-F). Moreover, I report that co-expression of dominant negative mutant of dynamin (Dyn^{K44A}) with either D1 or D5 receptors leads to inhibition of agonist-induced endocytosis (Figure 25).

Figure 23 - Intracellular sorting of wild-type D1 receptors expressed in HEK293 cells in the presence or absence of dopamine.

HEK293 cells transfected with wild-type D1 receptors were seeded on coverslips in a 24-well dish and treated without (A-C) or with (D-F) 10 μM dopamine (DA) for 30 min at 37 °C. Cells were then permeabilized and fixed as described in the Methods section. Confocal laser microscopy was used to visualize immunofluorescence and capture images.

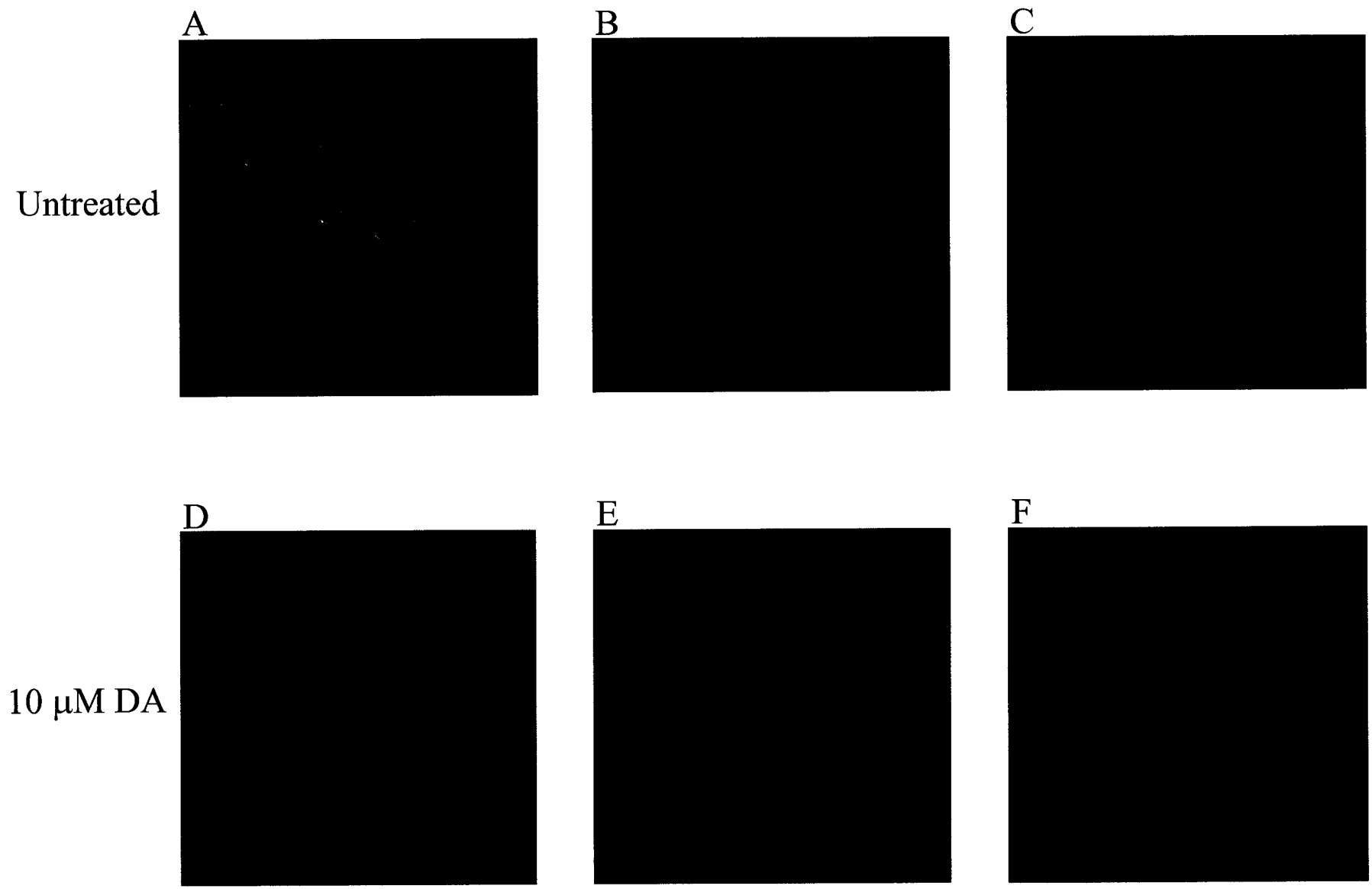
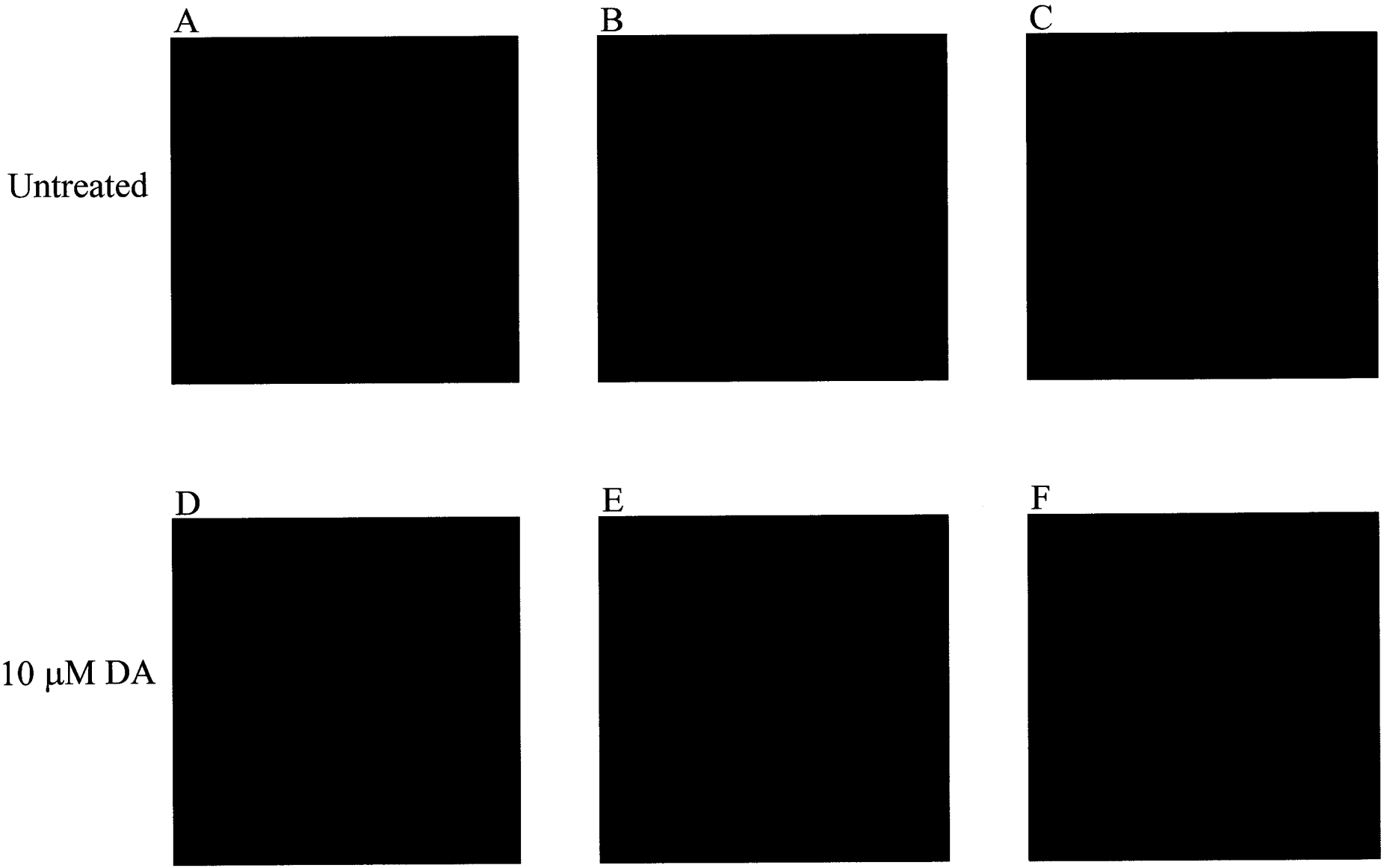

A
B
C
Untreated
D
E
F
10 μM DA

Figure 24 - Intracellular sorting of wild-type D5 receptors expressed in HEK293 cells in the presence or absence of dopamine.

HEK293 cells transfected with wild-type D5 receptors were seeded on coverslips in a 24-well dish and treated without (A-C) or with (D-F) 10 μM dopamine (DA) for 30 min at 37 °C. Cells were then permeabilized and fixed as described in the Methods section. Confocal laser microscopy was used to visualize immunofluorescence and capture images.


A
B
C
Untreated
D
E
F
10 μM DA

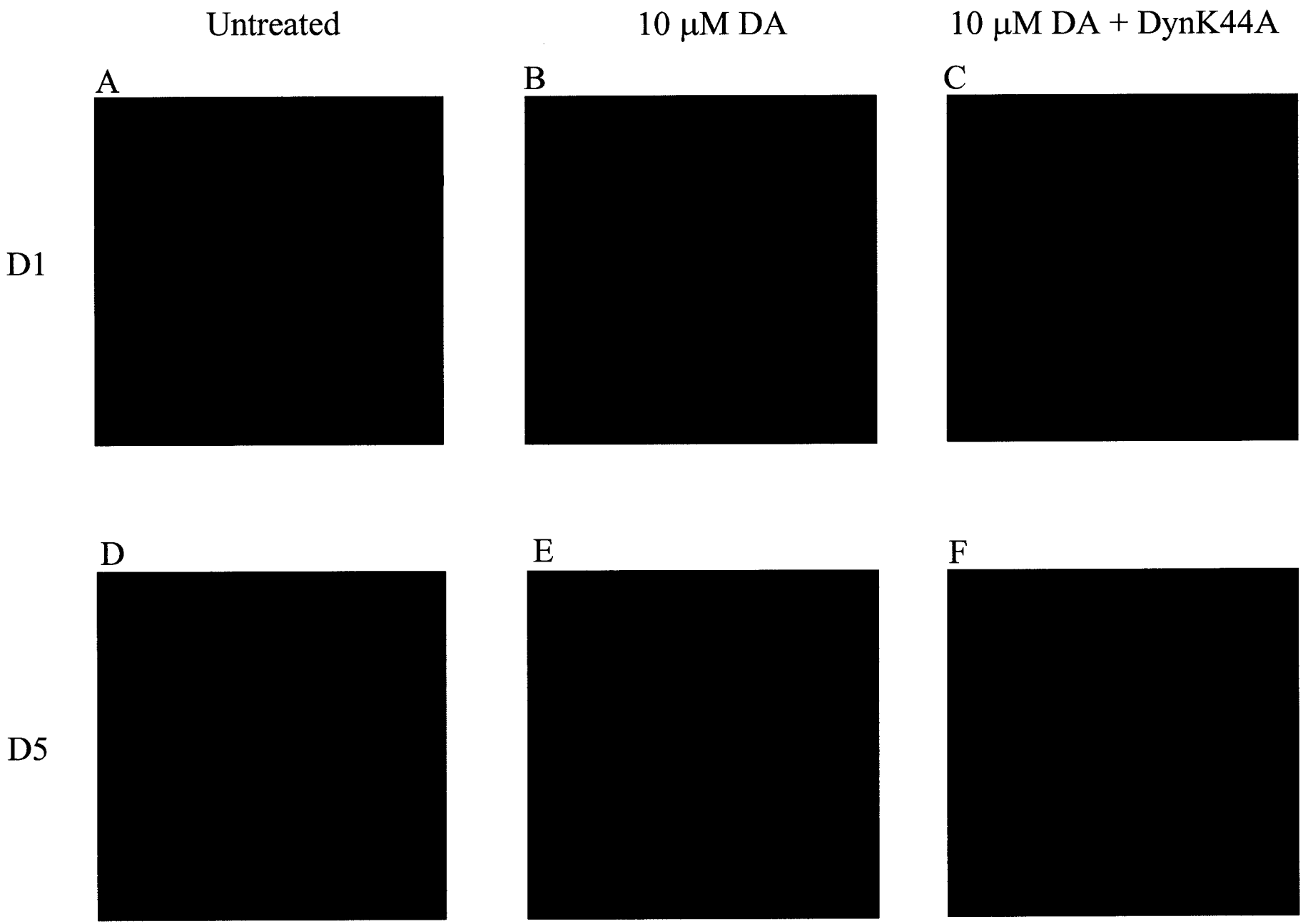
1.3 Conclusions

For the first time, this study reports that following acute agonist stimulation the D5 receptor undergoes phosphorylation-independent desensitization and dynamin-dependent endocytosis. Previous studies have shown that CT of the dopamine D1 receptor is involved in mediating receptor phosphorylation, desensitization, and endocytosis (Jackson et al., 2002; Lamey et al., 2002; Watanabe et al., 2002; Kim et al., 2004). In contrast to the D1 receptor, little evidence exists to describe the regulatory process of the D5 receptor responsiveness. Previous studies have reported that the D5 receptor undergoes desensitization in response to acute agonist treatment but whether this is a result of receptor phosphorylation and/or endocytosis was not evaluated (Jarvie et al., 1993; Callier et al., 2003).

The present study was unable to detect D5 receptor phosphorylation in the absence or presence of dopamine, or using overexpression of GRK2, 5, or 6. To explain the discrepancy between D5 receptors harboring numerous potential phosphorylation sites and lack of detectable phosphorylation, I propose that D5 receptors may be constitutively phosphorylated preventing the detection of basal and dopamine-dependent D5 receptor phosphorylation. Alternatively, the D5 receptor phosphorylation/dephosphorylation may be a rapid process that renders difficult the detection of D5 receptor phosphorylation. Moreover, the phosphorylation sites on D5 receptors may be blocked or concealed through an interaction with novel intracellular D5 regulatory proteins and/or D5 receptor homodimerization.

Figure 25 - Inhibitory effects of dominant negative dynamin on D1 and D5 receptor endocytosis in HEK293 cells.

Cells were grown on coverslips and transiently transfected with D1 or D5 receptors. Localization of D1 and D5 receptors is shown in: A,D, cells incubated in the absence of dopamine; B,E, cells incubated in the presence of 10 μM dopamine (DA) for 30 min at 37 °C. C,F, cells cotransfected with the either D1 or D5 receptor and dynamin-1(K44A), and incubated in the presence of 10 μM dopamine (DA) for 30 min at 37 °C. Confocal laser microscopy was used to visualize immunofluorescence and capture images.


Untreated
10 μM DA
10 μM DA + DynK44A
A
B
C
D1
D
E
F
D5

In addition to the D5 receptor, other GPCRs have been shown to undergo phosphorylation-independent desensitization and endocytosis (Murray et al., 1998; Lee et al., 2000a; Dhami et al., 2002; Jackson et al., 2002; Mukherjee et al., 2002; Pao and Benovic, 2002; Perroy et al., 2003; Qiu et al., 2003). For example, GABA(B) receptor is desensitized through the binding of GRK4, but this process is phosphorylation independent as demonstrated by using kinase deficient GRK4 constructs (Perroy et al., 2003).

My results clearly demonstrate that both D1 and D5 receptors undergo an agonist-dependent regulatory process that involves desensitization and endocytosis. The most striking difference between the two D1-like GPCRs is that the agonist-induced D5 subtype desensitization and endocytosis proceed through seemingly a phosphorylation-independent process that leads to a more robust loss of receptor responsiveness.

DISCUSSION

Symptoms of CNS disorders such as Parkinson's disease and schizophrenia are treated with therapeutics that mediate their actions at dopaminergic receptors. Drugs classified as dopaminergic agonists are used to replenish the loss of signaling observed in Parkinson's disease. However, treatment with agonists gives rise to side effects which manifest themselves as psychosis and delirium, and are reminiscent of symptoms observed in schizophrenia (Young et al., 1997). In contrast, symptoms associated with schizophrenia are alleviated using antipsychotic drugs that display inverse agonist properties at dopaminergic receptors. Treatment of schizophrenia with antipsychotic drugs, which have been shown to behave as inverse agonists at dopamine receptors (Tiberi and Caron, 1994; Cai et al., 1999), can give rise to side effects involving involuntary movements, symptoms reminiscent of Parkinson's disease (Gerlach et al., 1996).

The underlying basis of therapeutic benefits and/or side effects using dopamine receptor drugs remains largely unknown. The cellular co-localization of dopamine receptor subtypes and structural similarities shared between these receptors may explain the action of dopaminergic drugs. Structural similarities between these receptors, especially within transmembrane domains, contribute to the fact that drugs targeting one receptor can bind to and activate other members of the dopamine receptor family. This is best illustrated with the D1 and D5 receptors, two G_S-coupled GPCRs that share 82% amino acid identity within the transmembrane domains (Jarvie and Caron, 1993). In addition, these two D1-like receptors have been shown to be co-localized on the same neurons notably the

pyramidal cells of the prefrontal cortex (Bergson et al., 1995). The prefrontal cortex is a key brain region involved in the regulation of cognitive function and working memory (Seamans et al., 2001; Abi-Dargham et al., 2002; Lidow et al., 2003). In non-human primates, studies have shown that working memory is controlled by prefrontal cortex activation of D1-like receptors (Sawaguchi and Goldman-Rakic, 1994). Positron emission tomography (PET) imaging and post-mortem studies have reported a compromised D1-like receptor function in the prefrontal cortex of schizophrenic patients (Okubo et al., 1997; Lidow et al., 1998). Compromised D1-like function correlates with cognitive function and working memory deficits (Williams and Goldman-Rakic, 1995). Indeed, hypoactivity and hyperactivity of D1-like receptors lead to poor performance of working memory. The potential therapeutic benefit of D1-like drugs has been suggested by a study showing that antipsychotic D2-induced working memory deficits in monkeys is reversed by short-term dopamine D1-like receptor agonist stimulation (Castner et al., 2000).

In order to develop drugs that could target a specific D1-like receptor subtype, it is of paramount importance to understand the structural differences that differentiate the dopamine D1 and D5 receptors. Taking into consideration that D1-like receptors are highly conserved in the transmembrane domains, which have been shown to be responsible for ligand binding at GPCRs, it is likely that other domains outside the transmembrane boundaries play a critical role in distinguishing between the ligand binding and activation properties of D1 and D5 receptors. On the basis of my experimental data and the extended ternary complex

model, I propose that D1 and D5 receptors exist in different conformational states under basal and ligand occupancy. Indeed, I predict that the D1 receptor exists in a more constrained or inactive R state, while the D5 receptor exists in a less constrained (more relaxed) or active R* state. This notion is supported by experimental evidence showing that D5 receptors exhibit a higher constitutive activity, higher agonist affinities and lower inverse agonist affinities in comparison with D1 receptors, consistent with the presence of an active R* state.

GPCR transition between R and R* states is a dynamic activation process which has been described as the re-orientation of transmembrane domains. One key element of the transition towards an active state is the movement of the cytoplasmic end of TM6 away from TM3, which in turn exposes residues found in the third intracellular loop that are critical for binding G proteins (Gether and Kobilka, 1998). This transition process is likely to take place as well in D1 and D5 receptors and be of functional consequence as earlier studies have shown that Asp in TM3 and Ser/Ser in TM5 (which are conserved in all dopamine receptor subtypes) participate in the docking of dopamine (Tomic et al., 1993).

The pharmacological and functional differences observed at D1 and D5 receptors are a consequence of unique receptor domains responsible for constraining these receptors into specific structural conformations. The studies described in this thesis have identified numerous structural domains responsible for the distinguishing profiles of D1 and D5 receptors. Taking advantage of the well characterized pharmacological and functional properties of D1-like receptors I have studied the structural domains responsible for the unique profiles of D1 and

D5 receptors. Specifically through the use of chimera/mutations, I demonstrate that unique receptor conformations induced by specific domains/residues regulate the distinct functional properties of D1 and D5 receptors.

The TRL was identified as a large region encompassing structural domains responsible for the differences seen at D1 and D5 receptors. Within the TRL I have identified TM6, EL3 and CT as key determinants responsible for the differences in agonist and inverse agonist affinity and potency, constitutive activity, and dopamine-mediated maximal activation of adenylyl cyclase at D1 and D5 receptors.

Recently, the CT domain has been demonstrated as a key TRL structural determinant responsible for agonist affinity and constitutive activity at D1 and D5 receptors (Demchyshyn et al., 2000; Jackson et al., 2000). Because the intracellular CT can not be a direct binding determinant for the extracellular agonist, it seems to imply that the CT mediates receptor conformations involved in binding of agonists. Thus the receptor conformation induced by the CT of D5 shifts the receptor equilibrium towards R* and in turn results in increased binding affinity for agonists and an increased constitutive activity. In contrast, the CT of D1 constrains the receptor in an R inactive state and thereby lowering agonist binding affinities and decreasing receptor constitutive activity. Our data suggests that the D5-CT releases intramolecular constraints which lead to exposure of intracellular G protein binding determinants resulting in a greater propensity of D1-D5CT to couple to G_S in the absence of dopamine. These results are in agreement with the extended ternary complex model.

The extended ternary complex model postulates that the inactive R and active R* states display higher and lower affinity for inverse agonists, respectively (Leff, 1995). Inverse agonist binding data obtained with wild-type D1 and D5 receptors provide experimental evidence for the model. Similar results would then be expected with D1-D5CT (predominantly in R* state) and D5-D1CT (predominantly in R state). However, the inverse agonist affinity of D1-D5CT and D5-D1CT was not changed in comparison with wild-type D1 and D5 receptors, respectively. These results can be explained by the fact that GPCRs, including D1 and D5 receptors, can exist in multiple conformations not limited by the extended ternary complex model (Prather, 2004). Importantly, these data raise an important issue about the validity of the extended ternary complex model to explain the pharmacological behaviour of inverse agonists. Alternatively, it is possible that a swap of the CT between D1 and D5 receptors leads to chimeric receptors for which "inverse agonists" behave as neutral ligand, i.e. antagonists. Indeed, antagonists are postulated to leave the R $\leftrightarrow$ R* equilibrium intact and act solely as competitive blockers of other ligands binding to the receptor. Further studies are required to assess whether D1-like inverse agonists display inverse agonism at D1-D5CT and D5-D1CT chimera.

Studies described in my thesis identify EL3 as a key structural domain responsible for mediating a conformational receptor state implicated in the binding of inverse agonists and dopamine-mediated maximal activation of adenylyl cyclase at D1 and D5 receptors. I report that chimeric D1/D5 receptors harboring the counterpart EL3 (D1-EL3D5 and D5-EL3D1) unmask the structural

determinant involved in inverse agonist binding and dopamine-mediated maximal activation of adenylyl cyclase which remained concealed with the TRL chimera. Overall, the D1-EL3D5 and D5-EL3D1 chimera display dopamine-mediated maximal activation of adenylyl cyclase and inverse agonist affinity that is indistinguishable from wild-type D5 and D1 receptors, respectively. These observations support the notion that through conformational changes, the EL3 mediates the accessibility of ligands to the binding pocket and mediates the mechanism involved in agonist-mediated activation of G proteins (Colson et al., 1998; Dietrich et al., 1998).

At first, my published TRL chimera study, which encompassed the EL3 domain failed to demonstrate a role of EL3 in mediating inverse agonist binding or dopamine-mediated maximal activation of adenylyl cyclase (Iwasiow et al., 1999). However, when specific EL3 chimeras were studied the functional properties of this domain were revealed. These two studies comment on an elementary limitation with chimeric studies. We can see how swapping of large receptor regions results in the possibility that function associated to smaller domains/residues within the studied region could be masked. An alternative approach is the use of single-point mutation to investigate structure-function relationships. However, in contrasting fashion to using large chimeric regions, I would argue that single-point mutations do not take into consideration the interfering/synergistic actions of neighboring residues. This became evident in my EL3 proline single-point mutation study, which although demonstrated a partial role for these single residues in mediating inverse agonist binding at D1-like

receptors, they could not fully recapitulate the effects observed using the EL3 chimera. Thus, I believe that studies using both chimeric receptors and single-point mutation studies are extremely useful and beneficial in studying structure-function relationships of closely related GPCRs.

Using D1/D5 chimeric receptors, I identified the EL3 domain as playing an important role in mediating the inverse agonist binding affinity. In comparison to D5 receptors, D1 receptors are believed to exist more predominantly in an inactive R state displaying higher affinity for inverse agonists (Tiberi and Caron, 1994). Thus, the EL3 domain modulates the receptor conformational state in a way that D1-EL3D5 can display a lower affinity for inverse agonists. In accordance to the extended ternary complex model we would predict that this chimeric receptor should now be in a more active R* state displaying constitutive activity reminiscent of D5 receptors.

This is the first evidence for the EL3 in mediating inverse agonist binding affinity at GPCRs. In contrast, the extracellular domains of peptidergic GPCRs have been documented to play a key role in their function and chimeric studies of both the mu- and delta-opioid receptors have identified the third extracellular loop as a key determinant in ligand (agonist) binding (Varga et al., 1996; Dietrich et al., 1998). GPCR mutagenesis and modeling studies have proposed that fluctuations of extracellular loops may guide ligands to access the transmembrane binding pocket (Kobilka et al., 1988; Strader et al., 1994; Mizobe et al., 1996; Colson et al., 1998; Waugh et al., 2001). Dietrich et al. (1998) have hypothesized that the extracellular loops may constrain the accessibility to transmembrane

binding sites thus modulating ligand selectivity. In a similar fashion, I propose that the structural conformations induced by EL3 mediate inverse agonist accessibility to the transmembrane binding pocket and result in the observed binding affinity differences at D1 and D5 receptors.

Due to the homology among GPCRs, my studies have widespread implications on the structural requirements for inverse agonist selectivity and in turn could translate into development of more potent/discriminating therapeutic drugs for treating disease associated with constitutively active GPCRs. A defining property of inverse agonists is their ability to inhibit constitutively active receptors. Many constitutively active GPCRs have been implicated in syndromes of hyperfunction (Seifert and Wenzel-Seifert, 2002). They have also relevance in cancer (Shepard et al., 2001). Infection with Kaposi's sarcoma-associated herpes virus results in expression of a constitutively active chemokine receptor, which leads to cell proliferation and viral replication. Mutations in the thyroid stimulating hormone receptor resulting in elevated constitutive activity leads to hyperthyroidism (Polak, 1999). Similarly, a spontaneously occurring constitutively active mutant (CAM) in the rhodopsin receptor gives rise to retinitis pigmentosa (Arvanitakis et al., 1998; Smit et al., 2003). Importantly, the degree to which inverse agonists could be of therapeutic advantage depends on the extent of constitutive GPCR activity underlying the pathophysiology.

The studies described herein have addressed the role of EL3 as a potential target for development of new pharmaceuticals that would inhibit the activity of constitutively active receptors. However, our studies have demonstrated that the

receptor conformations induced by EL3 also play an important role in mediating the dopamine-mediated maximal activation of adenylyl cyclase by D1-like receptors. The fact that an extracellular domain can affect intracellular G protein coupling suggests that the conformational changes induced by EL3 have an effect on the activating properties of D1 and D5 receptors. The ability of EL3 to mediate dopamine-mediated maximal activation of adenylyl cyclase was not observed when studying the TRL chimera. Upon further investigation I identified two residues in TM6 as being crucial in mediating this effect. Proline point mutation studies indicate that secondary structures within these domains play a key role in mediating the inverse agonist binding affinity and dopamine-mediated maximal activation of adenylyl cyclase. I propose that the conformation of EL3 influences the TM6 tilt required for propagation of the G protein signal. Indeed, mutagenesis studies and three dimensional modeling of opioid receptors have revealed that the activation pathway originates from the EL3 and propagates through the transmembrane helices to a cytoplasmic switch (Decaillot et al., 2003).

Dissociation of constitutive activation and dopamine-mediated maximal activation of adenylyl cyclase using our chimeric receptors is very intriguing. In addition to the existence of fundamental differences in the intramolecular interactions at D1 and D5 receptors, I cannot rule out differences in receptor/$G\alpha_S$ interaction. Potentially, receptor conformational states may mediate the interaction of the D1 and D5 receptor with specific $G\alpha_S$ splice variants (short and long isoforms). Indeed, binding to and activation of different $G\alpha_S$ isoforms may contribute to the distinct activation properties observed between D1 and D5

receptors. Recent studies have reported that the β_2-adrenergic receptor fused to the long isoform of $G\alpha_S$ has the hallmarks of a constitutively activated GPCR (Seifert et al., 1998). $G\alpha_S$ subunit conformation may also play a role. Mutations in the $G\alpha_S$ (R258W or R258A) result in a reduced ability to stimulate adenylyl cyclase upon receptor activation (Warner et al., 1998). Another mutant of $G\alpha_S$ (S54N) demonstrates a selective coupling to receptors. The S54N $G\alpha_S$ mutant displays constitutive activity but lacks the ability to be stimulated upon agonist treatment (Cleator et al., 1999). Moreover, the composition of G proteins may dictate the receptor coupling efficiency and specificity. Using β_1-adrenergic receptors it was demonstrated that the coupling efficiency could be modulate by expressing different isoforms of the G protein β subunit (McIntire et al., 2001). Moreover, the D1 receptor was shown to couple G proteins containing a γ_7 subunit while D5/G protein coupling was independent of the γ_7 subunit (Wang et al., 2001).

In accord with this evidence the dissociation between constitutive activation and dopamine-mediated maximal activation of adenylyl cyclase observed in my studies may be explained by different G_S proteins exhibiting coupling preference for receptors in either R* or agonist-R* conformation. Recently, studies stemming from various GPCR systems have provided evidence for existence of multiple active conformations of GPCRs. Data from these studies suggests that GPCRs can activate multiple G proteins, depending on the receptor active state (Azzi et al., 2003; Gbahou et al., 2003). Such a mechanism would suggest that different agonists and inverse agonists could produce distinct

intracellular effects (Prather, 2004). This data supports that ligands can enrich a specific receptor conformation capable of differentially coupling to distinct G proteins.

In view that the distinct properties at D1 and D5 receptors are dependent on more than the structural determinants involved in ligand binding, I reason that G protein coupling and receptor regulation may contribute to these differences. Indeed, I have demonstrated that both D1 and D5 receptors undergo a dynamin-dependent endocytosis upon agonist stimulation. However, I report that this process is phosphorylation-dependent in the case of D1 receptors but phosphorylation-independent in the case of D5 receptors. Phosphorylation-independent regulation has been demonstrated in recent years in other GPCR systems. The luteinizing hormone receptor binds arrestin and is endocytosed in a process independent of receptor phosphorylation (Mukherjee et al., 2002). The opioid receptor has been demonstrated to undergo phosphorylation-independent desensitization and endocytosis (Murray et al., 1998; Qiu et al., 2003).

An abundance of recent studies challenge the classical paradigm of GPCR desensitization. Most notably the role of receptor phosphorylation has been challenged. In the classical paradigm, receptor conformational changes induced by stimulation with agonists result in a high affinity binding state for GRKs leading to the phosphorylation of the receptor and promoting an interaction with arrestin, which leads to uncoupling of the receptor from G protein (desensitization) and endocytosis. In recent years, it has become evident that desensitization upon activation of receptor by agonist does not exclusively require

phosphorylation (Ferguson, 2001). A new scheme of GPCR desensitization suggests that kinase activity of GRKs may be secondary to its role in attenuating signal transduction (Dhami et al., 2002). This scheme suggests that the active receptor conformation is a substrate for GRKs, however, GRK-mediated phosphorylation of the receptor is not necessary for desensitization. GRK affinity for the active conformation of the receptor can result in uncoupling of the receptor/G protein through steric interference (Dhami et al., 2002; Perroy et al., 2003). In fact, I have demonstrated that D5 receptors, in the absence of phosphorylation, undergo a more pronounced desensitization in comparison with D1 receptors. Recently, it has been demonstrated that in vitro the CT of D1 and D5 receptors interacts with N-ethylmaleimide-sensitive factor (NSF), which is involved in recycling of receptors back to the plasma membrane. Interestingly, the CT of D5 receptors was also shown to interact strongly with sorting nexin 1 (SNX1) (Heydorn et al., 2004). SNX1 is involved in targeting receptors to lysosomal degradation (Kurten et al., 1996). Potentially, targeting of D1 and D5 receptors to different intracellular compartments and the fate of internalized receptors may explain the differences in desensitization observed after treatment with dopamine.

Furthermore, in support of phosphorylation-independent desensitization, arrestin has been shown to bind IL3 of the active luteinizing hormone receptor with high affinity in the absence of receptor phosphorylation (Mukherjee et al., 2002). Moreover, our previous studies have implicated a phosphorylation-independent desensitization of the D1 receptor in HEK293 cells (Jackson et al.,

2002). Truncation of the CT of D1 receptors results in removal of phosphorylation sites, yet the receptor retains its ability to be desensitized. Similarily, a recent study has demonstrated that removal of CT serine/threonine residues in D1 receptors abolishes detectable phosphorylation, however the receptor retains its ability to undergo agonist-induced desensitization (Jackson et al., 2002; Kim et al., 2004). Interestingly, Kim et al. (2004) show that D1 receptors with mutated serine/threonine residues in IL3 displayed detectable levels of phosphorylation but impaired desensitization. This study dissociates the phosphorylation and desensitization properties of D1 receptors in HEK293 cells. The studies mentioned above address the significance of arrestin accessibility and binding to IL3 domains rather than CT phosphorylation as a key determinant of desensitization.

In contrast to the D1 studies, I report that no phosphorylation was detected at the wild-type D5 receptor, which contains putative serine/threonine GRK consensus sequences. I propose that the absence of detectable phosphorylation at the D5 receptor can potentially be explained by phosphorylation sites that are masked either through interaction with intracellular proteins and/or receptor dimerization. Protein interactions at both D1 and D5 receptors have been described. The CT of D1 receptors has been demonstrated to interact directly with the NR1-1a and NR2A subunits of NMDA glutamate receptors (Lee et al., 2002). Through this interaction D1 activation can mediate the signaling of NMDA receptors, a process known as receptor cross-talk. The D1 receptor has also been shown to interact with calcyon, a single transmembrane protein localized in

dendritic spines which allows D1 cross-talk to multiple effector systems (Lezcano et al., 2000). The D5 receptor has been demonstrated to cross-talk with $GABA_A$ receptors through a protein interaction involving the CT. This protein interaction is dependent on the agonist occupied D5 state and results in an inhibition of D5 signaling (Liu et al., 2000). Notwithstanding the reported protein interactions, the HEK293 cell system does not express native $GABA_A$ receptors. However, I cannot rule out the possibility that other natively expressed proteins could interact with either the D1 or D5 receptors. The electrophoretic mobility of D1 and D5 receptors suggests a striking difference in the migration pattern of these two structurally related receptors. The D1 receptor migrated at 75 – 85 kD, in contrast the D5 receptor migrated in a band ranging between 120 -140 kD. Potentially, these data support the view that the absence of phosphorylation observed at the D5 receptor results from a protein interaction. Alternatively, the discrepancy in D1 and D5 electrophoretic mobility may be explained by different post-translational modifications such as glycosylation.

Interestingly, the electrophoretic mobility of D5 receptors indicates a protein size approximately twice that of D1 receptors. Newly emerging topics regarding GPCR regulation have demonstrated that GPCRs can homo- and heterodimerize, a characteristic indication of dimerization is an increased overall protein size as indexed by a slower electrophoretic mobility (Rios et al., 2001). Indeed, members of the dopamine receptor family have been reported to dimerize. Specifically several groups have reported the dimerization of D2 and D3 receptors (Nimchinsky et al., 1997; Wurch et al., 2001; Lee et al., 2003). Moreover,

unpublished data from George et al. claimed that D1, D4, and D5 receptors can form homodimers (Lee et al., 2000b). Indeed, I have evidence suggesting that both D1 and D5 receptors can form homodimers. However, D5 receptor homodimers are resistant to SDS, while D1 receptor homodimers are sensitive to SDS (data not shown). These observations suggest that despite their structural similarities, D1 and D5 receptors display differing protein interactions involved in dimerization. Moreover, these observations may explain the different electrophoretic mobility of D1 and D5 receptors on SDS-page gels.

Other GPCR systems that have been studied in greater detail provide functional evidence for dimerization. Heterodimerization of opioid receptors results in ligand binding and functional properties that are different from those of either receptor (Jordan and Devi, 1999). Similarly, dimerization of D2 receptors may result in the formation of new binding sites capable of binding unique ligands (Ng et al., 1996; Zawarynski et al., 1998). Of more direct interest to us and an indication that dimerization can influence receptor signaling, GPCR dimers have been demonstrated to play a role in internalization and phosphorylation. Co-expression of wild-type α-mating factor receptors with internalization-defective mutants resulted in efficient internalization of the mutant receptors, suggesting that these receptors were internalized as dimers (Overton and Blumer, 2000). In the somatostatin receptor (SSTR) family, SSTR1 does not undergo agonist-dependent endocytosis while SSTR5 does. Co-expression of these two receptors resulted in endocytosis of both receptors, providing evidence for heterodimerization (Rocheville et al., 2000). Cross-phosphorylation and cross-

desensitization was observed when SSTR2A and the μ-opioid receptors were co-expressed. Agonist stimulation of either the SSTR2A or μ-opioid receptor results in the phosphorylation and desensitization of the non-stimulated receptor (Pfeiffer et al., 2002). Overall these results demonstrate the diversity of effects involving ligand binding and receptor regulation resulting from GPCR dimerization.

CONCLUSIONS

The studies reported in my thesis permit to draw conclusions on the stucture-function relationships of D1 and D5 receptors. Experimental data suggests that the extended ternary complex model is insufficient to describe the functional properties of D1-like receptors, which I demonstrated could adopt multiple conformational states. Specifically, I have reported for the first time that the third extracellular loop (EL3) mediates a conformational state responsible for inverse agonist binding and agonist-mediated maximal activation of adenylyl cyclase. Moreover, I have described a constraining interplay between EL3 and TM6 which is responsible for inducing subtype-specific functional phenotypes to D1 and D5 receptors. I propose that the TM6 and EL3 regions mediate accessibility of inverse agonists to the binding pocket and regulate the movement of TM6 relative to TM3 upon ligand binding. In contrast, other studies from our group (TRL and CT studies) have implicated the CT of D1-like receptors in mediating a conformational state regulating agonist binding and agonist-independent activity (Iwasiow et al., 1999; Jackson et al., 2000). I propose that the CT regulates structural constraints responsible for G protein accessibility and binding to a ligand-free receptor. Moreover, GPCR constitutive activity, resulting from increased coupling of an unoccupied receptor to G proteins induces a unique receptor state displaying increased agonist affinity.

Furthermore, my studies have revealed that the multiplicity of D1 and D5 receptors may be partially explained by differences in their regulation upon agonist stimulation. I propose that dimerization may represent a potentially novel mechanism that mediates phosphorylation, desensitization, and endocytosis of

dopamine D1-like receptors. In contrast with the D1 receptor, western blot analysis demonstrates that the D5 receptor migrates in the range consistent with a dimer. Despite the fact that both receptors are endocytosed in a dynamin-dependent manner, only D1 receptors can be shown to be phosphorylated. However, in the absence of phosphorylation, D5 receptors undergo a more robust desensitization in comparison with D1 receptors, suggesting that these two receptors may display distinct regulatory mechanisms in HEK293 cells.

FUTURE CONSIDERATIONS

Future studies designed to decipher the multiplicity of D1-like receptors must tackle the issue on several fronts, structure-function and regulation. In consideration of my assessment that D1 and D5 receptors can form multiple active conformations there is a need for molecular modeling. A better understanding of conformations induced by the EL3 domain would benefit the development of new inverse agonists with specificity for either D1 or D5 receptors. Another important aspect is what specific residues mediate the conformations induced by EL3. Identification of these residues could be assisted by accurate molecular modeling but until then a single-point mutation approach would be of great benefit. I have demonstrated that the proline residues play a partial role in mediating these effects. However, a more detailed study examining the synergy between proline residues, other residues within the EL3, and the spacing of EL3 may provide further insight into the structural requirements necessary to induce D1 and D5 specific conformations.

My observation that regulation of D1 and D5 receptors may contribute to their distinct functional properties provides reason to further study these processes. Of particular interest is the identification of the high molecular D5 receptor species observed in my immunoblotting studies, which may shed light onto the lack of phosphorylation detected at the D5 receptor. To further our understanding and confirm the phosphorylation-independent pathway several different avenues can be addressed. 1. Investigate whether or not the phosphorylation-independent pathway is specific to HEK293 cells or can it be recapitulated in other cellular systems and in vivo. 2. Use co-expression of kinase

deficient GRKs to demonstrate phosphorylation-independent desensitization of D1 and D5 receptors. Finally, in light of the results I have reported and recently published studies, it becomes apparent that investigating receptor endocytosis and recycling is of great importance. Specifically, calculating the rate of recycling and identification of intracellular compartments would provide insight into the different pathways involved in the regulation of D1 and D5 receptors.

Overall, the studies described herein provide new insights into the subtype-specific ligand binding, G protein coupling and signaling properties of dopamine D1 and D5 receptors. These studies may have widespread implications on the structural requirements for inverse agonist selectivity and development of new subtype-specific drugs. Moreover, I describe a differing agonist-induced regulation at D1 and D5 receptors which may provide a novel target for subtype-specific therapeutics.

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