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Functional Role of the Exofacial End of Transmembrane Domain 6 of the Human D1 and D5 Dopaminergic Receptors

by

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Abstract

The molecular basis underlying the distinct ligand binding and activation properties of the dopamine D1 and D5 receptors is not clearly understood. In the present study, I hypothesize that the variant residues of the exofacial end of the transmembrane 6 (TM6) region of D1 and D5 (Ile ^{294}/Leu^{295} in D1 and Met ^{318}/Val ^{319} in D5) regulate the ligand binding affinity and activation of these two receptors. Chimeric D1R and D5R harboring wild type counterpart variant TM6 residues along with the entire third extracellular loop (EL3) were engineered using PCR and expressed in HEK293 cells. My results show that variant TM6 residues play an important role in controlling the phenotypic expression of EL3-mediated regulation of ligand binding and G protein coupling properties of the human D1 and D5 receptors. These studies may prove useful in the design of subtype-selective drugs for the treatment of pathologies displaying D1-like receptor abnormalities.

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List of Abbreviations

AC	Adenylyl cyclase
ADHD	Attention-deficit/hyperactivity disorder
ANOVA	Analysis of variants
B_{MAX}	Maximal binding capacity
BP	Bipolar disorder
CAM	Constitutively active mutant
cAMP	cyclic 3', 5'-adenosine monophosphate
CNS	Central nervous system
CT	Cytoplasmic tail
DA	Dopamine
DNA	Deoxyribonoucleotide acid
DOPA	Dihydroxyphenylalanine
DRD1	Dopamine D1 receptor gene
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
EC50	Half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EL	Extracellular loop
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
Gi	Inhibitory G protein

Gs	Stimulatory G protein
G protein	GTP-binding protein
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
GTP	Guanine triphosphate
HBS	HEPES-buffered saline
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
[^{3}H]SCH	N-[methyl-^{3}H]SCH23390
IBMX	1-methyl-3-isobutylxanthine
ICD-10	International Statistics Classification of Diseases, 10th Revision
IL	Intracellular loop
K_D	Equilibrium dissociation constant
K_I	Equilibrium inhibition constant
7MSRs	Seven-membrane-spanning receptors
MEM	Minimal essential medium
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
R	GPCR inactive conformation
R*	GPCR active conformation
RGS	Regulation of G protein signaling
SCH	SCH23390
SCH23390	R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5 -tetrahydro-1H-3-benzazepine
TM	Transmembrane
TSH	Thyroid-stimulating hormone
TRL	Terminal receptor locus
TU	Total uptake

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Introduction

1. G protein-coupled receptors (GPCRs)

Receptors are fundamental biochemical components of the cell exterior that maintain homeostasis within a living organism. Various biological functions, responses, interactions, pathways and processes involved in the moment-to-moment existence of a cell are controlled by extracellular signals, which are then conveyed into the cell interior through various classes of integral plasma membrane receptors (Wess 1998). The vast majority of cell-surface receptors belong to the superfamily of G protein-coupled receptors (GPCRs) [also referred to as the seven-membrane-spanning receptors (7MSRs) or heptahelical receptors]. Genes encoding GPCRs represent approximately 1-2% (800-1000 receptors) of the human genome (Fredriksson et al. 2003; Lefkowitz 2004).

The broad variety of biologically active molecules or ligands – including hormones, neurotransmitters, ions, amino acids and lipids – acting through GPCRs underscores the physiological importance of these receptors. In fact, GPCRs mediate physiological processes that range from chemosensory recognition (perception of light, olfaction, taste), endocrine regulation, synaptic neurotransmission to complex behavioral events (Pierce and Lefkowitz 2001; Gainetdinov et al. 2004).

Abnormalities in GPCR-mediated signaling virtually affect most tissues and organs in human body, such as hyperfunctioning thyroid adenomas, nephrogenic diabetes insipidus and color blindness. Furthermore, over 90% of the

nonsensory GPCRs are expressed in the brain and their role is critical for normal brain function (Vassilatis et al. 2003). Importantly, dysfunction of individual GPCR systems is implicated in several pathological conditions, such as hypodopaminergic movement disorders, mania and depression. GPCRs are targets for a plethora of therapeutic agents and thus GPCRs are important in the current clinical practice of medicine (Karnik et al. 2003; Wise et al. 2004).

1.1 GPCR classification and basic architecture

The superfamily of GPCRs can be divided into three major subfamilies on the basis of the sequence homology and pharmacological nature of their ligands. This subdivision consists in the rhodopsin/β-adrenergic receptor (β-AR) family (Family A or class I), the secretin receptor family (Family B or class II) and the metabotropic glutamate receptor family (Family C or class III) which represent about 89%, 7% and 4%, respectively, of all known GPCRs in human (Chalmers and Behan 2002; Lawson and Wheatley 2004) (Table 1). Two additional classes, which are unrelated to any these receptor families, include the fungal pheromone and *Dictyostelium* GPCRs (Horn et al. 1998; Bockaert and Pin 1999).

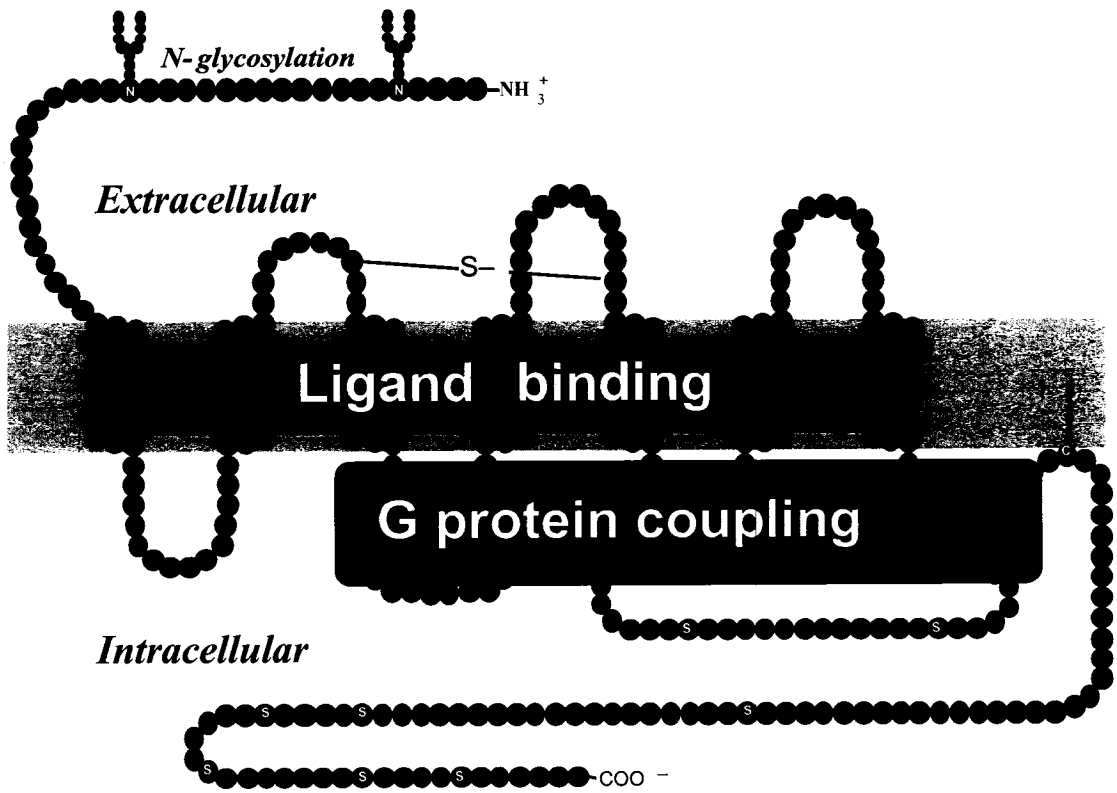
All GPCRs are predicted to share a common central core domain that consists of seven transmembrane helices (TM1 through TM7) linked by three intracellular (IL1, IL2 and IL3) and three extracellular (EL1, EL2 and EL3) loops (Figure 1). GPCRs also have an N-terminal region (harboring N-glycosylation

Table 1 – Family classification of important known G protein-coupled receptors in human.

Important known GPCRs in human		
Family A (class I)	**Family B (class II)**	**Family C (class III)**
Biogenic amine receptors •Acetylcholine •α-Adrenoceptors •β-Adrenoceptors •Dopamine •Histamine •Serotonin ***Peptide receptors*** •Angiotensin •Bradykinin •Interleukin-8 •Neuropeptide Y •Opioid •Endothelin •Vasopressin •Galanin ***Prostanoid receptors*** •Prostaglandin •Thromboxane ***Nucleotide-like receptors*** •Adenosine •Purinoceptors ***Non-calcified receptors*** •Cannabinoid •Platelet-activating factor •Gonadotropin-releasing hormone •Melatonin •Leukotriene B4 ***Olfactory receptors*** ***Receptors for light*** •Rhodopsin	***Secretin-like receptors*** •Secretion •Calcitonin •Corticotropin-releasing factor •Gastric inhibitory peptide •Glucagon •Growth-hormone-releasing-hormone •Parathyroid hormone •Vasoactive intestinal polypeptide •Diuretic hormone	***Metabotropic glutamatergic-like receptors*** •Metabotropic glutamatergic receptors •Extracellular calcium-sensing receptor •$GABA_B$ receptors •Taste receptors

Figure 1 – General topology of G protein-coupled receptors.

GPCRs have a central common core domain made of seven transmembrane (TM) helices connected by three intracellular and three extracellular loops. Aside from sequence variations, GPCRs differ in the length and function of their N-terminal extracellular region, C-terminal intracellular domain and intracellular loops. Two cysteine residues form a disulfide bond which is predicted to be important for the packing and stabilization of TM helix conformations.


N-glycosylation
N
N
NH 3 +
Extracellular
S–
Ligand binding
G protein coupling
C
S
S
Intracellular
S
S
S
S
S
S
S
COO –

sites) located extracellulary and a C-terminal region (containing phosphorylation sites) extending into the cytoplasm. Depending on the subfamily of GPCRs, each of these domains has distinct properties that confer on individual receptors their specific ligand binding and G protein coupling properties. The extracellular receptor surface (including the extracellular N-terminal domain, extracellular loops and exofacial ends of various TM domains) critically contributes in the ligand binding properties of GPCRs (Strader et al. 1994; Wess 1996). Moreover, the intracellular receptor surface (including the different intracellular loops, C-terminal domain and the cytoplasmic ends of TM helices) plays a crucial role in G protein recognition and activation (Dohlman et al. 1991; Hedin et al. 1993; Wess 1997).

1.2 Molecular diversity of G proteins and GPCRs downstream signaling

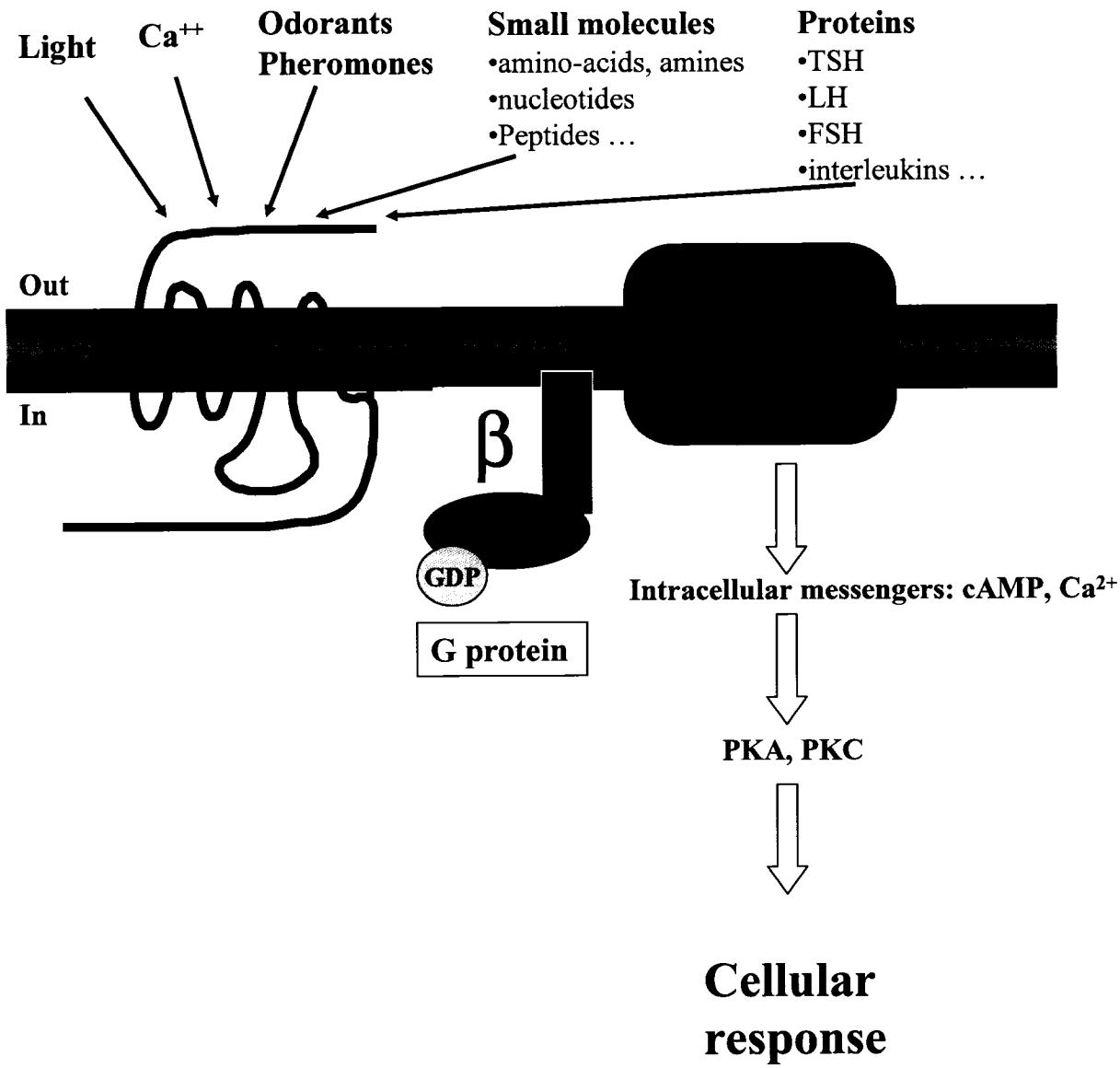
GPCRs have been named based on their ability to interact with and stimulate intracellular heterotrimeric G proteins, which upon their activation can modulate several cellular pathways (Gether 2000). Generally, in the absence of the appropriate activating ligand or agonist, the receptor and G proteins are both maintained in the inactive states. In the presence of activating ligands, GPCRs respond by coupling to and activating intracellular G proteins. Each receptor subtype can only activate a specific subset of G proteins, which mediate distinct

downstream signals (Figure 2). G proteins are composed of three associated protein subunits (called α, β and γ) and classified based upon their amino acid sequence as well as functional similarities of α subunits (Gudermann et al. 1997). There are 16 known mammalian α subunits that have been categorized into four distinct subfamilies: G_s proteins couple to the stimulation of adenylyl cyclases; G_i proteins couple to the inhibition of adenylyl cyclases; G_q proteins couple to the activation of phospholipase Cβ; and G_{12} proteins couple to the activation of Rho GUANINE-NUCLEOTIDE EXCHANGE FACTORS (RhoGEFs). Additionally, 5 β and 14 γ proteins have been cloned. The α subunit contains the guanine nucleotide binding site and is responsible for GTP-GDP binding cycle and GTP hydrolysis promoted by GPCR-mediated activation, whereas the β and γ subunits are tightly associated into a stable βγ complex, which is also regarded as a separate functional unit (Clapham and Neer 1997; Preininger and Hamm 2004).

In the inactive state, the GDP-bound α subunits is attached to the βγ dimer to form a heterotrimeric G protein complex. Agonist occupancy of GPCRs induces a conformational change that promotes an interaction between the activated receptor and heterotrimeric G proteins. In this scheme, receptors thus serve as guanine nucleotide exchange factors (GEF) i.e. promoting GDP release from and GTP binding to α subunits. Subsequently, the activated α subunit dissociates from the βγ dimer. The GTP-bound α subunits and free βγ dimers have an independent capacity to bind to and modulate separate intracellular or plasma membrane effectors, including stimulation or inhibition of membrane-associated adenylyl

Figure 2 – Schematic diagram representing key steps in G protein-coupled receptors signaling.

GPCRs are involved in the recognition and transductions of large diverse extracellular messages that activate these receptors. GPCRs control the activity of enzymes and ion channels via the catalysis of the GDP-GTP exchange on heterotrimeric G proteins ($G\alpha\beta\gamma$).


Light
Ca++
Odorants
Pheromones
Small molecules
•amino-acids, amines
•nucleotides
•Peptides …
Proteins
•TSH
•LH
•FSH
•interleukins …
Out
In
β
GDP
G protein
Intracellular messengers: cAMP, Ca2+
PKA, PKC
Cellular response

cyclase, activation of phopholipases and regulation of potassium and calcium ion channel activity. The hydrolysis of GTP to GDP promotes the re-association of GDP-bound α-subunits and βγ dimers into inactive G proteins and hence leads to the termination of G protein activation and signaling cycle. GTP hydrolysis is known to be regulated by the intrinsic GTPase activity of α subunits, a process that is catalyzed by the actions of a specific GTPase-activating protein family called the Regulators of G protein Signaling (RGS) or by G protein-regulated effectors themselves (Berman and Gilman 1998; Neubig and Siderovski 2002; Pierce et al. 2002). The diversity of the proteins involved in this transduction system can potentially account for the known specificity of G protein-mediated signaling in different cells.

1.3 Principal modes of GPCRs regulation

GPCRs are subject to three main modes of regulation: desensitization, internalization (sequestration) and down regulation. Desensitization is a biological process that makes a receptor refractory to continued agonist stimuli. Internalization is regulated by the agonist-mediated translocation of receptors from cell surface to an intracellular cytosolic compartment via clathrin-coated vesicles. The down regulation refers to a decrease of the receptor levels in cells or tissues induced over a period of hours to days after prolonged or repeated exposure to agonists (Penn and Benovic 1998; Ferguson 2001; Tsao et al. 2001).

Receptor desensitization, a rapid wane in a responsiveness of a signaling system in the presence of continued agonist or stimulus exposure is an important physiological feedback mechanism that protects against both acute and chronic receptor activation (Ferguson 2001). There is substantial evidence that the receptor desensitization is controlled by phosphorylation of serine and threonine residues located in the C-terminal domain and the third intracellular loop of GPCRs (Willets et al. 2003). Although, desensitization of G protein-regulated signaling systems can be mediated by a reduction of receptor coupling to G proteins or by changes in G protein or effector activity, the inhibition of the GPCR-mediated G protein activation especially within seconds to minutes of agonist stimulation seems to account for most of the desensitization (Pitcher et al. 1998; Pao and Benovic 2002).

The phenomenon of desensitization can be subdivided into two types: the heterologous (agonist nonspecific) and homologous (agonist specific) types (Hausdorff et al. 1990; Krupnick and Benovic 1998). Two main families of protein kinases participate in the phosphorylation of GPCRs: the second messenger-dependent kinases [the cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC)] and the G-protein-coupled receptor kinases (GRKs). PKA and PKC can phosphorylate either agonist-bound/activated GPCRs (homologous desensitization) or agonist-free/inactive GPCRs (heterologous desensitization). GRKs phosphorylate only agonist-occupied or activated GPCRs to initiate homologous receptor desensitization (Daaka et al. 1997; Krupnick and Benovic

1998; Pitcher et al. 1998; Lawler et al. 2001). Following GRK-mediated phosphorylation, phosphorylated receptors bind to cytosolic proteins called arrestins. Arrestins act in concert with GRKs to promote the full inactivation of phosphorylated receptors and thus to arrest intracellular signaling. Indeed, during the continued agonist-mediated activation of the receptor, arrestins have the ability to discriminate GRK-phosphorylated GPCRs by blocking the receptor G protein interaction and thus preventing the receptor-mediated GDP-GTP exchange on G protein α subunits (Hall et al. 1999; Luttrell and Lefkowitz 2002).

There are seven known mammalian GRK isoforms, which are classified in three subfamilies (GRK1/7, GRK2/3 and GRK4/5/6) on the basis of their sequence and functional similarity. GRK1 (formerly known as rhodopsin kinase) and GRK7 are expressed almost exclusively in retinal rods and cones, respectively. GRK2 (formerly known as β-adrenergic kinase 1 or βARK1), GRK3 (formerly known as β-adrenergic kinase 2 or βARK2), GRK5 and GRK6 are widely distributed and account for the regulation of most of the GPCRs found through the body, whereas GRK4 is restricted to the spermatogonia cell lineage (Pierce and Lefkowitz 2001; Lefkowitz 2004). Four members of the vertebrate arrestins have been identified. Two visual arrestins (rod arrestin or arrestin 1 and cone arrestin) have been identified and shown to play an important role in the modulation of phototransduction. β-arrestin 1 (arrestin 2) and β-arrestin 2 (arrestin 3) are expressed ubiquitously and account for the regulation of the vast majority of GPCRs (Carman and Benovic 1998; Miller and Lefkowitz 2001).

It is now well established that the GRK-arrestin system, in addition to their role in the receptor desensitization, has also the ability to promote internalization, which is another important aspect of the regulation of GPCR activity (Sterne-Marr and Benovic 1995; Bohm et al. 1997; Ferguson and Caron 1998). The concept that GPCRs are removed away from the cell surface following agonist activation by endocytosis via an intracellular compartment (endosomes) enriched with a GPCR-specific phosphatase activity, originated from the observation that β-adrenergic stimulation resulted in a loss of β-adrenergic receptor recognition sites on the surface of frog erythrocytes (Chuang and Costa 1979). Internalization, even though initially, considered solely as part of the process of desensitization, has subsequently been shown to promote receptor resensitization and recycling of resensitized receptors back to the cell surface in order to protect cells against prolonged receptor desensitization. Therefore, GRKs and arrestins are important components of GPCR signaling associated with GPCR desensitization and resensitization following agonist stimulation (Krupnick and Benovic 1998; Ferguson 2001).

Furthermore, GRKs and arrestins play a direct role in the positive regulation of GPCR signaling (Hall et al. 1999; Luttrell and Lefkowitz 2002). In recent years, a large volume of data revealed that GRKs and arrestins, by virtue of binding to additional cellular proteins, provide signal transduction scaffolds that bring specific molecules into proximity of the activated receptor and the cell membrane to generate new signaling pathways (Pierce et al. 2002; Gainetdinov et

al. 2004). In fact, recent studies have demonstrated that arrestins serve as agonist-dependent adaptors that ferry a wide variety of signaling proteins to activated receptors (Miller and Lefkowitz 2001; Lefkowitz 2004).

Most of the information about GRKs and arrestins has been obtained from in vitro studies and genetically altered mice. These studies have provided a unique insight into the potential physiological roles of these proteins. In this regard, these various experimental approaches have underscore a crucial role of GRKs and arrestins in regulating the cardiac and brain function. Additionally, intriguing findings obtained from transgenic and knockout mice suggest also that GRKs and arrestins may have a critical role in the regulation of growth and development (Carman and Benovic 1998; Pitcher et al. 1998; Gainetdinov et al. 2004).

1.4 Ligand-induced activation of GPCRs

Activation of GPCRs by diverse ligands is initiated by the binding of an agonist to the receptor. The model postulates that agonist binding triggers a cascade of structural changes in the receptor molecule, which leads to the activation of the associated G proteins and subsequent stimulation of a wide range of intracellular signal transduction pathways.

Mutagenesis and biophysical studies have revealed that conformational changes in the transmembrane regions, the receptor core domain, are probably responsible for GPCR activation and formation of the ligand binding pocket

(Scheer and Cotecchia 1997; Bockaert and Pin 1999; Gether et al. 2002). The majority of biophysical studies exposing the molecular mechanisms for ligand-mediated receptor activation were initially carried out with rhodopsin (the visual receptor). The relatively easy purification procedure of rhodopsin from retina (a plentiful source of rhodopsin) and the inherent stability of rhodopsin have allowed obtaining high quantities of receptors for crystallography studies. In fact, rhodopsin is the only GPCR for which a high resolution structure is available (Palczewski et al. 2000; Gether et al. 2002; Karnik et al. 2003). Importantly, the high resolution structure of rhodopsin has confirmed the presence of the seven TM regions and unraveled new structural features such as the presence of a α-helical region (H8) in the proximal region of the C-terminal domain located near the TM7. The crystal structure suggests also a potential role for the extracellular domains in the regulation of the covalent link between the 11-*cis*-retinal chromophore and rhodopsin (Palczewski et al. 2000).

Numerous biochemical studies with rhodopsin, together with an array of different indirect approaches, have demonstrated that the switch from the inactive to active conformation is associated with the rotation and tilting of TM6 relative to TM3 (Farrens et al. 1996; Javitch et al. 1997). The TM3 and TM6 movements are thought to affect the overall conformation of the intracellular loops that constitute the key sites for G protein recognition and activation (Spengler et al. 1993; Pin and Bockaert 1995; Preininger and Hamm 2004). Functional studies suggest that G protein recognition and activation depends upon a change in the relative

orientation of TM3 and TM6 with a significant rigid-body movement of TM6 in a counter-clockwise direction and a separation from TM3 by a movement of the cytoplasmic end of TM6 away from TM3 (Farrens et al. 1996). Furthermore, the existence of movements of TM3/TM6 along with a rigid-body motion has been detected for the β_2-adrenergic receptors (β_2AR) in a similar fashion to that observed upon light activation of the 11-*cis*-retinal chromophore, the ligand covalently linked to rhodopsin. Interestingly, structural studies with the muscarinic M3 receptor provide evidence for the movement of the cytoplasmic ends of TM5 and TM6 toward each other after agonist-induced activation (Bockaert and Pin 1999).

While interactions between aminergic GPCRs and their ligands seem to share similar TM orientations and positions in the binding-site crevice, it is difficult to put forward a general mechanism that could explain all the ligand interactions with every receptor. Many aminergic ligands can form critical interactions with TM5. Moreover, it is likely that for some GPCRs, EL2 and/or EL3 will participate in ligand binding. In fact, studies have shown that some GPCRs can be activated by interaction of the N-terminal extracellular domain with the extracellular loops as well (Rana et al. 2001; Shi and Javitch 2002).

1.5 Structural basis of receptor-G protein coupling selectivity

Over the past few years, the G protein coupling profiles of most GPCRs have been studied in more detail using different experimental approaches. Most GPCRs, after being activated by the appropriate ligands, can recognize and activate only a limited set of the many structurally similar G proteins expressed in a cell (Savarese and Fraser 1992; Hedin et al. 1993; Milligan 1995). However, it has become clear that GPCRs can also couple to multiple classes of G proteins with different efficiencies (Raymond 1995). Besides the structural information encoded by the receptor and G protein primary sequences, other factors may also contribute to the observed degree of coupling selectivity, including: receptor and/or G protein density, and restricted localization of heterotrimeric G proteins and receptors in specialized domains of plasma membrane (Neubig 1994; Gudermann et al. 1996; Gudermann et al. 1997).

Studies aimed at identifying domains which determine the selectivity and efficiency of receptor-G protein interactions indicate that the amino acid sequences of the intracellular loops and the proximal part of the C-terminal domain appear to contribute and mediate receptor-G protein coupling (Scheer and Cotecchia 1997). Several lines of evidence indicate that residues within the second intracellular loop (IL2) represent one of the key regions in determining the selectivity of receptor-G protein interactions and the efficiency of G protein activation. Meanwhile, the third intracellular loop (IL3) plays a fundamental role

for the proper G protein recognition. In fact, IL3 acts in a co-operative fashion with other receptor domains, primarily the IL2 loop, to allow for the optimum G protein coupling selectivity (Blin et al. 1995; Nasman et al. 1997).

1.6 Constitutive activity of GPCRs

Most GPCRs display constitutive activity, which is the ability of a receptor to mediate intracellular signaling in the absence of agonist activation. This is a natural phenomenon by which receptors undergo a spontaneous allosteric transition leading to the formation of an active state that can bind to and activate G proteins in the absence of agonists (Seifert and Wenzel-Seifert 2002).

The first indication for the existence of GPCR constitutive activity occurring naturally in a cellular milieu was obtained in NG108-15 neuroblastoma-glioma cells expressing endogenously delta opioid receptors (Costa and Herz 1989). In this study, cells incubated with some opioid antagonists display a reduction in the basal GTPase activity of Gi proteins (Costa and Hertz, 1989). However, the notion that GPCRs exhibit agonist-independent activity or constitutive activity was fully appreciated with studies using heterologous cells expressing mutated forms of the α- and β-adrenergic receptors (Cotecchia et al. 1990; Kjelsberg et al. 1992; Samama et al. 1993; Scheer and Cotecchia 1997; Lefkowitz 2000). It is now widely accepted that GPCRs for biogenic amines, nucleosides, lipids, amino acids, peptides and hormones can display various

degrees of constitutive activation in their wild type and/or mutated forms (Seifert and Wenzel-Seifert 2002).

The molecular mechanisms regulating the allosteric transition between the inactive and active states of GPCRs in the absence of agonists remain ill defined. Studies suggest that changes in the core 7TM regions of GPCRs underlie constitutive activity (Chalmers and Behan 2002). Experiments using rhodopsin, the only GPCR for which a crystal structure has been resolved under condition of "*ligand attachment*", indicate that the transition from inactive to active states of GPCRs may involve changes in the relative orientation of TM3 and TM6 with a concomitant rotation of TM6. A movement and tilting of TM6 produce a change in the positioning of the third intracellular loop (IL3), which exposes residues involved in G protein coupling. Mutagenesis studies indicate that amino acid substitutions in various GPCR regions (TM, extracellular and intracellular domains) can induce conformational changes resulting ultimately in a receptor activation in the absence of agonists (Palczewski et al. 2000).

Experimental findings suggest that mutations that trigger ligand-independent signaling can influence ligand-receptor and receptor-G protein interactions (Lefkowitz et al. 1993). In fact, studies using constitutively active mutant (CAM) receptors have led to the notion that these receptors have general pharmacological properties. The hallmark features of CAM receptors are 1) increased ability to generate an intracellular signal in an agonist-independent fashion, 2) increased affinity for agonists, 3) increased potency of agonists for

activating intracellular effectors, and 4) enhanced efficacy of partial agonists, when respective values at mutant versus corresponding wild-type GPCRs are compared, and 5) decreased affinity for antagonists/inverse agonists (Lefkowitz et al. 1993; Tiberi and Caron 1994; Scheer and Cotecchia 1997).

The physiological relevance of GPCR constitutive activity in normal conditions has been demonstrated using ligands that not only block agonist binding to GPCRs but are also capable to reduce the spontaneous activity of GPCRs in the absence of agonists. Ligands that promote and stabilize the inactive state of GPCRs under basal conditions are termed inverse agonists (Chidiac et al. 1994; Kenakin 2001).

The physiological relevance for GPCR constitutive activity was first demonstrated with native histaminergic H3 autoreceptors expressed in rodent brain (Morisset et al. 2000). The use of specific H3 receptor inverse agonists was shown to control histaminergic neuron activity (Morisset et al. 2000), which promotes arousal, attention and improving learning (Ligneau et al. 1998). Prior to this study, a body of evidence suggested that alterations in the constitutive activity of GPCRs may be associated with the etiology of phenotypic expression of several diseases (Arvanitakis et al. 1998a; Seifert and Wenzel-Seifert 2002; Kenakin 2004). Indeed, several human diseases are caused by natural activating mutations in GPCRs such as retinitis pigmentosa (Robinson et al. 1992) and familial hypoparathyroidism (Chattopadhyay et al. 1996). However, the precise mechanism underlying CAM-mediated diseases remains to be clearly established.

Interestingly, it has been clearly shown that mutations occurring in various domains of GPCRs can lead to agonist-independent activity and they can be linked to severe debilitating diseases (Arvanitakis et al. 1998b). For instance, a constitutively activating mutation in the second transmembrane domain of rhodopsin causes congenital night blindness (Rao et al. 1994), whereas a mutation in the TM7 is the probable cause of retinal cell degeneration in one form of retinitis pigmentosa (Robinson et al. 1992). Moreover, several activating mutations leading to hyperthyroidism have been found in various regions of the thyroid-stimulating hormone (TSH) receptor (Van Sande et al. 1995).

Other pathologies are also associated with virus expressing constitutively active forms of GPCRs. For instance, infection with Kaposi's sarcoma-associated herpes virus leads to expression of a constitutively active chemokine receptor (a GPCR), which results in cell proliferation and continued viral replication (Lyons et al. 1990; Weinstein et al. 1990; Spiegel et al. 1993). GPCR constitutive activity may also be involved in cancer as indicated by studies showing a link between GPCR overexpression, cell transformation and tumor growth. Indeed, the extreme level of receptors expressed in some tumors (e.g. 100,000-fold in pancreatic epithelial carcinoma compared to natural levels) may produce constitutive cellular responses that lead to and promote tumor growth (Virgolini et al. 1994).

1.7 Chemical targets and inverse agonists for GPCRs

In the past decade, research has generated an enormous amount of new information about binding and activation of GPCRs by their cognate ligands. As a result, changes in the prevailing classification of ligands acting at GPCRs have led to a rethinking of rational design of drugs targeting wild type and CAM receptors. Until 1989, chemical targets for GPCRs were simply classified as agonists, partial agonist and antagonists (Stephenson 1956; Prather 2004). It was demonstrated that agonists have both affinity and intrinsic activity, whereas antagonists show affinity without intrinsic activity. Moreover, partial agonists produce submaximal receptor activation leading to a submaximal response in cells or tissues (Kenakin 2001). Although this simple classification scheme could be sufficient to describe the regulation of GPCRs by most ligands, the exhibition of spontaneous receptor activity by these receptors has led to the discovery of drugs that display inverse agonism. In fact, several ligands that were originally described as antagonists, were re-evaluated in systems where constitutive activity of GPCRs could be measured. Most of these drugs were shown to inhibit the constitutive activity of GPCRs and thus were re-classified as ligands with negative intrinsic activity or inverse agonists (de Ligt et al. 2000).

The demonstration of ligands with negative efficacy was first demonstrated by Costa and Hertz (1989) in cells expressed naturally the delta opioid receptor and the opioid antagonist ICI 174864. Inverse agonistic effects of ligands

previously classified as antagonists (neutral ligands) have now been reported in various GPCR systems such as the adrenergic and dopaminergic receptors (Samama et al. 1993; Tiberi and Caron 1994; de Ligt et al. 2000; Kenakin 2004). Importantly, inverse agonism is a pharmacological behavior that can only be observed in systems in which GPCR constitutive activity can be detected. Therefore, ligands tested in such systems that do not reduce or increase GPCR activity are considered to be competitive antagonists or neutral ligand i.e drugs that do not possess negative efficacy (Kenakin 2004; Prather 2004).

The demonstration of the physiological relevance of inverse agonism (Bond et al. 1995; Morisset et al. 2000) suggests that this class of pharmacological agents will likely be useful in clinic. In fact, a limited survey indicates the predominance of inverse agonists versus natural antagonists, which are the minority class of drugs (around 15 %) used in the pharmacological space (Kenakin 2004). However, the therapeutic efficacy of inverse agonists for treating conditions remains unclear. For instance, the acute physiological effects mediated inverse agonists on organ or tissue function affected by elevated GPCR constitutive activity in conditions such as retinitis pigmentosa, hyperthyroidism, cancer growth or autoimmune diseases have yet to be tested in clinical trials. Additionally, the long-term use of inverse agonists to treat CAM-mediated pathological conditions raise an issue about whether adaptation mechanisms will take place in cells leading to undesirable side-effects or drug tolerance (de Ligt et al. 2000; Kenakin 2004).

2. The dopaminergic system

2.1 The classical view

Dopamine (3,4-dihydroxyphenylethylamine; DA) is a chemical messenger which can be found in a variety of organisms from simple invertebrates to humans. DA is a constituent of monoaminergic (dopaminergic, norepinephrinergic, epinephrinergic, serotoninergic) cell groups localized in the lower brainstem; a region supplying various regions of the nervous system with monoamines (Halasz 1985). Until the mid-1950s, the only biological role for DA was to serve as a precursor for the norepinephrine biosynthesis. Subsequently, Montagu, Carlsson and coworkers proposed additional functions for DA based on their findings showing that DA and norepinephrine were found at similar concentrations in the brain and as well as displaying a distinct distribution in the CNS and peripheral tissues (Cooper et al. 2003).

DA is involved in the control of a variety of physiological functions. In the CNS, DA participates in the control of locomotion, cognition, emotion and neuroendocrine secretion. In the periphery, DA is found most prominently in the kidney and vasculature, where it modulates cardiovascular function, catecholamine release, hormone secretion, vascular tone and renal function (Missale et al. 1998; Emilien et al. 1999).

During the past three decades, the dopaminergic system was of particular interest because studies have demonstrated that dysregulation of dopaminergic

transmission underlies several pathological conditions such as Parkinson's disease (PD), schizophrenia, hyperprolactemia/pituitary adenomas and hypertension. Furthermore, reward and addiction effects elicited by drugs of abuse such as cocaine and amphetamine are mediated by the dopaminergic system (Jarvie and Caron 1993; Missale et al. 1998; Wong et al. 2000).

Dopaminergic neurons are localized in most parts of the CNS but in particular in the three major neural systems of the midbrain (Bjorklund and Lindvall 1964). The first neural system, the nigrostriatal pathway originates from dopamine-synthesizing neurons in the substantia nigra and degeneration of this pathway in brains of patients afflicted with PD involvement leads to DA extrapyramidal dysfunction (Poirier and Sourkes 1965). The second neural system, the mesolimbic/mesocortical pathway, arises in the midbrain ventral tegmental area and innervates part of the frontal cortex, which regulates some aspects of cognitive function and motivated behavior, including activity related to reward (Willner and Scheel-Kruger 1991). The mesocorticolimbic pathway plays also a role in learning and memory as well as in the pathogenesis of psychosis (Creese et al. 1976; Carlsson 1988). The third neural system, the tubular-infundibular pathway originates in the hypothalamus and plays an important role in the control of neuroendocrine regulation (Emilien et al. 1999).

2.2 Classification of dopamine receptors

The existence of central DA receptors was for the first time proposed in 1972 when biochemical studies showed that DA was able to stimulate adenylyl cyclase (AC) activity (Brown and Makman 1972; Kebabian et al. 1972). Based on anatomical, electrophysiological and pharmacological evidence, studies suggested that there might be more than one type of receptor for DA in the brain (Cools and Van Rossum 1976). Additional studies investigating the mechanisms of DA actions demonstrated that DA effects were mediated through an interaction with at least two biochemically distinct DA receptors termed D1 (linked to AC stimulation) and D2 (not linked to AC stimulation) (Kebabian and Calne 1979). After a decade, with the introduction of gene cloning, five DA receptor subtypes have been identified and characterized: D1R (Dearry et al. 1990; Sunahara et al. 1990; Zhou et al. 1990), D2R (Sibley et al. 1982; Giros et al. 1989), D3R (Sokoloff et al. 1990), D4R (Van Tol et al. 1991) and D5R (Sunahara et al. 1991; Tiberi et al. 1991).

Notwithstanding the existence of five human genes coding for DA receptors, the D1/D2 classification concept originally developed in the late 1970s remains still valid today. Nowadays, the DA receptors are grouped into two subfamilies, whose features resemble the biochemically and pharmacologically distinct D1 and D2 receptors originally described. Specifically, the DA receptors are grouped into the D1-like (D1AR and D1BR in rats; D1R and D5R in humans)

and D2-like subfamilies ($D2_{short}R$, $D2_{long}R$, D3R and D4R). This receptor classification is based on the degree of identity within their primary amino acid sequences, ligand binding properties and ability to stimulate or inhibit AC activity (Jaber et al. 1996; Emilien et al. 1999; Neve et al. 2004) (Table 2).

2.3 Common properties of different dopamine receptor subtypes

2.3.1 Structural homology

The notion that the DA receptors mediate their effects through the interaction with members of the family of heterotrimeric guanine-nucleotide binding proteins (G-proteins), suggested that these receptors belong to the large family of G protein-coupled receptors or GPCRs (Dohlman et al. 1987). Consequently, all DA receptors are characterized by the presence of seven membrane-spanning alpha helices forming the hydrophobic core, interspersed by three intra- and extracellular connecting loops which vary in length among the different receptors. The amino terminus of DA receptors has a similar number of amino acids and contains one putative site for N-linked glycosylation. There are additional N-linked glycosylation sites on different extracellular regions of DA receptor subtypes notably the second extracellular loop (Civelli et al. 1993; Gingrich and Caron 1993; Jackson and Westlind-Danielsson 1994). The carboxy terminus, which is significantly longer in D1-like receptors (about seven times), is

Table 2 – Pharmacological and biochemical characteristics of the dopamine receptor subtypes.

Receptor / Property	D1-Like		D2-Like		
	D1	**D5**	**$D2_{(short)/(long)}$**	**D3**	**D4**
Amino acids	446(h,r)	447(h) 475(r)	414/443(h) 415/444(r)	400(h) 446(r)	387(h,r)
Agonist (high affinity)	Fenoldopam SKF38393 SKF82526	Fenoldopam SKF38393 Dopamine	Bromocriptine Dopamine Apomorphine	Quinpirole 7-OH-DPAT Apomorphine	Apomorphine Quinpirole Dopamine
Antagonists (high affinity)	SCH23390 (+)-Butaclamol *cis*-Flupentixol	SCH23390 (+)-Butaclamol *cis*-Flupentixol	Spiperone Raclopride Sulpiride	Spiperone Raclopride Sulpiride	Spiperone Clozapine Sulpride
Localization (Brain)	Striatum, nucleus accumbans, olfactory tubercles, hypothalamus, frontal cortex	Striatum, hippocampus, thalamus, lateral mamillary nucleus, cerebral cortex	Striatum, cerebral cortex, nucleus accumbans, olfactory tubercles	Nucleus accumbans, olfactory tubercles, islands of Calleja	Frontal cortex, midbrain, amygdala, hypothalamus, hippocampus
Localization (Periphery)	Cardiovascular system, kidney, parathyroid gland, ovaries	Kidney, blood lymphocytes	Pituitary gland, adrenal gland, kidney	Kidney, blood lymphocytes	Heart, kidney, retina
Biochemical response	AC ↑	AC ↑	AC ↓	AC ↓	AC ↓

predicted to be located into the intracellular space and anchored to the plasma membrane through palmitoylation (Ovchinnikov Yu et al. 1988; O'Dowd et al. 1989)

The mechanisms regulating DA binding to the dopaminergic receptors are similar to those described for agonist binding to adrenergic receptors (Dohlman et al. 1991). Notably, transmembrane segments carry the critical amino acid residues that are present in the core of the protein and define a narrow binding pocket for the DA (agonist) binding site (Hibert et al. 1993). In particular, the third transmembrane domain (TM3) through a conserved asparatic acid residue is involved in the binding to the amine group of the catecholamine side chain (Strader et al. 1989; Hibert et al. 1993). Additionally, the fifth transmembrane domain (TM5) through two conserved serine residues binds to the hydrohyl groups of catechol ring of catecholamines. Studies show that mutations of these key residues strongly reduce or completely abolish the binding of both DA agonists and antagonists (Cox et al. 1992; Mansour et al. 1992; Tomic et al. 1993; Civelli and Zhou 2001). Moreover, it has been shown that a phenylalanine residue in TM6 and an aspartic acid residue in TM2 play a crucial role in D1R and D2R activation and mutations of these residues affect agonist binding (Neve et al. 1991; Tomic et al. 1993).

A comparison of the primary amino acid sequence between the D1 and the D2 receptor subtypes shows a 29% homology throughout the entire protein while a higher degree of sequence homology (44%) can be observed within their seven

transmembrane domains (Jarvie and Caron 1993; Jaber et al. 1996). The D1-like receptor subfamily share a 60% overall identity and a high degree of amino acid similarity (~82%) in their TM domains whereas the D2-like receptor subfamily display lower degree of amino acid identity, both overall (60% between D2R and D3R; 40% for D4R when compared with D2R and D3R) and in TM regions (79% for D2R and D3R; 55% for D3R and D4R; 54% for D2R and D4R) (Jarvie and Caron 1993).

2.3.2 Receptor / G protein interactions

Site-directed mutagenesis studies for catecholamine receptors have identified the residues that play a role in receptor activation (Strader et al. 1989; Kjelsberg et al. 1992). Studies investigating the structural properties of catecholamine receptors suggest that critical residues regulating receptor coupling to and activation of G proteins resided primarily in the third intracellular loop (IL3). D1-like receptors are characterized by a short IL3 that couples to G_s proteins, whereas the D2-like receptors have a longer IL3 relative to D1R/D5R, which is a common feature of GPCRs inhibiting AC activity through an interaction of IL3 with G_i proteins (Civelli et al. 1993; Gingrich and Caron 1993; O'Dowd 1993). Furthermore, variant forms of D2R and D3R (short and long isoforms) exist based on the amino acid insertion in the third intracellular loop mediated by a gene splicing process (Giros et al. 1989; Fishburn et al. 1993).

Studies suggests that these D2-like receptor variants may exhibit different abilities to activate G proteins (Castro and Strange 1993; Guiramand et al. 1995; Cravchik et al. 1996) and display different pharmacological properties (Castro and Strange 1993; Malmberg et al. 1993).

2.4 Individual properties of different dopamine receptor subtypes

The DA receptor subtypes show different properties in terms of their location; pharmacological profiles and mechanisms of action, which are summarized in the following sections (See also Table 2).

2.4.1 Distribution and localization of dopamine receptors

The study of the distribution of DA receptors has helped clarify the distinct roles of the individual subtypes in regulation of the multiple DA-associated functions in the human body. The cloning and molecular characterization of DA receptors showed that the receptor distribution pattern in the CNS and peripheral tissues are essentially similar among different species (Missale et al. 1998).

2.4.1.1 Central dopamine receptors

The most widespread DA receptor in brain is D1R. D1R is expressed at a higher levels than any other dopaminergic receptors (Dearry et al. 1990; Fremeau

et al. 1991; Weiner et al. 1991). D1R is abundant in areas known to be under dopaminergic control such as striatum, nucleus accumbens and olfactory tubercles. D1R is also found in the limbic system, hypothalamus and thalamus (Mengod et al. 1989).

The D5R displays a more restricted expression pattern when compared with D1R. In fact, the D5R distribution is mostly limited to the hippocampus and to two sets of nuclei: the lateral mamillary nucleus and the parafascicular nucleus of the thalamus, where the D1R is not significantly expressed (Tiberi et al. 1991; Meador-Woodruff et al. 1992). Interestingly and of potential significance for neuroimmunology, the D5R expression has been reported in lymphocytes (Takahashi et al. 1992; Ricci et al. 1999).

The D2R is detected mainly in the striatum, olfactory tubercles and core of the nucleus accumbens (Bouthenet et al. 1991; Jackson and Westlind-Danielsson 1994), where it is expressed by dopaminoceptive GABAergic neurons co-expressing enkephalins (Le Moine et al. 1990; Le Moine and Bloch 1995). In addition, D2R is found in the substantia nigra pars compacta and ventral tegmental area, where it is expressed by dopaminergic neurons (Meador-Woodruff et al. 1989; Weiner et al. 1991).

The distribution of D3R is associated with the limbic area such as the shell of the nucleus accumbens, olfactory tubercles and islands of Calleja whereas its expression in the striatum is significantly lower than D2R. These findings have underscored the functional importance of the D3 subtype in regulating the brain

limbic function and suggested that D3R may represent a novel molecular target for the treatment of schizophrenia (Sokoloff et al. 1990; Bouthenet et al. 1991).

The D4R is expressed at a significantly lower density (10 to 100-fold) than D1R and D2R in the striatum (Rappaport et al. 1993; Patel et al. 1996; Schlachter et al. 1997). However, D4R is highly expressed in the frontal cortex, hypothalamus, and mesencephalon (Van Tol et al. 1991; O'Malley et al. 1992).

2.4.1.2 Peripheral dopamine receptors

DA modulates cardiovascular functions such as cardiac contractility, heart rate and arterial blood pressure through the activation of both D1-like and D2-like receptor subtypes located at various sites within the cardiac, vascular and renal regions (Goldberg et al. 1978; Lokhandwala and Amenta 1991). Functional and pharmacological evidence, confirmed by radioligand binding studies, suggest the presence of D1-like (postjunctional) and D2-like (prejunctional) receptor expression in the walls of the systemic arteries (Amenta et al. 1993). In systemic arteries, D1-like receptors have been identified within the smooth muscle of the tunica media (Amenta and Ricci 1990; Missale et al. 1998), where the D5R is the predominant subtype (Amenta et al. 1995). The vascular D2-like receptors are localized in the adventitial and intimal layers of systemic and pulmonary arteries (Amenta et al. 1990). Molecular biology studies have identified the presence of D4R in the heart and arterial tissue (O'Malley et al. 1992). Findings have also

demonstrated the presence of small amount of D1R expression in the rat heart (Ozono et al. 1996).

Of the cloned DA receptor subtypes, D1R (D1AR), D5R (D1BR), D2R and D3R have been found in kidneys (Felder et al. 1989; Sibley and Monsma 1992; Nash et al. 1993; Yamaguchi et al. 1993). D1-like receptors exhibit a vascular and tubular localization while D2-like receptors localize in the zona glomerulosa of renal cortex and tubules (Felder et al. 1989; Sokoloff et al. 1990; Lokhandwala and Amenta 1991). The presence of D1R in renin-containing vesicles within the kidney juxtaglomerular apparatus suggest that this DA receptor subtype potentially regulates renin secretion, which may explain the effect of DA on blood pressure, cardiac output and regional blood flow (O'Connell et al. 1995).

Autoradiographic analysis of [^{3}H]-spiperone binding (D2-like ligand) revealed the existence of D2-like subtypes in adrenal cortex with the majority of receptors concentrated in the zona glomerulosa and to a lesser extent in the zona reticularis (Amenta et al. 1994). This study supported the notion that aldosterone production in adrenal gland is controlled by a maximal tonic dopaminergic inhibition mediated through D2R (Carey et al. 1979; Sowers et al. 1981; Malchoff et al. 1986; Lombardi et al. 1988). Moreover, the D2R is expressed in the pituitary where it plays a major role in mediating the tonic inhibitory control of hypothalamic prolactin (PRL) secretion (Creese et al. 1976; Caron et al. 1978; Munemura et al. 1980; Enjalbert and Bockaert 1983; Ben-Jonathan 1985).

Studies have reported that DA can be detected in human ovaries (Bahr and Ben-Jonathan 1985; Fernandez-Pardal et al. 1986), in particular, in follicular fluid (Bodis et al. 1993). However, the physiological relevance of DA in the ovary requires further studies. Interestingly, the detection of specific binding sites for D1-like ligands and stimulatory effect of DA on the steroidogenesis in rat ovarian cells suggest the existence of functional D1-like receptors in ovaries (Mori et al. 1994). Recent studies suggested that the D1-like system likely participates in the regulation of the complex events associated with follicular development, possibly ovulation, and/or the regulation the corpus luteum (Mayerhofer et al. 1999).

2.4.2 Pharmacological properties of dopamine receptors

Overall, the D1-like and D2-like receptors exhibit pharmacological properties similar to those that were originally described for the two distinct pharmacological and biochemical D1R and D2R entities (Kebabian and Calne 1979). Even though highly selective agonists and antagonists can discriminate between the D1 and D2 receptor subfamilies, there are relatively few drugs that are selective for individual members within each subfamily. Currently, for the D1 subfamily, ligands can not be more than approximately 10-fold selective for either the D1 or D5 subtype (e.g. dopamine and (+)-butaclamol) (Sunahara et al. 1991; Tiberi et al. 1991; Seeman and Van Tol 1994). Recently, some progress has been made with the D2 subfamily, where some antagonists are at least 1000-fold

selective for the D4R versus both the D2R and D3R (Merchant et al. 1996; Tallman 1998).

The D1-like receptor subfamily shows a high affinity for the benzazepine ligands SCH23390 and SKF83566 that are defined as selective D1-like antagonists (Cross et al. 1983; Iorio et al. 1983). SKF38393, which was the initial tool to study D1-like receptor subtypes, is a selective D1-like agonist (Pendleton et al. 1978; Setler et al. 1978). There are significant differences in the affinities of D1-like compounds between the D1R and D5R. In brief, D5R exhibits higher agonist and lower antagonist affinities when compared with D1R, but in spite of these differences, no truly selective compounds are yet available to distinguish these two receptors (Sunahara et al. 1991; Tiberi and Caron 1994).

The D2-like receptor subfamily has a pharmacological profile, which closely resembles that of the original D2 receptors described prior to the cloning of DA receptors. In fact, they all show high affinities for neuroleptic drugs such as the haloperidol, spiperone and clozapine. These drugs provide selective antagonists for the D2-like receptors in a way that haloperidol and spiperone show a 10- to 20-fold higher affinity at the D2R (compared with D3R and D4R), whereas clozapine displays a significantly higher affinity for the D4R in comparison with D2R and D3R (Sokoloff et al. 1990; Van Tol et al. 1991) (Table 2).

3. Insight into the physiological relevance of D1-like receptors

Recent studies in the field of cellular and molecular neuroscience have focused on the important functional roles of the D1-like receptors mainly because of their potential impact on the design of therapeutics for the treatment of several pathological conditions associated with impairment in D1-like receptor functions.

3.1 D1-like receptor functions

3.1.1 Neurophysiological functions

D1-like receptors modulate the working memory function of the prefrontal cortex (PFC) (Funahashi et al. 1993; Goldman-Rakic 1996; Robbins 1996); a brain region controlling behaviors and thoughts, which is affected in many neuropsychiatric disorders including conditions such as stress-induced impairment of working memory (Murphy et al. 1996), age-related cognitive decline (Goldman-Rakic and Brown 1981; Wenk et al. 1989) and schizophrenia (Goldman-Rakic 1991; Rajkowska et al. 2001). The role of the D1-like receptors in controlling the performance of working memory tasks and cognitive behavior has been supported by localization studies reporting the D1R and D5R expression in PFC and pharmacological data obtained with D1-like dopaminergic ligands (Sawaguchi and Goldman-Rakic 1991; Levey et al. 1993; Murphy et al. 1996; Zahrt et al. 1997; Goldman-Rakic et al. 2000).

Studies also suggest that D1-like receptors in hippocampus play an important role in the modulation of long-term potentiation (LTD), a neural process implicated in the initial memory consolidation (Huang and Kandel 1995; Matthies et al. 1997; Otmakhova and Lisman 1998). Studies using genetic approaches have generated homozygous D1R-deficient mice, which exhibit impairment in learning and memory paradigms underscoring an important role of the D1R in memory formation/retention process (Smith et al. 1998). It is also worth mentioning that the expression of D5R in the hippocampus (which is higher than D1R and D2R expression) is likely to mediate the effects of D1 agonists on learning and memory (Missale et al. 1998). In fact, the hippocampal DA-mediated acetylcholine release involved in regulating learning and memory processes is significantly reduced in D5R knockdown and knockout mice (Hersi et al. 2000; Laplante et al. 2004).

The importance of the D1-like receptors in modulating locomotor activity is well established. However, it has been assumed that activation of D1R has little or no effect on controlling the degree of forward locomotion. However, studies have clearly shown that concomitant stimulation of D1R is essential for D2 agonists to produce maximal locomotor activity stimulation (Breese et al. 1987; Clark and White 1987; Dreher and Jackson 1989; Jackson and Westlind-Danielsson 1994).

Motivated behaviors including feeding and drinking are mediated by dopaminergic pathways and observations made in D1R knockout animals suggest a possible role of this DA receptor subtype in the motivation to seek food and/or experience reward from eating (Koob 1996; Sibley 1999). Reward and

reinforcement behavior mechanisms, which are controlled by mesolimbocortical DA, can be observed with the administration of psychostimulants and drugs of abuse (Le Moal and Simon 1991; Self and Stein 1992; Wise 1994; Di Chiara 1995). Studies have demonstrated that D1-like receptors play a permissive role in stimulant drug reinforcement using the drug-self administration paradigm (Beninger et al. 1989; Maldonado et al. 1993; Phillips et al. 1994). Initial studies examining behavioral mechanisms underlying cocaine administration suggested that D1R is required for specific actions of cocaine, including locomotor hyperactivity and stereotyped behaviors. However, recent observations suggest that D1-like receptors mediate a reduction in the drive to seek further cocaine reinforcement and rewarding properties of this drug of abuse (Self et al. 1996).

3.1.2 Cardiovascular and renal functions

D1-like receptors, which mediate direct vasodilatation, can be found in the medial layer of the renal, superior mesenteric and splenic arteries (Goldberg et al. 1978; Missale et al. 1988; Amenta and Ricci 1990). Based on the pharmacological characterization and autoradiographic localization of D1-like receptors, D5R is potentially associated with regulation of arterial blood pressure and renal and femoral blood flow (Goldberg et al. 1978; Amenta et al. 1995).

In the kidney, D1R is lozalized in renin-containing granules within the juxtaglomerular apparatus (O'Connell et al. 1995). These findings support

previous studies showing that fenoldopam (a D1-like agonist) stimulates the secretion of renin from renal cortical tissue slices (Kurtz et al. 1988; Antonipillai et al. 1989). In proximal tubules, DA inhibits sodium reabsorption *via* the activation of D1-like receptors (Jadhav and Liu 1992). Moreover, the inhibitory effect of DA on NA^+ reabsorption demonstrated in the ascending limb of loop of Henle (Meister et al. 1989), outer renal medulla (Amenta and Ricci 1990) and cortical collecting duct is also mediated by D1-like receptors (Ohbu and Felder 1991; Takemoto et al. 1992).

3.1.3 Gastrointestinal functions

Recently, D1R expression has been detected in the gastroesophageal junction, stomach, small intestine and colon. Although, their exact physiological functions still need more investigations, they may be involved in modulation of motility, fluid and electrolyte balance, and blood flow (Vaughan et al. 2000).

3.1.4 Ovarian and sexual functions

The presence of DA and D1R has been documented in the ovary. They have been proposed to play a role in the regulatory mechanisms controlling follicular development, possibly ovulation and corpus luteum function (Bahr and Ben-Jonathan 1985; Fernandez-Pardal et al. 1986; Mayerhofer et al. 1999). Behavioral evidence suggest that administration of D1-like agonists induces

lordosis in estrogen-primed female rats and gene knockdown and knockout studies suggest that D5R play an important role in mediating certain aspects of sexual behaviors in mammals (Apostolakis et al. 1996; Holmes et al. 2001; Kudwa et al. 2005).

3.2 Parkinson's disease and role of D1-like receptors

Parkinson's disease (PD) is primarily an age-related disease of the brain motor system with slowly progressing to ultimate severe disability. Diverse motor symptoms include muscle rigidity, tremor at rest, bradykinesia and postural instability. In addition, patients with PD frequently have cognitive and mood disturbances (Korczyn 1989; Jenner 1995).

The pathophysiology of PD requires more investigation. Meanwhile, PD has been clearly associated with a degeneration of the dopaminergic cells in the substantia nigra pars compacta leading to the loss of dopamine nerve terminals in the striatum, which underlie the movement abnormalities. Moreover, there is also reduction in the density of DA uptake sites (transporter) in the striatum (Marsden 1982; Bergman et al. 1990; Kaufman and Madras 1991; Jenner 1995). From a neuropathology aspect, PD is associated with intracellular inclusions named Lewis bodies, which presumably occur inside DA-containing cells in the substantia nigra (Hornykiewicz 1988; Scherman et al. 1989; Paulus and Jellinger 1991). In spite of severe striatal DA loss, other neuropathological damages has been detected in the

amygdala, hippocampus, magnocelluar cholinergic cell bodies and other monoaminergic cells, leading to norepinephrine, serotonin, acetylcholine, and neuropeptide deficiencies. These deficiencies probably contribute to the affective and cognitive changes as well as motor dysfunction observed in PD (Braak et al. 1995).

Pharmacotherapy is the most widely used treatment for PD. Using the amino acid L-3,4-dihydroxyphenylalanine (L-dopa or levodopa, the DA precursor) as DA replacement, this therapeutic approach is dramatically effective for several years in early stage of the disease in controlling most of the motor problems. L-dopa efficacy can be improved by the combined administration of an aromatic amino acid decarboxylase inhibitor to prevent peripheral conversion of L-dopa to DA and reduce undesirable side effects such as orthostatic hypotension and nausea (Cedarbaum et al. 1991; Clough 1991; Kurth and Adler 1998).

It is important to note that, the efficacy of DA replacement approach is reduced after few years (3-5) concomitant with the manifestation of side effects. Dyskinesia (uncoordinated, spasmodic, or irregular movements) and "on/off" fluctuations (fluctuating control of motor symptoms) are the most problematic complications of long-term therapy (Nutt et al. 1984; Kostic et al. 1991; Nutt et al. 1992; Kopin 1993). It has been suggested that reduced L-dopa efficacy upon prolonged administration manifests itself because of the cumulative loss of DA cells as the disease progresses (Marsden and Parkes 1976; Spencer and Wooten

1984). Furthermore, evidence indicate that the L-dopa can exacerbate the loss of dopaminergic neurons (Zou et al. 1999).

It has been reported that the D1R and D2R, the most abundant DA receptor subtypes in the striatum, undergo an up-regulation as a compensatory response to DA denervation in PD (Emilien et al. 1999). Clinical and preclinical investigations suggest a potential role of D1-like receptors in dyskinesia induced by L-dopa (Boyce et al. 1990). These investigations suggest that the incidence of dyskinesia may correlate with the ability of anti-parkinsonian drugs to stimulate D1-like receptors and increase intracellular cAMP formation. These effects can be attenuated by co-administration of antagonists that have higher affinity for D1R than D2R without reducing the anti-parkinsonian efficacy of DA agonists (Bennett et al. 1993; Jenner 1995; Fici et al. 1997; Williams et al. 1997).

3.3 Drug addiction and D1-like receptors

Drug addiction is a long-lasting condition involving compulsive drug-seeking and drug-taking, which can lead to neuroadaptations (Koob et al. 1998; Hyman and Malenka 2001; Nestler 2001; Laakso et al. 2002). Acute administration of psychostimulants such as cocaine and amphetamine, with adequate dose and frequency of these drugs, produces both short-term and long-term behavioral changes in rodents including sensitization and tolerance which can lead to addiction (Kuczenski and Segal 1989; Gawin 1991; Hyman 1996; Koob and Le Moal 1997).

The major target for the neurobiological mechanisms underlying the psychostimulant-induced behavioral changes is believed to be the brain mesolimbic DA pathway, which projects from the midbrain ventral tegmental area to the nucleus accumbens (Roberts et al. 1980; Delfs et al. 1990; Koob 1992). It has been hypothesized that most drugs of abuse potentially producing dependence, increase DA transmission in this specific brain region (Diana 1998). Moreover, studies investigating drugs of abuse such as morphine suggest also that the mesolimbic DA system is profoundly affected by withdrawal of addictive drugs. Thus, DA system can be consider as the key neural target for potential new therapies to improve the psychological discomfort or "dysphoria" produced by drug withdrawal (Devine et al. 1993; Diana 1998).

D1R plays a central role in mediating both acute and chronic effects of psychostimulants in rodents and non-human primates (Henry and White 1991; Hiroi and White 1991). Experiments aimed at testing locomotor responses and stereotyped behaviors in wild-type and D1R mutant mice after cocaine and amphetamine treatment indicated a prominent role of D1R in promoting changes in behavioral responses following both acute and repeated administrations of these two drugs (Xu et al. 1994b; Xu et al. 2000). In addition, alterations of gene expression mediated by D1R has been suggested to be implicated in drug-induced neuroadaptation in the brain (Konradi et al. 1994; Moratalla et al. 1996). Studies using wild-type and D1R mutant mice have identified chronic cocaine-induced gene expression changes mediated via the D1R, which potentially contribute to

persistent cocaine-induced behavioral changes (Zhang et al. 2004; Zhang et al. 2005). Although D1-like agonists increase the latency for initiating cocaine self-administration in rats and non-human primates (Koob et al. 1987; Self et al. 1996), these D1R drugs can also reduce the subjective and craving effects of cocaine in a dose-response manner (Haney et al. 1999). These findings data may support the potential use of D1R agonists in the treatment of drug abuse and in particular cocaine addiction.

3.4 D1-like receptors in neuropsychiatric disorders

3.4.1 Schizophrenia

Schizophrenia is a neuropsychiatric disorder that manifests itself with at least three distinct symptom domains such as auditory and visual hallucinations, paranoia, delusion and thought disorder (positive symptoms); blunted affect, avolition, anhedonia, emotional withdrawal and thought poverty (negative symptoms); cognitive dysfunction especially in attention and working memory, and bizarre behaviors (disorganized symptoms) (Liddle 1987; Carpenter and Buchanan 1989; Lenzenweger et al. 1991; Grube et al. 1998).

Schizophrenia still remains a life-long course illness without known pathophysiology. It has a complex etiology with both genetic and environmental components (Lewis and Lieberman 2000). Two major neurochemical theories, the

dopaminergic and glutamatergic hypotheses, have been proposed to explain the etiology of schizophrenia (Seeman 1987; Coyle 1996; Carlsson et al. 2001).

Classically, it has been believed that DA plays a crucial role in the generation of schizophrenia symptoms. The dopaminergic hypothesis was postulated based on early pharmacological observations showing that blockade of brain DA receptors reduces psychotic symptoms. Moreover, indirect DA agonists such as amphetamine can incite psychotic reactions in normal human beings that closely resemble those observed in schizophrenia as well as exacerbating psychotic symptoms in schizophrenics. These studies thus suggested that hyperactivity of the dopaminergic system or an increased sensitivity to DA may underlie schizophrenia symptoms (Carlsson and Lindqvist 1963; Breier et al. 1997; Laruelle 1998; Carlsson et al. 2001).

More recently, glutamate and its interactions with DA system have become a focus of intense investigations mainly because of its abundance in CNS and also the schizophrenic-like symptoms produced by the administration of phencyclidine (PCP), ketamine and other noncompetitive NMDA (*N*-methyl-D-aspartate) glutamatergic receptor antagonists in humans (Javitt and Zukin 1991; Coyle 1996; Tamminga 1998; Krystal et al. 1999). Additionally, studies support the notion that NMDA receptor dysfunction would be sufficient to account for the pattern of dopaminergic hyperactivity within PFC and striatum observed in schizophrenia (Balla et al. 2001; Balla et al. 2003).

Increased occupancy of D2-like receptors by endogenous DA has been observed in neuroleptic-naïve and neuroleptic-free schizophrenic patients (Wong et al. 1986; Pearlson et al. 1995; Abi-Dargham et al. 2000). However, subsequent studies did not support this view. Instead, it has been proposed that fluctuations in D2-like receptor density may contribute to clinical characteristic of different groups of patients, perhaps for those with a long duration of illness (Hietala et al. 1994; Knable et al. 1997). From a pharmacological point of view, D2-like agonists such as haloperidol, which exhibits a high affinity for these receptors, are classical antipsychotic drugs that can treat positive schizophrenic symptoms effectively (Farde et al. 1986; Marder and Van Putten 1995).

The insufficient D1-like receptor stimulation in prefrontal cortex (PFC) has been associated with the cognitive dysfunctions, especially working memory, in schizophrenic patients (Marder and Van Putten 1995; Goldman-Rakic et al. 2000; Castner and Goldman-Rakic 2004). D1-like receptors are at least 20-fold more abundant than the D2-like receptors in the PFC of non-human primates and an optimal level of D1-like receptors activation is necessary for normal cognitive function and working memory (Goldman-Rakic 1990; Lidow et al. 1991; Williams and Goldman-Rakic 1995). The correlation between the reduction of these receptors density in PFC and the severity of negative symptoms and cognitive disturbance has been found in both medicated and non-medicated schizophrenic patients (Okubo et al. 1997; Abi-Dargham et al. 2002; Karlsson et al. 2002). Clinical and preclinical trials demonstrated that selective D1-like antagonists, such

as SCH39166 and NNC 01-068, not only show lack of antipsychotic activity but may also aggravate psychoses in some patients (Den Boer et al. 1995; Karle et al. 1995; Karlsson et al. 1995). In contrast, it seems that low dose of selective full D1-like agonists such as dihydrexidine, A77636 and SKF81297 have cognitive-enhancing actions in non-human primates (Arnsten et al. 1994; Schneider et al. 1994; Cai and Arnsten 1997). Overall, findings showing that working memory can be improved by D1-like agonists has led to the notion that novel compounds targeted at stimulating D1R activity are likely to be of immense value in treating cognitive deficits and substantially improving the outcome in schizophrenia (Castner et al. 2000; Miyamoto et al. 2005).

3.4.2 Bipolar disorder

Bipolar (BP), or manic-depressive, disorders represent clinically episodic, lifelong severe mental illnesses associated with considerable morbidity and mortality. The diagnosis of BP makes several challenges mainly because of overlapping symptomatology and comorbodity with other diseases, as well as the somewhat restrictive and categorical approach taken by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV)* and the *International Statistics Classification of Diseases, 10th Revision (ICD-10)* diagnostic criteria. Currently, BP disorders can be divided into four entities: bipolar I, bipolar II, cyclothymic disorder and bipolar disorder not otherwise

specifies, based on a spectrum of severities, frequencies and duration of manic and depressive symptoms, as described in DSM-IV (Kraeplin 1921; Dunner et al. 1982). Recent findings demonstrate that BP illnesses are highly recurrent, malignant disorders with estimated lifetime prevalence rates ranging from 2.8 to 6.5% (Bauer and Pfennig 2005) which is far more frequent than previously thought (1%) (American Psychiatric Association 1994).

Several lines of evidence suggest an important role of the dopaminergic system activity in the pathophysiology of BP. It has been demonstrated that DA abnormalities are involved in the hyperactivity associated with severe stages of mania (Goodwin and Jamison 1990; Manji and Lenox 2000); Pharmacological data support this notion, as demonstrated in mania induction using direct and indirect DA agonists (L-dopa, amphetamine and bromocriptine) or blockers of DA reuptake (Silverstone and Romans-Clarkson 1989; Keck and McElroy 1996; Vacheron-Trystram et al. 2004).

The possible association between the etiology of BP disorder and dopamine D1R gene (*DRD1*) has interested investigators for decades. Some studies found no close linkage or association of BP disorder and *DRD1* gene in a case-control studies (Mitchell et al. 1992; Cichon et al. 1994; Savoye et al. 1998). On the other hand, medications used in BP therapy have significant effects on central DA activity through D1R. Indeed drugs such as lithium can reduce dopaminergic turnover and modify D1R responsiveness to dopaminergic agents (Gottberg et al. 1988). Moreover, it has been shown that clozapine may be effective in the

treatment of the manic phase of BP through interaction with DA receptor subtypes, including D1R (Suppes et al. 1992). Recent findings from linkage disequilibrium, pathophysiological, pharmacological and genetic studies has provided a new impetus for studies investigating the dopamine D1R gene (*DRD1*) as a good candidate gene for BP (Ni et al. 2002; Severino et al. 2005).

3.4.3 Attention-deficit/hyperactivity disorder (ADHD)

Attention-deficit/hyperactivity disorder (ADHD) is a multifactorial neurodevelopment disorder that is associated with tremendous financial burden, stress to families, and adverse academic and vocational outcomes. AHDH is highly prevalent in childhood, affecting roughly 5-10% of school-age children worldwide (Barkley 1990; Swanson et al. 1998; Scahill and Schwab-Stone 2000; Brown et al. 2001), with male being affected three times more than females (Anderson et al. 1987; Baumgaertel et al. 1995).

ADHD is characterized by age-inappropriate and impairing levels of inattention, hyperactivity and impulsivity, thus affected patients exhibit behavioral problems such as carelessness, restlessness, disobedience and failure to stay quiet in class (Swanson et al. 1998; Wolraich et al. 1998; Shastry 2004). It has been estimated that 30% to 70% of children diagnosed with ADHD have persisting psychopathology in adulthood. The prevalence rate of adult with ADHD demonstrates that 4.5% of these patients continue to exhibit this disorder from

childhood and the majority of them show high proportion of other serious psychiatric comorbidities, especially anxiety, substance abuse, or BP disorders (Gittelman et al. 1985; Weiss and Hetchman 1986; Barkley 1990; Swanson et al. 1998; Brown 2000; Montano 2004; Clarke et al. 2005).

However, the exact etiology of ADHD has not been clearly identified, but family ((Biederman et al. 1990; Biederman et al. 1992; Faraone et al. 1994), twin (Thapar et al. 1995; Silberg et al. 1996; Levy et al. 1997) and adaptation studies (Alberts-Corush et al. 1986) have strongly supported the existence of genetic factors for ADHD. In addition, data from several twin studies indicate that the heritability of this disorder is as high as 80-90% due mainly to shared gene effects (Gillis et al. 1992; Thapar et al. 1995; Sherman et al. 1997; Faraone and Biederman 1998).

A dysregulation of dopamine and/or noradrenaline neurotransmission is probably underlying the pathophysiological mechanisms of ADHD (Faraone and Biederman 2002). According to several lines of evidence obtained from three different research areas, dysfunction of the dopaminergic system plays an extensive role in the etiology of ADHD. First, psychostimulant medications (methylphenidate and amphetamine), which are by far the most widely studied and commonly prescribed treatments for ADHD, can increase synaptic levels of DA through the inhibition of the DA transporter activity and DA release (Levy 1991; Amara and Kuhar 1993; Seeman and Madras 1998). Second, abnormalities in neuroanatomical regions with high DA innervations have been reported in ADHD

children (Ernst et al. 1999), which have also been observed in neuroimaging studies in ADHD adults (Dougherty et al. 1999; Krause et al. 2000). Third, behavioral and biochemical studies in animal models suggest that hyperactivity is potentially associated with perturbations in the brain motor control caused by an increased DA neurotransmission (Giros et al. 1996; Gainetdinov et al. 1999; Jaber et al. 1999; Russell 2000). Therefore, these studies suggest that DA-related genes can be considered as potential candidate genes for ADHD. So far, genes encoding the DA transporter or DAT1 (Cook et al. 1995; Volkow et al. 1998; Curran et al. 2001) D4R and D5R (Benjamin et al. 1996; Smalley et al. 1998; Holmes et al. 2000; Faraone et al. 2001) have been investigated but an association between these genes and ADHD remains to be firmly established.

Recently, the *DRD1* gene has attracted interest as a potential candidate gene for genetic susceptibility to ADHD. Neuropsychological observations indicated that individuals with PFC lesions exhibit ADHD-like behaviors (Arnsten et al. 1996; Arnsten 2001). Moreover, working memory impairment in ADHD patients (Denney and Rapport 2001; Castellanos and Tannock 2002) and correlation between working memory capacity and attentional ability in the individuals from the general population (Conway et al. 2001; de Fockert et al. 2001) have been reported. In addition, the combination of hyperactivity and impaired motor coordination observed in the D1R-knockout mice (Xu et al. 1994a; Clifford et al. 1998) resembles the prevalent deficit in motor coordination in children with ADHD (Barkley 1997; Kadesjo and Gillberg 1998). Thus, on the basis of the

known importance of D1R in working memory function and evidence from both animal and human studies, the *DRD1* gene has been suggested to play an important role in the phenotypic expression of ADHD (Misener et al. 2004)

However, the potential role of the *DRD5* gene in the etiology of ADHD is considerably less understood. The pivotal role of DA in mediating attentional processes has been demonstrated (Nieoullon 2002). Attentional deficits are a prominent feature of ADHD. Recent studies has proposed that D5R may contribute to activation of dopaminergic pathways relevant to exploratory locomotor, startle, and prepulse inhibition (Holmes et al. 2001). Interestingly, a recent genetic study suggest a possible etiological role of the *DRD5* gene in ADHD (Manor et al. 2004).

3.5 Hypertension and renal D1-like receptors

Hypertension, the leading cause of morbidity and mortality worldwide, still remains the most powerful risk factor for cardiovascular disease (stroke), coronary artery disease (acute myocardial infarction), congestive heart failure (both systolic and diastolic dysfunction), and renal dysfunction (Kuller 1978; MacMahon et al. 1990; Palmer et al. 1992; Joint National Committee report on high blood pressure 1997).

Essential hypertension, also known as primary or idiopathic hypertension, is a heterogeneous disorder with different causal factors in patients and accounts

for 95% of all hypertension cases (Carretero and Oparil 2000; Dosh 2002). Kidney is the primary locus for the initiation and subsequent maintenance of hypertension process mostly caused by sodium intake and genetic factors (Goldblatt et al. 1934; Guyton et al. 1971; Dudley et al. 1992; Dominiczak et al. 2000; Iwamoto 2006; Mullins et al. 2006).

Our understanding of the DA-mediated effects on renal and cardiovascular functions has made tremendous progress since 1964, when the DA-mediated regulation of natriuresis and diuresis was first reported in humans. There is clear evidence that DA is an important modulator of renal sodium excretion. A defective renal DA synthesizes within proximal tubule and renal DA receptor functions have been observed in human primary hypertension as well as in genetic models of animal hypertension (McDonald et al. 1964; Aoki et al. 1989; Iimura and Shimamoto 1990; Jose et al. 1992).

Administration of DA and selective D1-like receptor agonists reduce the after load and augment blood flow to certain organs as well as increasing urine flow rate and sodium excretion, which cause hypotension (Felder and Carey 1987). Moreover, in rat models of hypertension the D1-like receptors located on the renal proximal tubule are defective, which results in impaired inhibition of tubular sodium reabsorption caused by a reduction of the DA-mediated inhibition of the Na,H-exchanger and Na,K-ATPase activity (Debska-Slizien et al. 1994; Albrecht et al. 1996; Hussain and Lokhandwala 1996).Notably, the association between a defective regulation of renal proximal tubule sodium transport and renal

D1-like receptors has been suggested in human essential hypertension (Saito et al. 1994; O'Connell et al. 1997).In fact, a study has reported that the defective G protein coupling function of the D1R subtype observed in the primary cells cultured from hypertensive human proximal tubules was associated with an increased basal phosphorylation of D1R (Sanada et al. 1999).

Overall, these physiological, and biochemical, and molecular studies emphasize the importance of renal D1-like receptors in the regulation of sodium and body volume homeostasis.

3.6 Pharmacological characteristics of D1-like receptors

Despite of the high degree of sequence identity within their putative transmembrane regions, D1R and D5R display noticeable different pharmacological properties. D5R binds drugs with a pharmacological profile similar to D1R. However, D5R exhibits higher affinity for agonists and lower affinity for antagonist drugs (Sunahara et al. 1991; Tiberi et al. 1991; Tiberi and Caron 1994). Furthermore, D5R can be distinguished from D1R subtype by a higher agonist-dependent activity (also referred to as basal, spontaneous or constitutive activity) (Sunahara et al. 1991). Based upon a functional comparison with D1R, D5R was the first receptor to display naturally the ligand biding and G protein coupling properties of constitutively active mutant GPCRs (Tiberi et al. 1991).

In a similar fashion to other GPCRs, ligands that bind to D1-like receptors be classified as full and partial agonist, full and partial inverse agonists and neutral antagonists (Schutz and Freissmuth 1992; Lefkowitz et al. 1993; Milligan et al. 1995; Seifert and Wenzel-Seifert 2002). The D1-like receptor-based therapeutic agonists include candidate antiparkinsonism (Neumeyer et al. 2003) and antihypertensive drugs (Singh and Goyal 1999; Mathur 2003). Meanwhile, D1-like receptor-based therapeutic antagonists include candidate antipsychotic (Karlsson et al. 1995). However, the clinical efficacy of D1-like antagonists in the treatment of schizophrenia remains debatable (Den Boer et al. 1995; Karle et al. 1995; Karlsson et al. 1995). In addition, it has been suggested that D1-like receptor-based therapeutics may be useful in the treatment of psychostimulant abuse and dependence (Bergman et al. 2000). Interestingly, D1-like receptor partial agonists have been shown to be better therapeutic approaches to reduce abuse-related behavioral effects of stimulants in comparison with antagonists and full agonist, which are associated with behavioral-disturbing effects and hypotension, respectively (Bergman and Goldberg 1998; Khroyan et al. 2000).

The worsening of symptoms in some patients with schizophrenia, following administration of a D1-like selective antagonist, is potentially explained by studies showing that antipsychotics are not just simply neutral antagonists but they display negative activity intrinsic activity or inverse agonism at the D1-like receptors (Schwarzkopf et al. 1996; Zhang et al. 2000). Additionally, the lack of D1-like subtype-selective antagonists or inverse agonists hinders our ability to

assess the clinical relevance of blocking D1R or D5R in schizophrenia. Studies showing that antipsychotic drugs can modulate the expression of D1R and D5R in the cortex of primates, may be of relevance for their clinical efficacy in the treatment of neuropsychiatric illnesses displaying compromised D1-like function (Karlsson et al. 1995; Martin et al. 2001).

3.7 Structure-function relationship of D1-like receptors

The identification of structural domains and amino acid residues involved in the regulation of ligand binding and activation properties of D1-like receptor subtypes remain to be fully appreciated. Towards the evaluation of the structural determinants of dopamine receptors functions, two approaches have been used in mutagenesis studies: single amino acid mutations and construction of chimeric receptors using domains of two receptors exhibiting particular functional characteristics (Neve and Neve 1997).

The molecular basis underlying the differences in the activation properties of the D1-like receptors, and more specifically the role of amino acids in agonists and antagonists binding, is not clearly understood. A previous study has shown that potential interactions between specific amino acid residues in TM2 (Asp^{70}) and TM3 (Cys^{106} and Ser^{107}) of D1R may contribute to the binding affinities of both agonist and antagonist (Tomic et al. 1993). Moreover, mutations of residues located in TM5 (Ser^{199} and Ser^{202}) and TM7 (Trp^{321}) domains impair agonist and

antagonists binding, respectively (Tomic et al. 1993). Another study demonstrated that the carboxyl-terminal region of the third intracellular loop (IL3), and in particular the residue Ile288, is one of the structural determinants involved in the constitutive activation of the D5R (Charpentier et al. 1996). Additionally, an amino acid substitution (Ser^{56} to Leu) in the first intracellular loop (IL1) of D1R did not change the ability of this D1-like subtype to bind ligand or couple to G protein (Jin et al. 1998). Mutational analysis has shown that an amino acid substitution in the TM7 (Asn^{351} to Asp) of the D5R leads to a 3- and 10-fold decrease in the binding affinity to SKF38393 and DA, respectively (Cravchik and Gejman 1999). Moreover, an amino acid substitution in the TM2 (Leu^{88} to Phe) of D5R promotes a small increase in DA biding affinity (Cravchik and Gejman 1999). Amino acid sequences within the cytoplasmic tail (CT), and in particular one amino acid (Gln^{439}), has been shown to play an important role in the high affinity binding for DA and expression of functional properties of D5R (Demchyshyn et al. 2000).

Prior to the work of Demchyshyn et al. (2000), a study using chimeric D1R/D5R has identified a receptor region in D1R and D5R referred to as the terminal receptor locus (TRL), which includes sequences from the TM6, and third extracellular loop (EL3), TM7 and CT (Iwasiow et al. 1999). This study demonstrated that the TRL was the predominant structural regulating the phenotypic expression of ligand binding, constitutive activation and G protein coupling properties of D1-like receptors (Iwasiow et al. 1999). Subsequently,

more refined chimeras were generated to assess the underlying structural determinants of TRL involved in the expression of D1R and D5R functional phenotypes (Jackson et al. 2000; Tumova et al. 2003). Data obtained using these refine chimeras suggest that the CT plays fundamental role in the regulation of DA affinity and constitutive activity of D1R and D5R (Jackson et al. 2000). Furthermore, these studies have demonstrated that a molecular interplay between EL3 and CT must take place to coordinate the full expression of DA biding affinity, agonist-independent and dependent G protein-coupling properties of D1R and D5R (Tumova et al. 2003).

4. Objective and Hypothesis

Previous studies in our lab have put forward the notion that the TRL cassette contains the structural determinants for the ligand binding and G protein coupling properties of D1R and D5R. Notably, unpublished studies from our lab suggest that other structural determinants located in the TRL cassette act in concert with EL3 to regulate the ligand binding (e.g. selectivity profile of inverse agonists and antipsychotic) and activation properties of D1R and D5R. Recently, data obtained from our lab using chimeras in which the EL3 region of D1R and D5R was exchanged, suggest that EL3 is a crucial domain in conferring to D1R and D5R their subtype-selectivity profiles for antipsychotic drugs (which act as inverse agonists at D1R and D5R). Moreover, data from our lab showed that a

substitution of the two residues located on the exofacial end of TM6 (Ile^{294}/Leu^{295} in D1R and Met^{318}/Val^{319} in D5R) of the EL3 chimeras, does not allow the full expression of the binding affinity of inverse agonists and selectivity profile displayed by the EL3 chimeras and wild-type receptors.

The objective of my master thesis is to investigate the coordinated role between EL3 region and two variant residues of the exofacial end of TM6 (Ile^{294}/Leu^{295} in D1R and Met^{318}/Val^{319} in D5R) in regulating the signaling properties of human D1-like receptors.

I hypothesize that Ile^{294} of D1R and Met^{318} of D5R are the TM6 residues regulating negatively the EL3-mediated affinity and selectivity of inverse agonists.

Experimental procedures

1. Materials

N-[*methyl*-^{3}H]-SCH23390 (66-90 Ci/mmol), [^{3}H]-adenine (27 Ci/mmol), [^{14}C]-cAMP (250-275mCi/mmol), RediVue [^{35}S]-dATP (1000 Ci/mmol) and Biodegradable Counting Scintillant were from Amersham Biosciences (Baie d'Urfé, Québec, Canada). Dopamine (DA), SKF38393, Apomorphine, SCH23390, *cis*-flupentixol, (+)-butaclamol, fluspirilene, thiothixene, thioridazine and 1-methyl-3-isobutylxanthine (IBMX) were purchased from Sigma/Research Biochemicals International (Oakville, Ontario, Canada). Human embryonic kidney 293 (HEK293) cells were from American Type Culture Collection (Manassas, VA, USA). Minimum essential media (MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), gentamicin, trypsin, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic] acid (HEPES) buffer solution (1M), agarose and *Taq* DNA polymerase were obtained from Invitrogen (Burlington, Ontario, Canda).

2. Construction of Chimeric Human D1 and D5 Receptors

The third extracellular loop (EL3) and two variant residues of the sixth transmembrane (TM6) domain of the human D1 and D5 receptor were swapped by gene splicing using a polymerase chain reaction (PCR)-based overlap extension approach. The wild-type receptor sequences were swapped between the junction of TM6 and EL3 and the junction of EL3 and the seventh transmembrane (TM7)

domain. This region corresponds to amino acid residues 290 to 315 in D1R and residues 314 to 345 in D5R. In this region, the two variant residues of TM6 are Ile^{294} and Leu^{295} in D1R and Met^{318} and Val^{319} in D5R. In the present study, four chimeric receptors have been constructed, including: D1-TM6B^{Met318}-EL3B; D1-TM6B^{Val319}-EL3B; D5-TM6A^{Ile294}-EL3A; and D5-TM6A^{Leu295}-EL3A (Figure 3). Four additional chimeric receptors D1-EL3B; D1-TM6B$^{Met318/Val319}$-EL3B; D5-EL3A; and D5-TM6A$^{Ile294/Leu295}$-EL3A; readily available in our lab (Iwasiow et al. 1999) were also used (Figure 3).

In the case of D1-TM6B^{Met318}-EL3B chimera, the first round of PCR generated two overlapping fragments: the first fragment coding for EL3 of D5R and the second fragment coding for TM6-Met^{318} (indicated in bold) and EL3 region of D5R. The first fragment was amplified using primers D1-P1 (HD1A-3ELB3) (forward primer): 5' CCC GAA GGC CCT CCG GCC GGC TTC CCC TGC GTC AGT GAG ACC ACC TTT GAC GTG 3'; and T3 (reverse primer): 3' GA AAT CAC TCC CAA TTA 5'. The second fragment was amplified using primers T7 (forward primer): 5' TAA TAC GAC TCA CTA TA 3'; and a different reverse primer which was designed and named D1-P2 (HD1AEL3B-M) (reverse primer): 3' TAG AAC TTG ACG **TAC** AAC GGG AAG ACG TCA CCT GTG GGA CTT CCG GGA GGC 5'; the nucleotide sequence corresponding to D1 receptor are underlined.

In the case of D1-TM6B^{Val319}-EL3B chimera, the first round of PCR was similar to D1-TM6B^{Met318}-EL3B chimera with the exception that the second

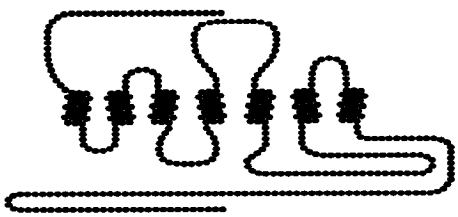
Figure 3 – Primary structure of the putative sixth transmembrane region (TM6) and third intracellular loop (EL3) of the human D1-like subtypes and schematic representation of the wild-type and chimeric receptors.

(A) Alignment of the primary structure of TM6, EL3 and TM7 between human D1R and D5R is shown. Identical amino acids found between the primary structures are shown with an *asterisk*. **(B)** Putative topology of the wild-type D1R (red circles) and D5R (blue circles), D1-EL3B; D1-TM6B$^{Met318/Val319}$-EL3B; D1-TM6B^{Met318}-EL3B; D1-TM6B^{Val319}-EL3B; D5-EL3A; D5-TM6A$^{Ile294/Leu295}$-EL3A; D5-TM6A^{Ile294}-EL3A; and D5-TM6A^{Leu295}-EL3A chimeras is represented.

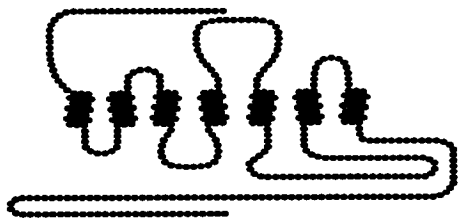
A


```
Human D1R  VIMGVFVCCWLPFFILNCILPFC-GSGETQP--F-CIDSNTFDVFVWFGWANSSLNPIIYAFN
Human D5R  VIMGVFVCCWLPFFILNCMVPFCSGHPEGPPAGFPCVSETTFDVFVWFGWANSSNLPVIYAFN
           ******************  *** *  *  *  * *    ***************** *****
```

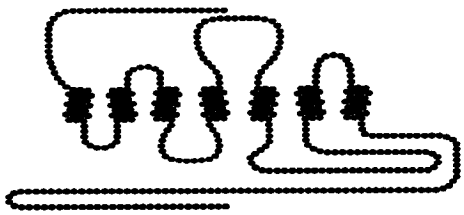
B



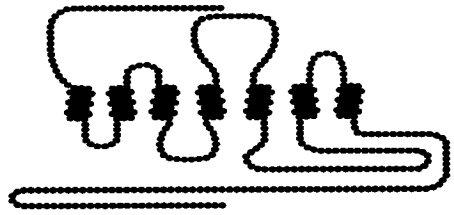
D1 / D1A



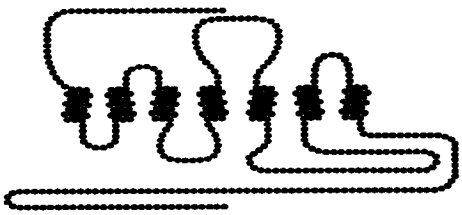
D5 / D1B



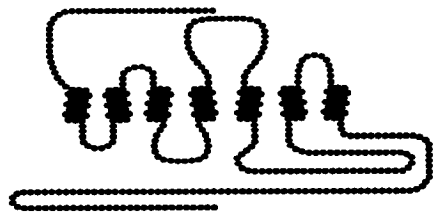
D1-EL3B



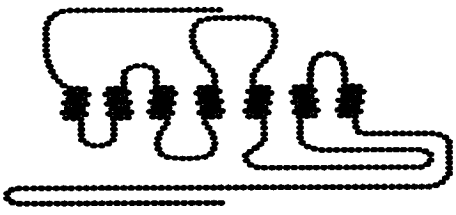
D5-EL3A



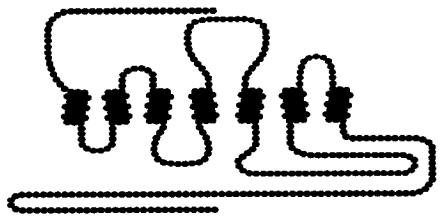
D1-TM6B$^{Met318/Val319}$-EL3B



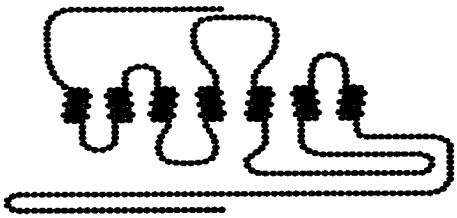
D5-TM6A$^{Ile294/Leu295}$-EL3A



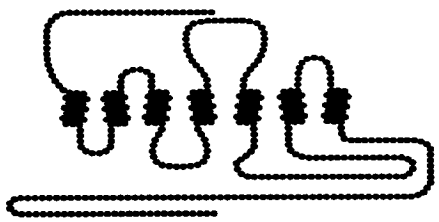
D1-TM6B^{Met318}-EL3B



D5-TM6A^{Ile294}-EL3A



D1-TM6B^{Val319}-EL3B



D5-TM6A^{Leu295}-EL3A

fragment encoded the TM6-Val^{319} (indicated in bold) and EL3 region of D5R. This fragment was amplified using primers T7 (forward primer): 5' TAA TAC GAC TCA CTA TA 3'; and a specific designed primer called D1-P3 (HD1AEL3B-V) (reverse primer): 3' TAG AAC TTG ACG TAA **CAG** GGG AAG ACG TCA CCT GTG GGA CTT CCG GGA GGC 5'; the nucleotide sequence corresponding to D1 receptor are underlined.

In the case of D5-TM6A^{Ile294}-EL3A chimera, the first PCR round generated two overlapping fragments: the first one coding for EL3 of D1R and the second fragment coding TM6-Ile^{294} (indicated in bold) and EL3 of D1R. Primers D5-P1 (HD1B-3ELA3) (forward primer): 5' GGG GAG ACG CAG CCC TTC TGC ATT GAT TCC AAC ACC TTC GAC GTC 3'; and HD5-1410R (reverse primer): 3' TCA GAC AGA CCC TCG 5' were used to amplify the first PCR fragment. For the second fragment, HD5-640F (forward primer): 5' ACC TGG CCA ACT GGA 3'; and a unique reverse primer which was prepared and called D5-P2 (HD1BEL3A-I) (reverse primer): 3' TAG GAA TTG ACG **TAA** CAG GGA AAG ACG CCT AGG CCC CTC TGC GTC GGG 5' were used to amplify the PCR fragment; the nucleotide sequence corresponding to D5 receptor are underlined.

In the case of D5-TM6A^{Leu295}-EL3A chimera, the first round of PCR generated the same first fragment as described for D5-TM6A^{Ile294}-EL3A chimera. The second fragment coding for TM6-Leu^{295} (indicated in bold) and EL3 and of D1R was amplified using the following primers: HD5-640F as the forward primer

and a different designed reverse primer called D5-P3 (HD1BEL3A-L) (reverse primer): 3' TAG GAA TTG ACG TCA **AAC** GGA AAG ACG CCT AGG CCC CTC TGC GTC GGG 5'; the nucleotide sequence corresponding to D5 receptor are underlined.

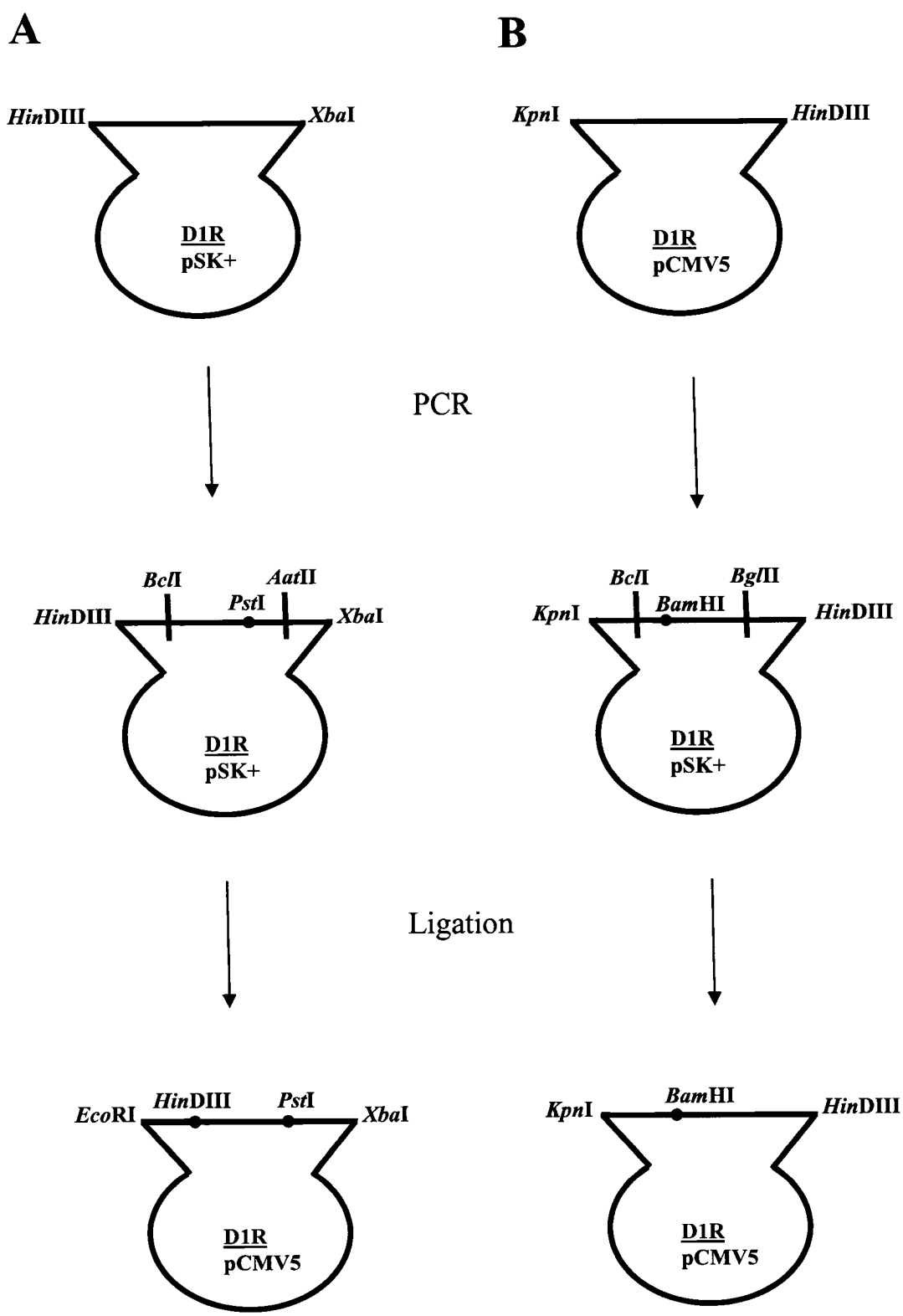
To facilitate the construction and identification of these chimeras, an additional restriction endonuclease site (silent mutation) was created in each chimeric construct. For the D1-TM6B^{Met318}-EL3B and D1-TM6B^{Val319}-EL3B chimeras, a *Pst*I site was engineered in the nucleotide sequence encoding amino acid residues 298 and 299 (5'-CTACAG-3'→ 5'-CTGCAG-3'; modified nucleotide is underlined). The *Pst*I restriction site is located at the 3' end of the junction between the TM6 and EL3 regions of chimeric receptor sequences (Figure 4A). Likewise, for D5-TM6A^{Ile294}-EL3A and D5-TM6A^{Leu295}-EL3A chimeric receptors, a *BamH*I restriction site was introduced in the nucleotide sequence coding for amino acid residues 323 and 324 (5'-GGGTCT-3'→ 5'-GGATCC-3'; modified nucleotide is underlined). The *BamH*I restriction site was located near the 3' end of TM6 region, immediately at the beginning of EL3 sequence of D1R (Figure 4B).

PCR reactions were run and separated on a 1% agarose gel and optimum bands removed by cutting gel slices and DNA fragments purified by QIAEX II gel extraction method (Qiagen, Valencia, CA, USA). Each pair of purified DNA fragments (mega primers) for the mutant chimeras were diluted to optimal concentrations, combined and subjected to overlap PCR using appropriate sets of

Figure 4 – Schematic representation of the strategy to construct the chimeric D1-like receptors.

A. Schematic representation of construction of D1-TM6B^{Met318}-EL3B and D1-TM6B^{Val319}-EL3B chimeras. **B.** Schematic representation of construction of D5-TM6A^{Ile294}-EL3A and D5-TM6A^{Leu295}-EL3A chimeras.

Black lines indicate vectors (pSK+ and pCMV5); red lines indicate D1R DNA; blue lines indicate D5R DNA; italic letters indicate diagnostic restriction enzymes.


A
B
HinDIII
XbaI
D1R
pSK+
KpnI
HinDIII
D1R
pCMV5
PCR
BclI
AatII
PstI
HinDIII
XbaI
D1R
pSK+
BclI
BglII
KpnI
BamHI
HinDIII
D1R
pSK+
Ligation
EcoRI
HinDIII
PstI
XbaI
D1R
pCMV5
KpnI
BamHI
HinDIII
D1R
pCMV5

specific 5' and 3' flanking primers to generate chimeric D1/D5 DNA product. The resulting PCR products for D1 chimeras were cut with *Aat*II and *Bcl*I and subcloned into pBluescript II SK+ (Stratagene) containing *Hind*III / *Xba*I fragment of the wild-type D1 receptor. Prior to subcloning, the D1-pBluescript II SK+ construct was linearized with *Aat*II and *Bcl*I. The chimeric constructs for D5 receptor were excised with *Bcl*I and *Bgl*II and subcloned in pBluescript II SK+ containing the full-length coding sequence of D5R (linearized with *Bcl*I and *Bgl*II). The identity of the chimeras in pBluescript II SK+ was confirmed by dideoxy sequencing using Sequenase version 2.0 kit (U. S. Biochemical Corp.). Mammalian expression constructs for the wild-type and chimeric D1R and D5R were engineered into the expression vector pCMV5 in a 2-piece ligation process. The integrity of nucleotide sequence for cloning sites (*Hind*III / *Xba*I for D1R chimeras and *Kpn*I / *Hind*III for D5R chimeras) was also verified by dideoxy sequencing (Figure 4).

3. Cell culture and Transfection

Human embryonic kidney 293 (HEK293) cells were cultured in minimal essential medium (MEM) containing heat-inactivated FBS (10% (v/v)) and gentamicin (10 μg/ml) at 37°C in a 5% CO_2 environment. Cells were seeded into 100-mm dishes (2.5×10^6 cells/dishes) and transiently transfected by a modified calcium-phosphate method (Didsbury et al. 1991) using 0.05–5 μg DNA/dish. In

brief, a plastic tube containing 10 μg of DNA in 900 μl of sterile milli-Q-water was supplemented with 100 μl of 2.5 M $CaCl_2$ and gently mixed by finger flicking. Then, 1 ml of 2X HEPES-buffered saline (HBS; 0.28 M NaCl, 0.05 M HEPES, and 1.5 mM Na_3PO_4, pH 7.1), was added drop wise to the DNA-calcium solution, mixed by gently tapping the side of tube and two 100-mm dishes transfected at the same time. Typically, 1 ml of transfection mixture (5 μg DNA) was added drop wise to one 100 mm-dish of cells containing 10 ml of complete MEM. When less than 5 μg receptor DNA per dish was employed to transfect cells, empty pCMV5 vector was added to normalize the total amount of DNA. Cells were incubated with the DNA-calcium phosphate precipitate overnight before reseeding. All experiments were done with cells from 38 to 52 passages.

4. Membrane preparation

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with 5 ml of PBS, trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Subsequently, transfected HEK293 cells were washed with cold PBS, scraped off from the dish in ice-cold lysis buffer (10 mM Tris-HCL, pH 7.4, 5 mM EDTA), transferred into 35-ml tube and cell membrane preparations centrifuged twice at 40 000 x g for 20 min at 4°C. The final membrane pellets were resuspended in 2 ml of lysis buffer using a Brinkman polytron (17 000 rpm for 15 s). Fresh aliquots of 0.5 ml of membranes

were used immediately in saturation studies and remaining membrane preparations dispensed in microcentrifuge tubes, frozen in liquid nitrogen and stored at –80°C until needed for competition studies.

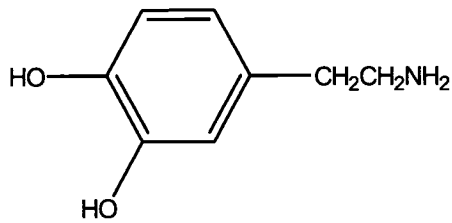
5. Radioligand binding assay

Fresh or frozen membranes (thawed on ice) from transfected cells were diluted in binding buffer (final concentrations in assay: 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1 mM EDTA) using a Brinkmann polytron. Binding assays were carried out with 100 µl of membranes in a total volume of 500 µl in the presence of N-[*methyl*-^{3}H]-SCH23390 as radioligand. Saturation studies were performed using fresh membrane preparations and increasing concentrations of N-[*methyl*-^{3}H]-SCH23390 ranging between 0.01 and 6 nM. The non-specific binding was assessed using a series of tubes containing radioligand and 10 µM of cis-flupentixol (dissolved in milli-Q-water). For competition studies, membranes thawed on ice were diluted in appropriate volume of binding buffer and incubated with a constant concentration of N-[*methyl*-^{3}H]-SCH23390 (~0.6 nM) in the presence of increasing concentrations of competing agonist ligands (DA, SKF38393, Apomorphine) (Figure 5) and antagonist/inverse agonist ligands (*cis*-flupentixol, (+)-butaclamol, fluspirilene, thioridazine, thiothixene) (Figure 6). All drugs tested in competition studies were prepared in milli-Q-water with exception of dopamine, which was made in

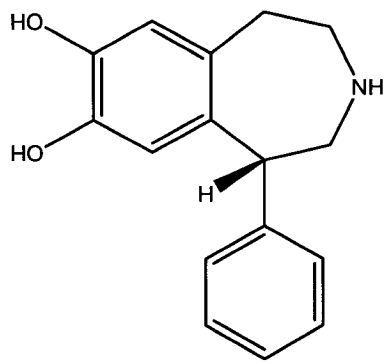
Figure 5 – Chemical structure of D1-like tested agonist ligands.

Structural representation of tested dopamine agonists.

Dopamine



SKF-38393



Apomorphine

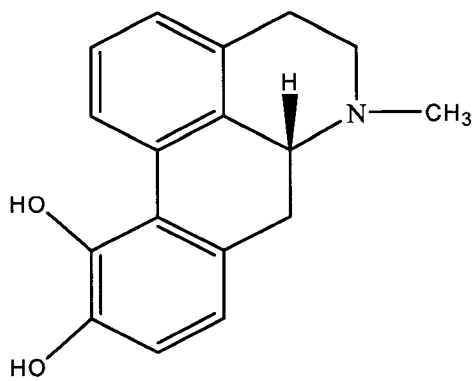
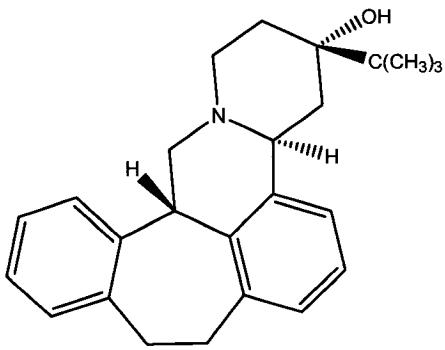


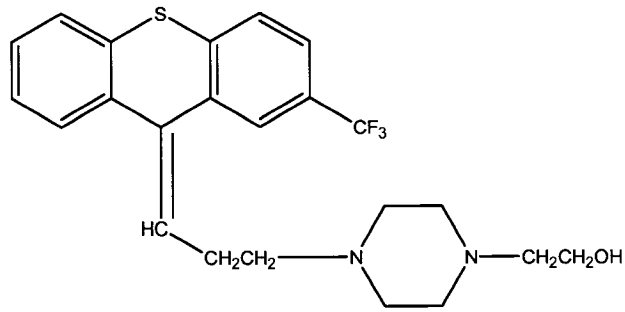
Figure 6 – Chemical structure of D1-like tested antagonist ligands.

Structural representation of tested dopamine antagonists and inverse agonists.

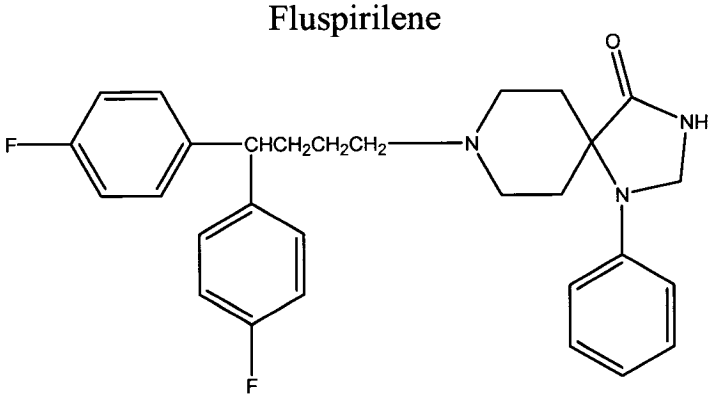
(+)- Butaclamol



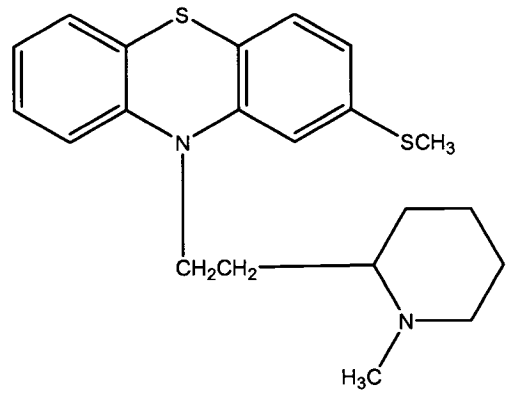
cis-Flupentixol



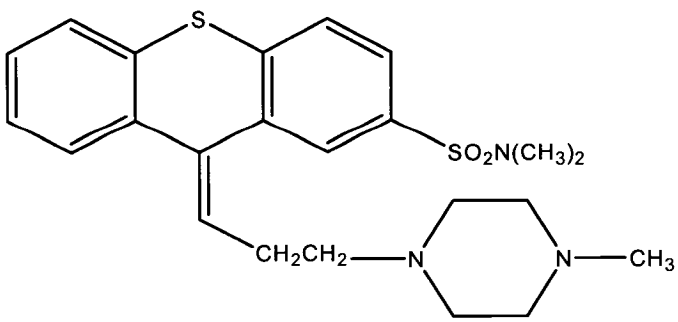
Fluspirilene



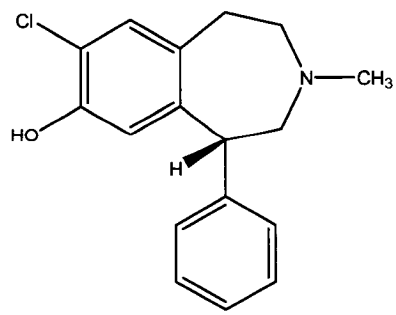
Thioridazine



Thiothixene



SCH23390



0.1 mM ascorbic acid (dissolved in milli-Q-water) to prevent its oxidation. Binding assays were incubated for 90 min at room temperature and stopped using rapid filtration through glass fiber filters (GF/C, Whatman). Filters were subsequently washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and bound radioactivity was calculated by liquid scintillation counting (Beckman Coulter Counter, LS6500). Protein concentrations were determined using the Bio-Rad assay kit with bovine serum albumin as standard.

6. Whole cell cAMP assay

Regulation of adenylyl cyclase (AC) activity by wild-type and chimeric D1R and D5R was determined using a whole cell cAMP assay as described previously (Tumova et al. 2003; Tumova et al. 2004). Whole cell cAMP assays were utilized to assess dopamine-independent and dependent G protein-coupling properties of wild-type and chimeric receptors. Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12-well dishes to perform agonist-independent activity (constitutive activity) studies and dose-response curves for dopamine, respectively. The next day, the culture medium was replaced with fresh labeling MEM containing 5% (v/v) FBS, gentamicin (10 μg/ml) and [^{3}H]-adenine (2 μCi/ml) and cells were metabolically labeled for 16–18 h at 37°C in a 5% CO_2 environment. At the end of metabolic

labeling period, the medium was removed and HEK293 cells incubated in 20 mM HEPES-buffered MEM containing 1 mM IBMX (phosphodiesterase inhibitor) in the presence or absence of dopamine for 30 min at 37°C (in the presence of 0.1 mM ascorbic acid). At the end of 30-min incubation, the medium was aspirated and each well supplied with 1 ml of lysis solution containing 2.5% (v/v) perchloric acid, 1 mM cAMP and [^{14}C]cAMP (2.5–5 nCi, ~5000–10 000 cpm). Cells were lysed 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 (1500 rpm) at 4°C. The amount of intracellular [^{14}C]-cAMP was assessed from supernatants purified by sequential chromatography using Dowex (AG 50W-X4) and alumina columns as described before (Johnson and Salomon 1991). The amount of intracellular [^{3}H]-cAMP (CA) over the total amount of intracellular [^{3}H]-adenine (TU) multiply by 1000 was calculated to define the relative intracellular cAMP levels (CA/TU x 1000). The receptor expression (in pmol/mg of membrane proteins) of wild-type and chimeric D1R and D5R was assessed using a saturating concentration (~6 nM) of N-[*methyl*-^{3}H]-SCH23390.

7. Statistics

Binding isotherms were analyzed using the non-linear curve-fitting program to determine the equilibrium dissociation constant (K_D, expressed in nM)

and maximal binding capacity (Bmax, expressed in pmol/mg prot.) of N-[*methyl*-^{3}H]-SCH23390 assessed in saturation studies and inhibitory constants (K_I, expressed in nM) of drugs tested in competition studies. Dose-response curves to dopamine were analyzed by a four-parameter logistic equation (DeLean et al. 1978), to determine bottom or basal activity, top or maximal stimulation, EC_{50} or effective concentration that displays 50% of maximal stimulation response and slope (Hill factor) values. K_D values are expressed using the arithmetic mean ± standard error (S.E.). Prior to the statistical analysis of data, homogeneity of variances was determined using either Fmax (N = 3) or Bartlett (N ≥4) test (Sokal and Rohlf 1981). One sample *t*-test and analysis of variance (one-way ANOVA) with Newman-Keuls post test were performed to assess statistical significance between different experimental conditions. The level of significance was established at $P < 0.05$. Non-linear curve fitting and statistical analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

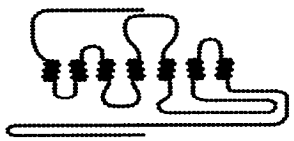
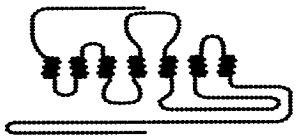
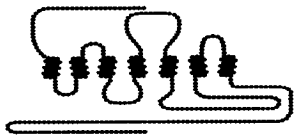
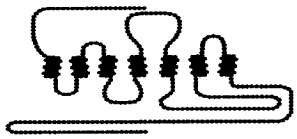
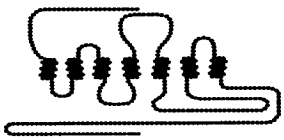
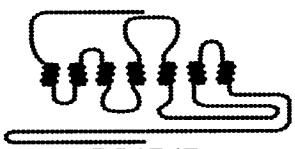
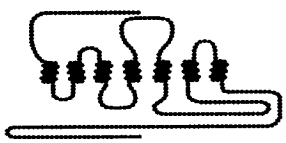
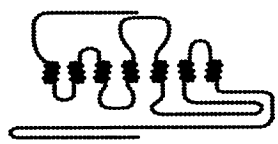
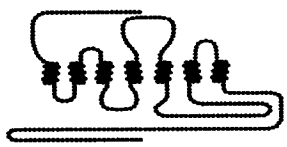
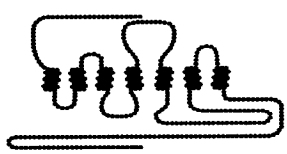
Results and Discussion

1. Ligand binding properties of the D1-like selective radioligand N-[methyl-^{3}H]SCH23390 for wild-type and chimeric receptors

The equilibrium dissociation constant (K_D) values of the radioligand N-[methyl-^{3}H]SCH23390 ([^{3}H]SCH) for wild-type and chimeric receptors obtained using saturation studies are summarized in Table 3. The similarity of K_D values between wild-type and chimeric receptors suggests that the chimeras keep their ability to bind [^{3}H]-SCH with high affinity. Additionally, these results indicate that protein folding of chimeric receptors required for [^{3}H]SCH binding is not altered significantly. Similar results were obtained with unlabeled SCH using competititon studies (data not shown). Importantly, as previously reported by our group (Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al. 2003), [^{3}H]SCH displays a lower affinity for D5 in comparison with D1. In the present study, we show that wild-type and chimeric D1 receptors exhibit a similar affinity for [^{3}H]SCH. Additionally, the [^{3}H]SCH affinity obtained with the D1 chimeras remains significantly higher than that of the wild-type D5. In an opposite manner to results obtained with wild-type and chimeric D1 receptors, all D5 chimeras (with the exception of D5-EL3A) bind [^{3}H]SCH with a higher affinity. Interestingly, these values were reminiscent of the affinity obtained with the wild-type D1. Overall, these results suggest that TM6 and EL3 residues may play a more important role in D5 than D1 with respect to shaping intramolecular

Table 3 – Equilibrium dissociation constants (K_D) and maximal binding capacity (B_{MAX}) values for binding of *N*-[*methyl*-^{3}H]SCH2339 ([^{3}H]SCH) to wild-type and chimeric D1-like receptors.

K_D (nM) and R (pmol/mg membrane protein) values are expressed as the arithmetic means ± S.E. Means are from six to eight experiments done in duplicate determinations using saturation studies. *****, $p < 0.05$ when compared with D1; **#**, $p < 0.05$ when compared with D5; **Ψ**, $p < 0.05$ when compared with D1-EL3B.

Wild type and Chimeric receptors	K_D (nM)	B_{MAX}(Pmol/mg Protein)
 D1 / D1A	**0.46** ±0.03	**15.6** ±1.65
 D1-EL3B	**0.48** ±0.04 (#)	**35.9** ±4.43 (* #)
 D1-TM6B$^{Met318/Val319}$-EL3B	**0.46** ±0.08 (#)	**28.8** ±2.83 (*)
 D1-TM6B^{Met318}-EL3B	**0.53** ±0.06	**31.3** ±3.29 (* #)
 D1-TM6B^{Val319}-EL3B	**0.44** ±0.08 (#)	**31.7** ±3.33 (* #)
 D5 / D1B	**0.72** ±0.05 (*)	**22.7** ±2.06 (*)
 D5-EL3A	**0.54** ±0.08	**5.4** ±0.43 (* # Ψ)
 D5-TM6A$^{Ile294/Leu295}$-EL3A	**0.38** ±0.03 (#)	**3.3** ±0.23 (* # Ψ)
 D5-TM6A^{Ile294}-EL3A	**0.46** ±0.04 (#)	**3.8** ±0.29 (* # Ψ)
 D5-TM6A^{Leu295}-EL3A	**0.42** ±0.03 (#)	**2.8** ±0.20 (* # Ψ)

interactions involved in SCH binding. In fact, earlier studies using D1/D2 chimeras and single-point mutations suggest that the TM7 region may participate in D1 binding to SCH (MacKenzie et al. 1993; Tomic et al. 1993; Kozell et al. 1994). However, it remains unclear whether differences in TM7 of D1 and D5 may underlie the distinct requirement of TM6 and EL3 in SCH binding. Indeed, D1 and D5 differ only by one amino acid (Leu↔Val) in the TM7 region. While further studies are required to assess the role of TM7 in the TM6/EL3-regulated intramolecular interactions of D1 and D5, we made another interesting observation about the Bmax values obtained in our saturation studies with the different chimeras. These results are described in the next section.

2. Variant TM6 and EL3 residues regulate receptor expression of the human D1 and D5 subtypes in HEK293 cells

All chimeric receptors constructed for the purpose of the present study showed a significant detectable expression ($\geq$3.5pmol/mg of protein) in HEK293 cells (Table 3). Previous studies from our lab have exposed the role of EL3 in the regulation of maximal binding capacities (B_{MAX}) of N-[methyl-^{3}H]SCH23390 in HEK293 cells expressing wild-type and chimeric D1 and D5R receptors (Iwasiow et al. 1999; Tumova et al. 2003). In agreement with these studies, our saturation studies indicate that B_{MAX} values for all chimeric D1 receptors displayed approximately 2-fold increase in comparison with wild-type D1. In contrast, B_{MAX}

values obtained with D5 chimeras showed a significant decrease (varying from 4- to 8-fold) relative to wild-type D5. Statistical analysis of our data using one-way anova suggests that D1-EL3B displays the highest cellular expression. In contrast, D5-TM6A$^{Ile294/Leu295}$-EL3A, D5-TM6A^{Ile294}-EL3A and D5-TM6A^{Leu295}-EL3A chimeras displayed B_{MAX} values that are the lowest relative to those measured with wild-type and other chimeric D1 and D5 receptors. Overall, these results strongly suggest that EL3-mediated effects on the B_{MAX} value of D1 are not modulated by the presence of the variant residues found in the exofacial end of D5-TM6. Meanwhile, the presence of the variant residues of the exofacial end of D1-TM6 seems to exacerbate the EL3-mediated effect on the B_{MAX} value of D5. These results highlight a potential role of intramolecular rearrangements in the TM6 and EL3 region in regulating the structural stability of GPCRs, which may also explain the higher and lower Bmax values measured in cells expressing chimeric D1 and D5 receptors, respectively. Indeed, studies have shown that GPCRs harboring constitutively activating mutations frequently display a lower Bmax value in comparison with their wild-type counterpart (Kjelsberg et al. 1992; Samama et al. 1993). The lower Bmax value of these CAM receptors has been attributed to a structural instability (Gether et al. 1997b; Samama et al. 1997; Rasmussen et al. 1999). This is an important issue with respect to the expression of wild-type D1 and D5 receptors in the same cellular context. Indeed, while D5 receptors exhibit a significantly higher constitutive activity than D1 receptors, the Bmax value in D5-expressing cells is not decreased in comparison with that of D1

receptors expressed in the same cellular context. Previously, we have shown that a chimeric D1 receptor harboring the full cytoplasmic tail of D5 has an increased constitutive activity (reminiscent of D5) while displaying a significantly lower Bmax value as compared with wild-type D1 and D5 receptors (Jackson et al. 2000). Interestingly, a recent study from our lab has demonstrated that a D1 chimera containing the EL3 and cytoplasmic tail of D5 remained constitutively active while displaying a similar Bmax value as compared with wild-type D1 and D5 receptors (Tumova et al. 2003). In the present study, we have shown that all the D5 chimeras harboring the EL3 region of D1 had a lower B_{MAX} values whereas, in striking contrast, all D1 chimeras containing the EL3 region of D1 exhibited higher receptor expression (Table 3). We previously reported similar findings with D1 and D5 chimeras harboring only the EL3 region (Iwasiow et al. 1999). Overall, our previous and current studies suggest that EL3 plays a potential role in the regulation of the structural stability of D1 and D5 receptor. Moreover, the present study indicates that the exofacial end of TM6 of D5 seems to reduce slightly the Bmax increase observed with D1-EL3B. In contrast, it looks like if the exofacial end of TM6 of D1 appears to exacerbate further the B_{MAX} decrease noted with D1-EL3A. Therefore, we would like to propose that EL3 of D1 and D5 contains structural determinants that control negatively and positively the structural stability of D1 and D5, respectively. Additionally, the molecular constraints imparted to D1 and D5 by the exofacial end of TM6 region have

potentially antagonistic effects on the EL3-regulated structural stability of these D1-like receptors.

3. TM6 and EL3 residues have a coordinated role in modulating the dopamine affinity of the human D1-like receptor subtypes

Competition studies were performed to investigate the role of TM6 and EL3 in coordinating the intramolecular interactions involved in the binding of agonists to D1-like receptors. For this purpose, DA (the natural endogenous agonist) and two synthetic agonists (SKF-38393 and apomorphine) were tested in competition studies using membrane preparations expressing wild-type of chimeric D1-like receptors. As shown in Table 4 and in agreement with previous studies (Sunahara et al. 1991; Tiberi et al. 1991; Tiberi and Caron 1994), DA has a higher affinity (~10-fold) for D5 in comparison with D1. Furthermore, previous radioligand binding studies in our lab indicated that an exchange of the EL3 region between wild-type D1 and D5 receptors, resulted in a partial increase and partial decrease of DA affinity of D1-EL3B and D5-EL3A, respectively, when compared with their cognate wild-type receptors (Iwasiow et al. 1999). Interestingly, an exchange of both EL3 and variant residues of the exofacial end of TM6 resulted in affinities that are similar to those observed with wild-type D1 and D5 receptors (Table 4). These data suggest that the exofacial end of TM6 plays an important role in

Table 4 – Equilibrium inhibition constants (K_I) for the binding of dopaminergic agonists to wild-type and chimeric D1-like receptors.

K_I (nM) values are expressed as arithmetic means ± S.E. of seven experiments done in duplicate determinations using competition studies. *N*-[*methyl*-^{3}H]SCH2339 was used as a radiotracer.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5; **Ψ**, $p < 0.05$ when compared with D1-EL3B; **Ω**, $p < 0.05$ when compared with D1-TM6B^{Met318}-EL3B.

Drugs / Wild-type & chimeric receptors	Dopamine	SKF-38393	Apomorphine
D1	4749 ± 561	98.3 ±10.3	452 ± 55
D1-EL3B	2697 ±99 (* #)	56.4 ± 6	332 ± 66
D1-TM6B$^{Met318/Val319}$-ELB	4167 ±872 (# Ψ Ω)	58.6 ±7.4	376 ±52
D1-TM6B^{Met318}-EL3B	5803 ±599 (Ψ)	91.3 ±12.6	356 ±58
D1-TM6B^{Val319}-EL3B	3205 ±307 (* # Ω)	44.8 ±6.6 (*)	268 ±46
D5	604 ±48 (*)	72 ±7.3	359 ±38
D5-EL3A	758 ±103 (*)	90.3 ±9.8	378 ±68
D5-TM6A$^{Ile294/Leu295}$-EL3A	482 ±94 (*)	76.3 ±13.2	213 ±47
D5-TM6A^{Ile294}-EL3A	594 ±63 (*)	72.7 ±6.3	302 ±68
D5-TM6A^{Leu295}-EL3A	729 ±102 (*)	85.4 ±17	360 ±43

controlling the EL3-mediated effect on the DA binding conformation of D1 and D5. Studies with chimeras harboring a single variant amino acid of the exofacial end of TM6 suggest that only one TM6 residue is inhibiting the EL3-mediated effect on DA affinity. Indeed, while the DA affinity measured for wild-type D1 and D1-TM6B^{Met318}-EL3B are similar; D1-TM6B^{Met318}-EL3B chimera displayed a significantly lower DA affinity when compared with D1-EL3B and D1-TM6B^{Val319}-EL3B chimeras. Thus, we suggest that the D5-TM6^{Met318} residue play a coordinated role with EL3 in the modulation of DA affinity of the D5 subtype. Reciprocal studies using chimeric D5 receptors show that the DA affinity of D5-TM6A^{Ile294}-EL3A and D5-TM6A$^{Ile294/Leu295}$-EL3A is unchanged in comparison with wild-type D5. In contrast, D5-EL3A and D5-TM6A^{Leu295}-EL3A exhibit a lower DA affinity than the one measured at wild-type D5. In spite of reaching statistical significance (when compared with wild-type D5), we propose that in a similar fashion to its D5 counterpart amino acid, the D1-TM6^{Ile294} residue plays a crucial role in controlling the EL3-mediated effect on the intramolecular interactions underlying the DA binding mechanisms of D1. Regardless of the lower discriminative property of SKF-38393 and apomorphine towards D1 and D5 (low selectivity ratio), a similar trend was also observed for these two agonists. These results reinforce our view that the variant TM6 residues and more specifically Ile^{294} of D1R and Met^{318} of D5R, coordinate the modulation of agonist affinity mediated by EL3.

4. Variant TM6 residues negatively regulate the EL3-mediated effect on antipsychotic affinity and selectivity of the human D1 and D5 receptors

Previous studies have shown that antipsychotic drugs interacting with D1-like receptors behave as inverse agonists binding (Tiberi and Caron 1994). Moreover, antipsychotic drugs bind to D5 with a lower affinity in comparison with D1 (Tiberi and Caron 1994; Charpentier et al. 1996). As our lab reported before, the TRL cassette (see "Introduction" section) potentially contain the structural determinants required for antagonist/inverse agonist binding to D1-like receptors (Iwasiow et al. 1999). Additionally, findings from our lab (Iwasiow and Tiberi, unpublished data) suggest that the EL3 domain may play a role in the binding of inverse agonists (antipsychotic drugs) and more specifically in the regulation of their selectivity towards D1 and D5. Here, we further explored the role of EL3. Specifically, we tested whether the variants amino acids of the exofacial TM6 region may also regulate the EL3-induced effect on the binding affinity of five antipsychotic drugs (Figure 6, Table 5).

Our competition studies indicate that the exchange of EL3 between wild-type D1 and D5 receptors leads to a full switch in the binding affinity of thioridazine, *cis*-flupentixol, and fluspirilene, while partially switching the affinity of thiothixene, and (+)-butaclamol (Table 5). Meanwhile, an exchange of EL3 and the two

Table 5 – Equilibrium inhibition constants (K_I) for inverse agonists binding at wild-type and chimeric D1-like receptors.

K_I (nM) values are expressed as the arithmetic means ± S.E. Means are from five to seven experiments done in duplicate determinations using competition studies. *N*-[*methyl*-^{3}H]SCH2339 was used as a radiotracer.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5; **Ψ**, $p < 0.05$ when compared with D1-EL3B.

Drugs / Wild-type & Chimeric receptors	(+) Butaclamol	*cis*-Flupentixol	Fluspirilene	Thioridazine	Thiothixene
D1	6.1 ±1.34	11.1 ±1.63	480 ±75	62 ±9	56 ±5.31
D1-EL3B	8.3 ±0.88 (#)	15.6 ±1.63	1772 ±309 (*)	196 ±30 (*)	128 ±11.88 (* #)
D1- TM6B$^{Met318/Val319}$-EL3B	8.8 ±0.81 (#)	11.8 ±1.30	1195 ±258 (#)	153 ±26 (#)	111 ±8.9 (#)
D1-TM6B^{Met318}-EL3B	9.1 ±1.41 (#)	14.5 ±1.46	1035 ±120 (#)	126 ±17 (#)	111 ±11.9 (#)
D1-TM6B^{Val319}-EL3B	8.2 ±0.74 (#)	15.8 ±1.67	1497 ±182 (*)	236 ±26 (*)	149 ±15.7 (*)
D5	32.9 ±4.06 (*)	17.9 ±2.13	2006 ±163 (*)	254 ±31 (*)	259 ±30.6 (*)
D5-EL3A	9.2 ±1.66 (#)	10.7 ±1.20	742 ±160 (# Ψ)	70 ±11 (# Ψ)	87 ±7.2 (#)
D5- TM6A$^{Ile294/Leu295}$-EL3A	13.6 ±2.9 (#)	13.1 ±2.51	864 ±95 (#)	117 ±27 (#)	88 ±3 (#)
D5-TM6A^{Ile294}-EL3A	11.4 ±1.5 (#)	9.8 ±0.96 (#)	735 ±56 (#)	141 ±47 (#)	84 ±1 (#)
D5-TM6A^{Leu295}-EL3A	14.9 ±2.06 (#)	14.6 ±1.60	798 ±152 (#)	106 ±9 (#)	108 ±2 (#)

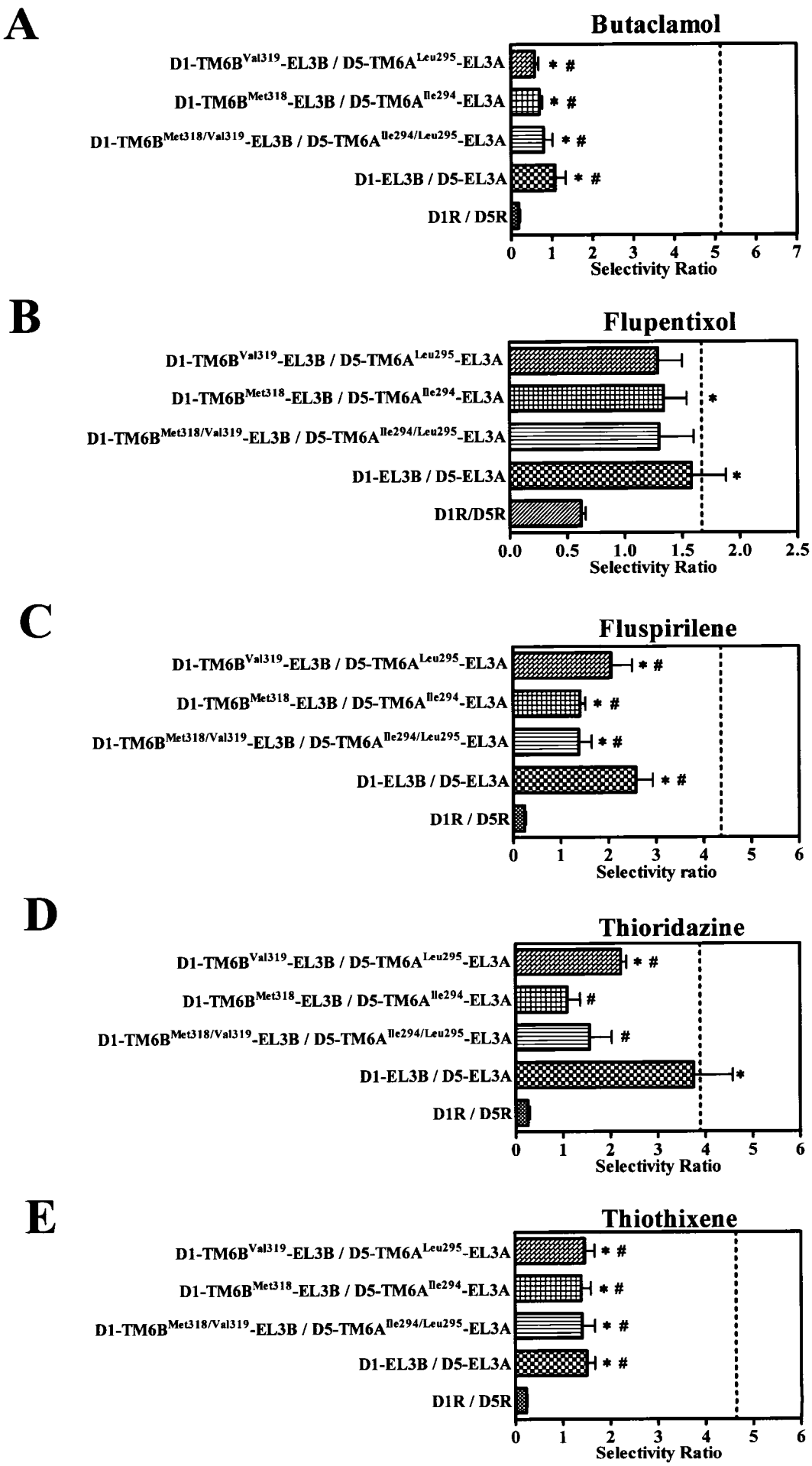
variants residues of the exofacial end of TM6 fully or partially prevented EL3-mediated effects on inverse agonist binding affinity. Overall, these results suggest that TM6 can inhibit the EL3-mediated swap of inverse agonist affinity. Interestingly and similar fashion to agonists, the TM6 inhibitory effect on EL3-induced swap of inverse agonist affinity can be attributed mostly to one of the variant residues of TM6: $TM6^{Ile294}$ of D1R and $TM6^{Met318}$ of D5R. We propose that these two amino acids regulate the EL3-mediating effect on inverse agonist binging affinity through negative intramolecular interactions.

Another important observation made with our competition studies pertains to the relationship between EL3 and TM6 on the D1/D5 selectivity ratios for different antipsychotic drugs. We investigated the role of EL3 and TM6 on the D1/D5 selectivity ratio of the antipsychotic drugs under study. The computed D1/D5 and chimeric D1 /chimeric D5 selectivity ratios of the five antipsychotic drugs are reported in Figure 7. It is worth mentioning that the dashed line shown in Figure 7 corresponds to a fully switched selectivity ratio between D1 and D5. As predicted from previous studies, antipsychotic drugs display different D1/D5 selectivity ratios. A swapping of the EL3 domain leads to a partial switch of the selectivity ratio of (+)-butaclamol, fluspirilene and thiothixene (Figure 7A, 7C, 7E). In striking contrast, the selectivity ratio of *cis*-flupentixol and thioridazine were fully switched upon the exchange of EL3 region (Figure 7B, 7D).

Figure 7 – D1R/D5R Selectivity ratios for different antipsychotic drugs (inverse agonists).

The computed D1R/D5R and chimeric D1 / chimeric D5 receptors selectivity ratios of the five tested antipsychotic drugs (all inverse agonists) are represented in **A.** (+)-Butaclamol; **B.** *cis*-Flupentixol; **C.** Fluspirilene; **D.** Thioridazine; **E.** Thiothixene. Dash line corresponds to a fully switched selectivity ratio between D1R and D5R. Data are expressed as arithmetic means ± S.E. of five to seven experiments done in duplicate determinations using competition studies.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5.


A
Butaclamol
D1-TM6B^{Val319}-EL3B / D5-TM6A^{Leu295}-EL3A
D1-TM6B^{Met318}-EL3B / D5-TM6A^{Ile294}-EL3A
D1-TM6B$^{Met318/Val319}$-EL3B / D5-TM6A$^{Ile294/Leu295}$-EL3A
D1-EL3B / D5-EL3A
D1R / D5R
* #
0 1 2 3 4 5 6 7
Selectivity Ratio
B
Flupentixol
D1-TM6B^{Val319}-EL3B / D5-TM6A^{Leu295}-EL3A
D1-TM6B^{Met318}-EL3B / D5-TM6A^{Ile294}-EL3A
D1-TM6B$^{Met318/Val319}$-EL3B / D5-TM6A$^{Ile294/Leu295}$-EL3A
D1-EL3B / D5-EL3A
D1R/D5R
*
0.0 0.5 1.0 1.5 2.0 2.5
Selectivity Ratio
C
Fluspirilene
D1-TM6B^{Val319}-EL3B / D5-TM6A^{Leu295}-EL3A
D1-TM6B^{Met318}-EL3B / D5-TM6A^{Ile294}-EL3A
D1-TM6B$^{Met318/Val319}$-EL3B / D5-TM6A$^{Ile294/Leu295}$-EL3A
D1-EL3B / D5-EL3A
D1R / D5R
* #
0 1 2 3 4 5 6
Selectivity ratio
D
Thioridazine
D1-TM6B^{Val319}-EL3B / D5-TM6A^{Leu295}-EL3A
D1-TM6B^{Met318}-EL3B / D5-TM6A^{Ile294}-EL3A
D1-TM6B$^{Met318/Val319}$-EL3B / D5-TM6A$^{Ile294/Leu295}$-EL3A
D1-EL3B / D5-EL3A
D1R / D5R
* #
#
*
0 1 2 3 4 5 6
Selectivity Ratio
E
Thiothixene
D1-TM6B^{Val319}-EL3B / D5-TM6A^{Leu295}-EL3A
D1-TM6B^{Met318}-EL3B / D5-TM6A^{Ile294}-EL3A
D1-TM6B$^{Met318/Val319}$-EL3B / D5-TM6A$^{Ile294/Leu295}$-EL3A
D1-EL3B / D5-EL3A
D1R / D5R
* #
0 1 2 3 4 5 6
Selectivity Ratio

These data thus suggest that distinct EL3-regulated structural determinants of D1 and D5 are probably required in controlling D1-like subtype-specific affinity and selectivity to different classes of heterocyclic antipscychotic drugs such as piperazine-substituted thioxanthenes (e.g. *cis*-flupentixol) and diphenylbutylpiperidines (e.g. fluspriliene). Additionally, our study using chimeras clearly indicates that the antipsychotic drugs belonging to the same chemical class do not necessarily bind to the same EL3-regulated receptor residues. This is best illustrated by results obtained with the piperazine-substituted thioxanthenes *cis*-flupentixol and thiothixene, which display full and partial switch, respectively. The demonstration of a full switch with cis-flupentixol (a piperazine-substituted thioxanthene) and thioridazine (a piperidine-substituted phenothiazine) suggests that the replacement of the nitrogen at position 10 by a carbon atom with a double bond to the side chain does not have an important role in mediating drug selectivity at D1 and D5. Similarly, the presence of a piperidine ring in the side chain (thioridazine) is not playing an important role in regulating selectivity towards D1 and D5. Instead, we propose that EL3-regulated residues mediating drug selectivity at D1 and D5 may be controlled by the bulkiness of the group at position 2 of the second benzene ring. Indeed, at position 2, thiothixene (partial switch) harbors a dimethylsufonamido ($SO_2N(CH_3)_2$) group while *cis*-flupentixol and thioridazine have trifluoromethyl (CF_3) and methylmercapto (SCH_3) groups, respectively. Globally, our results suggest that additional D1 and D5 residues (not regulated by EL3) are also important in shaping the binding

determinants for some thioxanthenes (e.g. thioridazine) and other heterocyclic compounds such as (+)-butaclamol and fluspirilene.

Interestingly, an exchange of the two variant TM6 amino acids and EL3 region reduces significantly the EL3-mediated swap of the selectivity ratios of thioridazine, and fluspirilene while a lesser or no effect was detected on the selectivity ratio of (+)-butaclamol, *cis*-flupentixol and thiothixene (Figure 7). Based on these results, we would like to propose that other structural determinants located outside of the exofacial end of TM6 and EL3 regions regulate the binding of these antipsychotic drugs. Notwithstanding this issue, our results also suggest a differential interplay between the variant residues of the exofacial end of TM6 and EL3 with respect to the regulation of the affinity and selectivity ratio of antipsychotic drugs. While our agonist binding studies highlight the functional importance of $TM6^{Ile294}$ of D1R and $TM6^{Met318}$ of D5R in regulating the EL3-induced intramolecular interactions, a different outcome is noted in our experiments with antipsychotic drugs. In fact, our data suggest that $TM6^{Leu295}$ of D1R and $TM6^{Val319}$ of D5R may also play a role in regulating the binding and selectivity ratios of inverse agonists. This is best highlighted with our studies using thioridazine (Figure 7D). Indeed, an exchange of these TM6 residues with EL3 interferes significantly with the EL3-mediated swap of the thioridazine selectivity ratio. Meanwhile, a swapping of the adjacent residues ($TM6^{Ile294}$ of D1R and $TM6^{Met318}$ of D5R) promotes a bigger inhibition of the EL3-mediated

effects (Figure 7D). Intriguingly, swapping both TM6 residues leads to a smaller inhibition of the EL3-mediated effect in comparison with the inhibition elicited by chimeras made from the exchange between $TM6^{Ile294}$ of D1R and $TM6^{Met318}$ of D5R. These results suggest that the two variant TM6 residues may exert antagonistic interactions on each other with respect to the regulation of D1-like binding conformation for thioridazine. In striking contrast to observations made with thioridazine, $TM6^{Ile294}$ of D1R and $TM6^{Met318}$ of D5R are the structural determinants of the exofacial end of TM6 that promote the negative regulation of EL3-mediated effect on the selectivity ratio of fluspirilene. Overall, these studies suggest a more complex interplay between the variant residues of the exofacial end of TM6 and EL3 in controlling the binding conformation for inverse agonists/antipsychotic drugs than anticipated. Meanwhile, one issue remains to be fully addressed with respect to the regulation of ligand binding pocket of D1 and D5. Is the spatial orientation of the D1 and D5 ligand binding determinants regulated their inactive and active conformational states?

5. Inactive and active conformational states of D1 and D5 and the regulation of ligand binding properties

Experimental data and computer simulations have helped formulating a revised and extended version of the ternary complex model for GPCRs referred to as the "two-state model" (DeLean and Lefkowitz 1980; Samama et al. 1993; Perez and

Karnik 2005). In the revised and extended version of the ternary complex model, an isomerization of the GPCR must take place prior to receptor coupling to G proteins (Samama et al. 1993). In fact, the two-state model postulates that GPCRs exist in a thermodynamic equilibrium between an inactive (R) and active (R*) state. According to the two-state model, inverse agonists bind preferentially to and stabilize R by virtue of a higher affinity for the inactive state. In contrast, agonists, by virtue of a higher affinity for the active state, bind preferentially to and stabilize R*, promoting further coupling to and activation of G proteins. Therefore, agonists and inverse agonists display lower affinity for R and R* states, respectively. Neutral ligands or antagonists have equal affinity for R and R* states and thus leave the thermodynamic equilibrium unchanged. As discussed earlier in the "Introduction", D5 exhibits a significantly higher level of constitutive activity (agonist-independent activity) than D1 (Tiberi and Caron 1994; Charpentier et al. 1996; Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al. 2003). This implies that D1 and D5 expressed in the plasma membrane adopt mostly an R and R* state, respectively. Alternatively, D1 and D5 could display similar amounts of R and R* states. If so, this particular scheme entails that these D1-like subtype-specific R and R* states must exhibit as well distinct affinity for agonists and inverse agonists in order to concur with our experimental data. Notably, we show that agonists bind to D1 and D5 with lower and higher affinities, respectively. In an opposite manner, inverse agonists display higher and lower affinity for D1 and D5, respectively. Notwithstanding the different scenarios describing the R and R*

states for D1 and D5, we believe that D1-like subtype-specific affinity for agonists and inverse agonists are also potentially modulated by the amount of R and R* found in membrane preparations as postulated by the two-state model.

This is an important issue that deserves further consideration with respect to our studies using chimeras. Indeed, in addition to structural determinants located outside of the exofacial end of TM6 and EL3 regions necessary for the full expression of D1-like subtype conformations for agonists and inverse agonists, the degree of constitutive activity of the chimeric D1-like receptors may play an important role in the modulation of ligand affinities and selectivity ratios measured in our studies. Importantly, the amount of R* expressed at the plasma membrane, and more specifically the amount of R*/G protein complex, correlates with the extent of GPCR constitutive activity. Consequently, the degree of constitutive activity of chimeras may play a role in determining the ligand affinities and selectivity ratios measured with our chimeras. In the next series of experiments we have addressed the role of TM6 and EL3 in the regulation of constitutive activity of D1-like receptors.

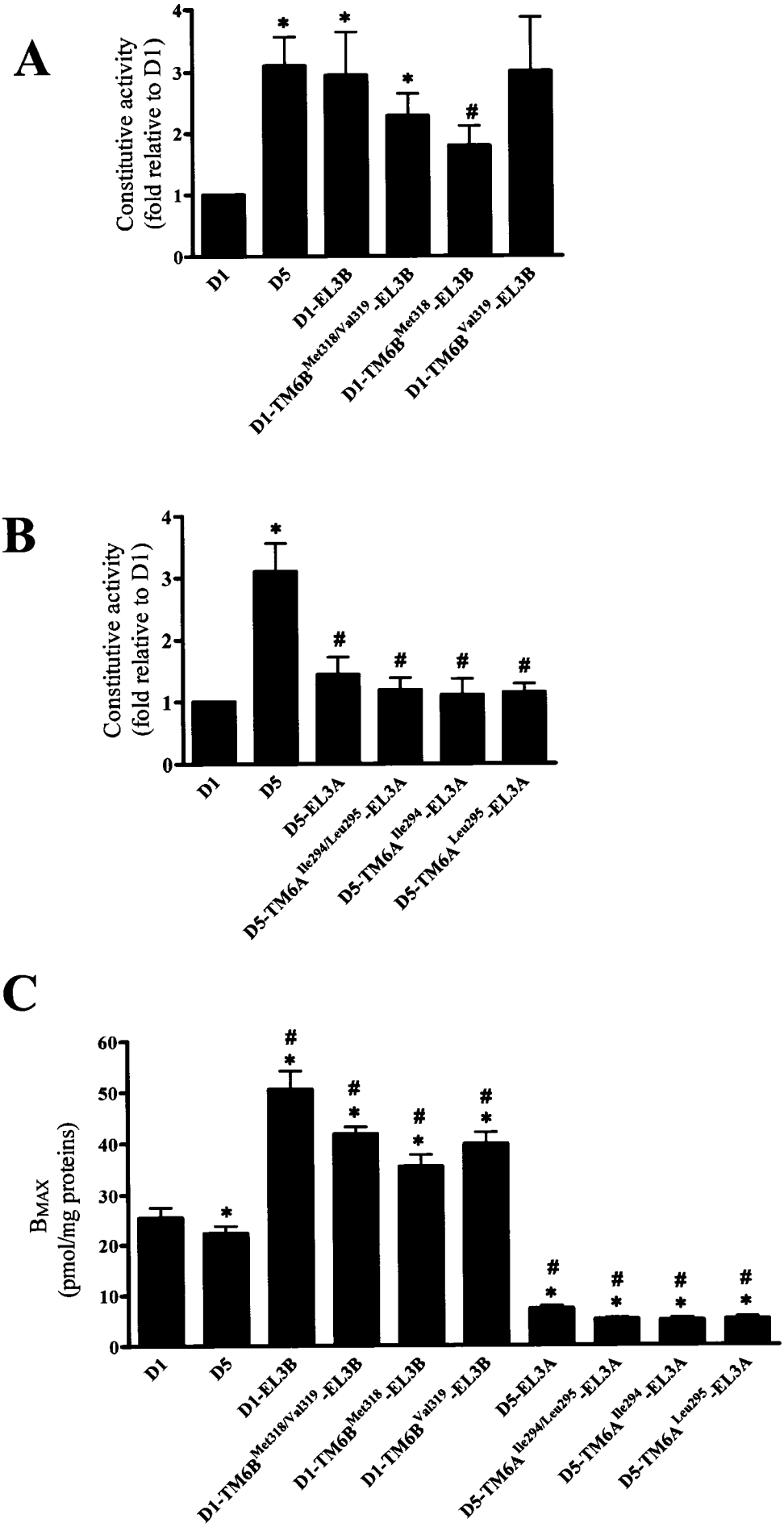
6. Variant residues of the exofacial end of TM6 and EL3 modulate the constitutive activity of the D1 and D5 receptors

To determine the role of TM6 and EL3 in agonist-independent activity (constitutive activity) of adenylyl cyclase (AC), whole cAMP assays were performed in intact HEK293 cells. We first assessed the constitutive activity of each wild-type and chimeric D1 and D5 receptors using 5 μg of DNA expression construct per transfected dish. This DNA quantity leads to the highest achievable receptor expression in HEK293 cells. Values were normalized relative to the D1 constitutive activity (Figure 8). As previously reported, we show that D5 display a higher constitutive activity. In this series of experiments, we hypothesize that a switch of D1 and D5-derived receptor domains would lead to chimeras exhibiting a constitutive activity reminiscent of their respective wild-type receptor counterpart. Intriguingly, we observe that all the D1 chimeras exhibit overall a higher constitutive activity relative to wild-type D1 (Figure 8A). In an opposite manner, all the D5 chimeras display a significantly lower constitutive activity (reminiscent of wild-type D1) when compared with the wild-type D5 (Figure 8B). However, a major drawback with this series of experiments pertains to the B_{MAX} values obtained with chimeras, which were all significantly different from the B_{MAX} values obtained with wild-type receptors. Importantly, in order to make valid conclusions about the role of TM6 and EL3 region in the regulation of D1 and D5 constitutive activity, one needs to ensure that B_{MAX} values of wild-type

Figure 8 – Constitutive activity (agonist-independent activity) of wild-type and chimeric D1-like receptors expressed in HEK293 cells.

Basal levels of AC activity were assessed in single wells of 6-well dish using whole cell cAMP assays. Data obtained using 5 μg DNA expression construct per transfected dish in the absence of DA. Values are expressed as arithmetic means ± S.E. of five experiments done in triplicate determinations and normalized relative to the D1R constitutive activity. **A.** Constitutive activity of wild-type and chimeric D1 receptors. **B.** Constitutive activity of wild-type and chimeric D5 receptors. **C.** B_{MAX} values of wild-type and chimeric D1 and D5 receptors.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5.

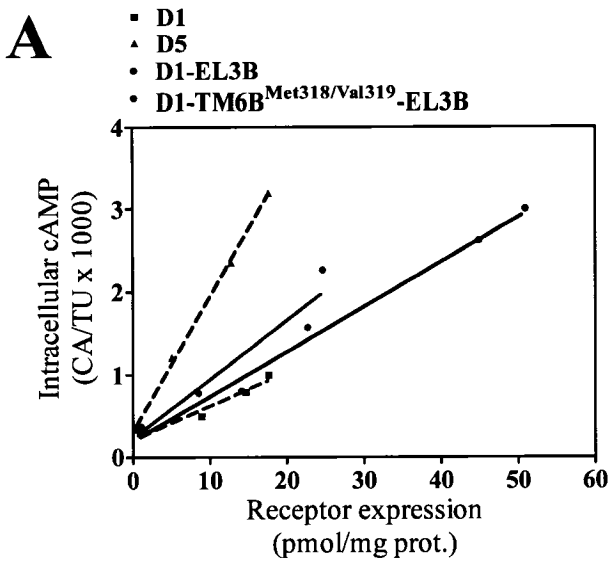

A
Constitutive activity
(fold relative to D1)
0
1
2
3
4
D1
D5
D1-EL3B
D1-TM6B Met318/Val319 -EL3B
D1-TM6B Met318 -EL3B
D1-TM6B Val319 -EL3B
B
Constitutive activity
(fold relative to D1)
0
1
2
3
4
D1
D5
D5-EL3A
D5-TM6A Ile294/Leu295 -EL3A
D5-TM6A Ile294 -EL3A
D5-TM6A Leu295 -EL3A
C
BMAX
(pmol/mg proteins)
0
10
20
30
40
50
60
D1
D5
D1-EL3B
D1-TM6B Met318/Val319 -EL3B
D1-TM6B Met318 -EL3B
D1-TM6B Val319 -EL3B
D5-EL3A
D5-TM6A Ile294/Leu295 -EL3A
D5-TM6A Ile294 -EL3A
D5-TM6A Leu295 -EL3A

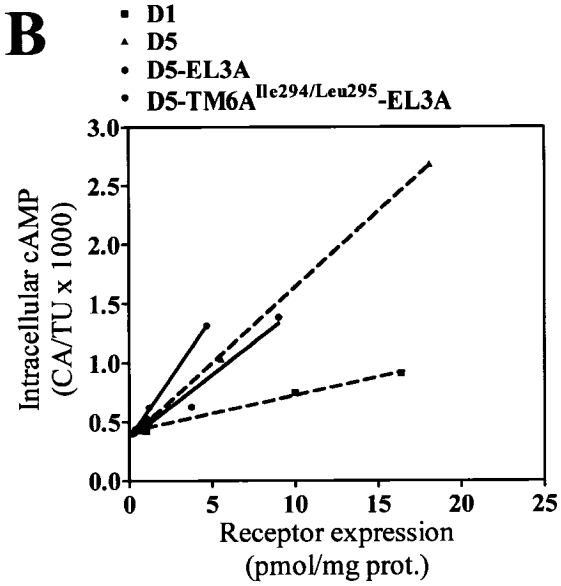
and chimeric receptors are similar. In fact, we can not conclude indisputably that EL3 alone or with TM6 modulate the constitutive activity of D1-like receptors.

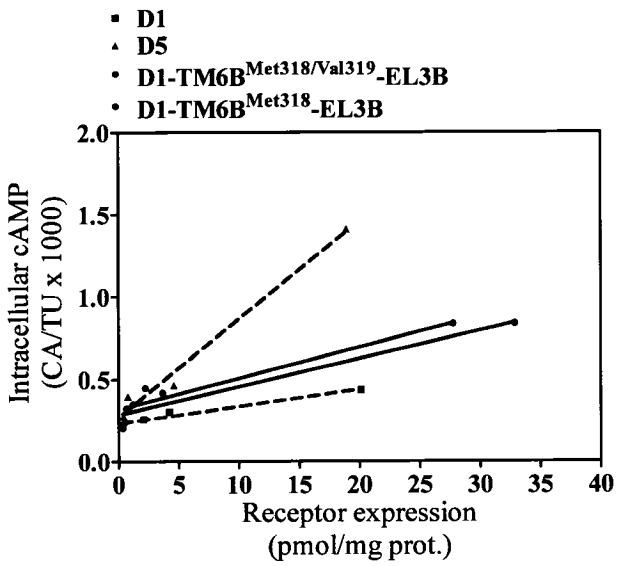
To circumvent the issue of discrepancy between Bmax values of wild-type and chimeric D1 and D5 receptors, we use a different experimental approach. This approach relied on the use of cells transfected with increasing amount of DNA (0.005, 0.05, 0.5 and 5 μg) coding for the different wild-type and chimeric receptors to obtain a range of Bmax values (receptor expression). Indeed, studies have shown that the amount of receptors expression increased proportionately with the amount of plasmid DNA utilized to transfect cells (Luttrell et al. 1993; Tiberi and Caron 1994). Maximal receptor expression for wild-type and chimeric D1-like receptors is achieved with 5 μg of plasmid DNA (data not shown). Most importantly, this approach has been used previously to demonstrate a linear relationship between the amount of receptor expression (B_{MAX}) and the extent of GPCR constitutive activity (Samama et al. 1993; Tiberi and Caron 1994; Jackson et al. 2000). The slope factor calculated from these linear curves can then serve as an indicator of constitutive activity and be used to compare constitutive activity of receptors that have different B_{MAX} values (Jackson et al. 2000). For each transfection condition, we measured the extent of constitutive activity and values were plotted as a function of receptor expression (Bmax). Curves were then best-fitted using a linear regression to determine the slope factor, an indicator of the constitutive activity. Representative examples of these experiments are shown in Figure 9. Slope factor values (constitutive activity) are summarized in Figure 10.

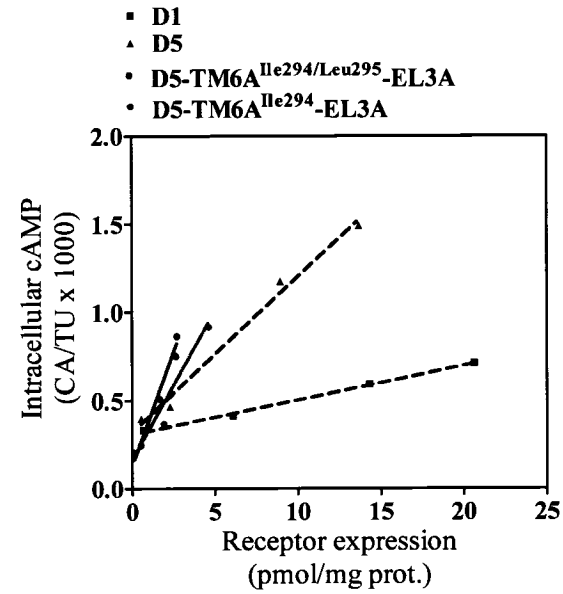
Figure 9 – Representative examples of linear relationships between B_{MAX} values and receptor constitutive activity for wild-type and chimeric D1-like receptors expressed in HEK293 cells.

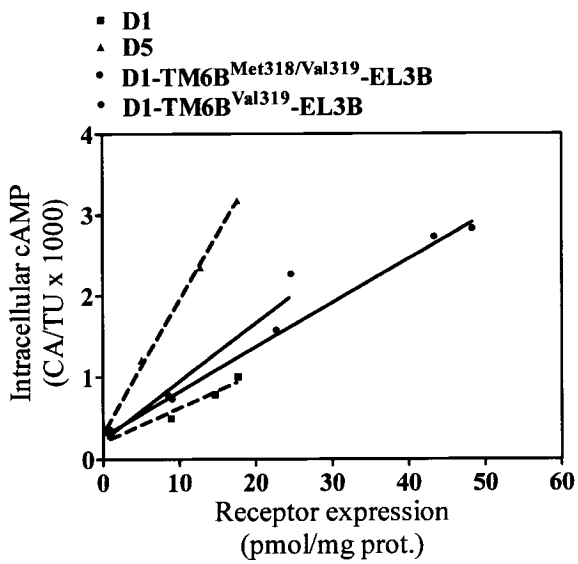
Basal intracellular levels of AC activity versus receptor expression (pmol/mg) were assessed in single wells of 6-well dish using whole cell cAMP assays. Data for linear curves obtained using increasing amount of DNA (0.005, 0.05, 0.5 and 5 μg) coding for wild-type and chimeric receptors. **A.** Linear curves of wild-type and chimeric D1 receptors. **B.** Linear curves of wild-type and chimeric D5 receptors.


A
D1
D5
D1-EL3B
D1-TM6B Met318/Val319 -EL3B
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)


B
D1
D5
D5-EL3A
D5-TM6A Ile294/Leu295 -EL3A
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)


D1
D5
D1-TM6B Met318/Val319 -EL3B
D1-TM6B Met318 -EL3B
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)


D1
D5
D5-TM6A Ile294/Leu295 -EL3A
D5-TM6A Ile294 -EL3A
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)


D1
D5
D1-TM6B Met318/Val319 -EL3B
D1-TM6B Val319 -EL3B
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)

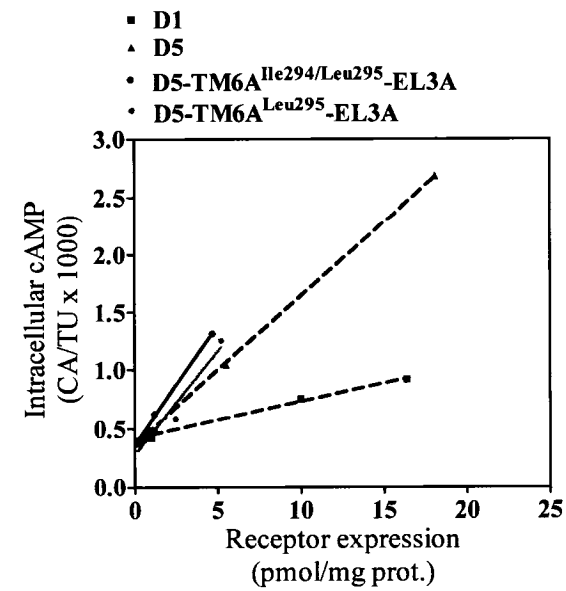
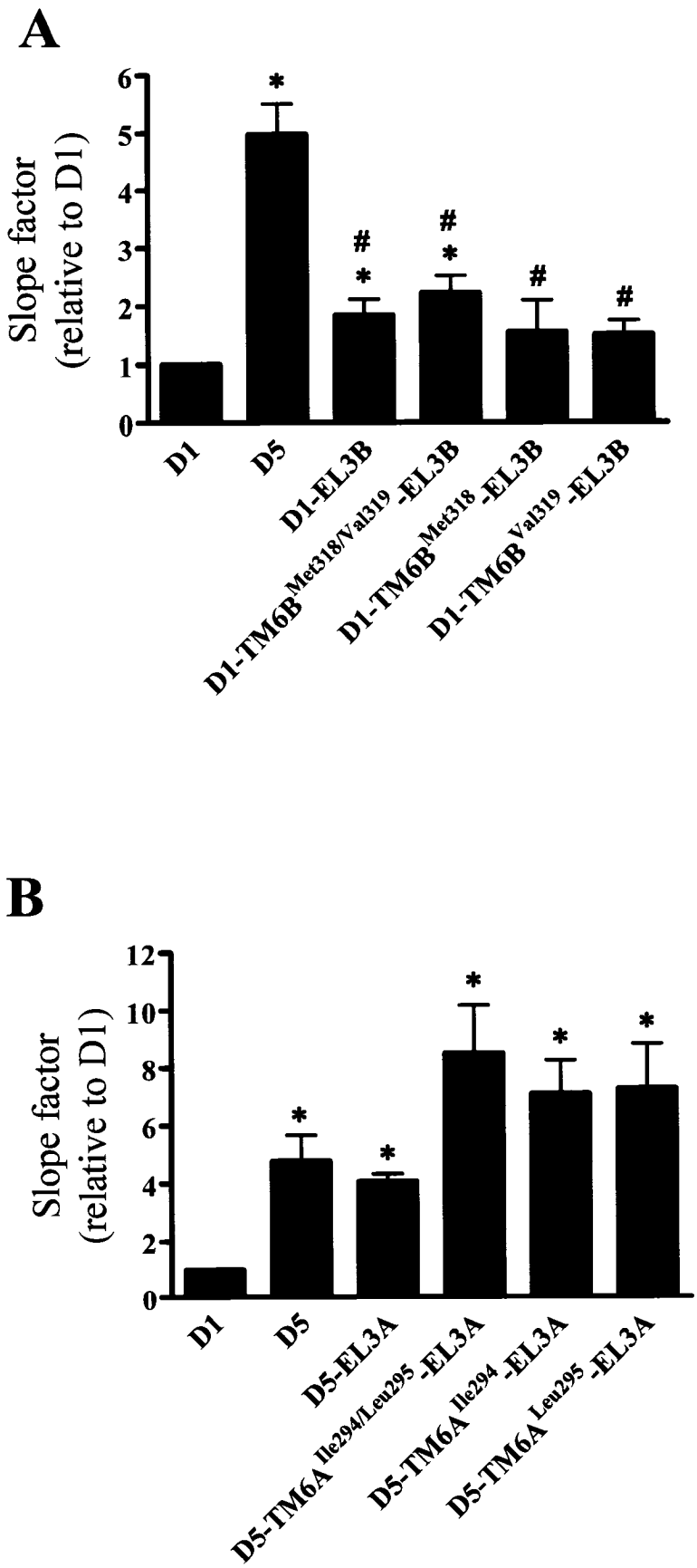

D1
D5
D5-TM6A Ile294/Leu295 -EL3A
D5-TM6A Leu295 -EL3A
Intracellular cAMP (CA/TU x 1000)
Receptor expression (pmol/mg prot.)

Figure 10 – The slope factor values (indicator of constitutive activity) of wild-type and chimeric D1-like receptors expressed in HEK293 cells.

Best-fitted values for the slope factor were obtained from linear regression of the individual curves and expressed relative to D1. Data are shown as the arithmetic means ±S. E. of five experiments. **A.** Constitutive activity of wild-type and chimeric D1 receptors. **B.** Constitutive activity of wild-type and chimeric D5 receptors.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5.


A
Slope factor
(relative to D1)
0
1
2
3
4
5
6
*
*
#
*
#
#
#
D1
D5
D1-EL3B
D1-TM6B Met318/Val319 -EL3B
D1-TM6B Met318 -EL3B
D1-TM6B Val319 -EL3B
B
Slope factor
(relative to D1)
0
2
4
6
8
10
12
*
*
*
*
*
D1
D5
D5-EL3A
D5-TM6A Ile294/Leu295 -EL3A
D5-TM6A Ile294 -EL3A
D5-TM6A Leu295 -EL3A

Importantly, in agreement with previous studies and results shown in Figure 8, we observe that D5R exhibits a higher (~5-fold) constitutive activity than D1R. Previous findings from our lab showed that a swapping of the EL3 region led to a partial modulation of the agonist independent activity of D1 and D5 (Iwasiow et al. 1999). Similar conclusions can be drawn from our experiments (Figure 10). Additionally, we observe that the reciprocal exchange of the two variant residues of the exofacial end of TM6 along with the EL3 region modulate differentially the constitutive activity of chimeras when compared with their respective wild-type parent (Figure 9 and 10).

While our data indicate that a D1 chimera harboring the EL3 region (D1-EL3B) exhibit a significantly higher constitutive activity than wild-type D1 (~2-fold), the swap of one or both of the variant residues of the exofacial end of TM6 along with the EL3 region did not alter significantly the level of receptor constitutive activity in comparison with D1-EL3B (Figure 9). The higher constitutive activity of D1-EL3B would be consistent with an increase in the amount of R* of this chimera and thus may also explain the higher and lower affinity of D1-EL3B for agonists and inverse agonists, respectively (Table 4 and 5). However, the fact that switching TM6 residues did not alter significantly the constitutive activity of D1-EL3A, and presumably the amount of R* for this chimera, we can assume that the TM6-induced inhibition of EL3-mediated effects on ligand affinities of D1-TM6B chimeras (Table 4 and 5) are not explained by a decreased amount of R* but rather by a TM6-induced modulation of the EL3-

mediated spatial orientation of residues involved in ligand binding. Similar conclusions can also be drawn from our studies using D5 chimeras. Indeed, D5-TM6A$^{Ile294/Val295}$-EL3A exhibits a higher constitutive activity than wild-type D5 and D5-EL3A. Interestingly, D5-TM6A^{Ile294}-EL3A and D5-TM6A^{Val295}-EL3A display as well a higher constitutive activity in comparison with wild-type D5 and D5-EL3A but not significantly different from D5-TM6A$^{Ile294/Val295}$-EL3A (Figure 10B). Additionally, these results suggest that in the context of D5-TM6A$^{Ile294/Val295}$-EL3A, as in the case of the selectivity ratio of thioridazine, effects mediated by Ile^{294} and Val^{295} are not additive but antagonistic. Regarding the impact of constitutive activity and increased amount of R* for D5-TM6 chimeras on ligand binding properties, we would like to propose that the TM6-induced modulation of the agonist and inverse agonist affinities of D5-EL3B (Table 4 and 5) is mostly explained by a regulation of the EL3-induced effect on the spatial orientation of ligand binding determinants within the R* conformation of the chimeras rather than by the greater propensity of these TM6 chimeras to adopt the R* state. However, the greater propensity of these chimeras to adopt the R* conformation suggest a potential role of TM6 and EL3 in the regulation of DA-promoted receptor coupling to Gs proteins and activation of AC. We have addressed this issue in the next series of experiments using dose-response curves for DA in intact cells.

7. Variant TM6 residues with EL3 domain are importantly involved in the D1 and D5 receptor coupling properties

Some of the molecular determinants involved in D1-like subtype specific agonist-mediated G protein coupling properties have been identified in our lab previously (Iwasiow et al. 1999; Jackson et al. 2000). Importantly, studies from our lab and others indicate that the cytoplasmic tail plays an important role in the activation of G proteins and AC (Iwasiow et al. 1999; Demchyshyn et al. 2000; Jackson et al. 2000). Recently, our lab showed that EL3 and CT play a coordinated role in modulating agonist-mediated G protein coupling properties (Tumova et al. 2003). To examine the role of variant amino acids of the exofacial end of TM6 and EL3 region in controlling DA potency, dose-response curves were done in HEK293 cells expressing wild-type and chimeric receptors at similar Bmax values (~3-4 pmol/mg protein). Averaged curves from raw and normalized data were analyzed with GraphPad Prism (v 4.03) nonlinear curve fitting program using unconstrained and constrained parameters (Figure 11). Best-fitted parameters derived from dose-response curves are summarized in Table 6. In agreement with previous studies (Tiberi and Caron 1994), DA potency (as indexed by EC_{50} values) is ~10-fold higher at the wild-type D5 receptors in comparison with the wild-type D1 receptors (Figure11 and Table 6). Overall, our results demonstrate that a swapping of the EL3 region lead to a significant increase in DA potency (i.e. decreased EC_{50} value) of D1 chimeras in comparison with wild-type D1

Figure 11 – Dose-response curves of DA for AC stimulation by wild-type and chimeric D1-like receptors expressed in HEK293 cells.

Dose-response curves were done in HEK293 cells expressing wild-type and chimeric D1 and D5 receptors at similar B_{MAX} values (~3-4 pmol/mg protein). AC stimulation levels for each condition were measured in single wells of a 12-well dish with increasing concentration of DA. Each point represents the arithmetic means ± S.E. form data obtained of five experiments done in triplicate determinations. Averaged curves from raw and normalized data were analyzed with GraphPad Prism (v 4.03) nonlinear curve fitting program. **A.** Averaged curves of raw and normalized data of DA for AC stimulation by wild-type D1R and D5R. **B.** Averaged curves of raw and normalized data of DA for AC stimulation by chimeric D1 receptors. **C.** Averaged curves of raw and normalized data of DA for AC stimulation by chimeric D5 receptors.

Red and blue dashlines represent curves for wild-type D1 and D5, respectively.

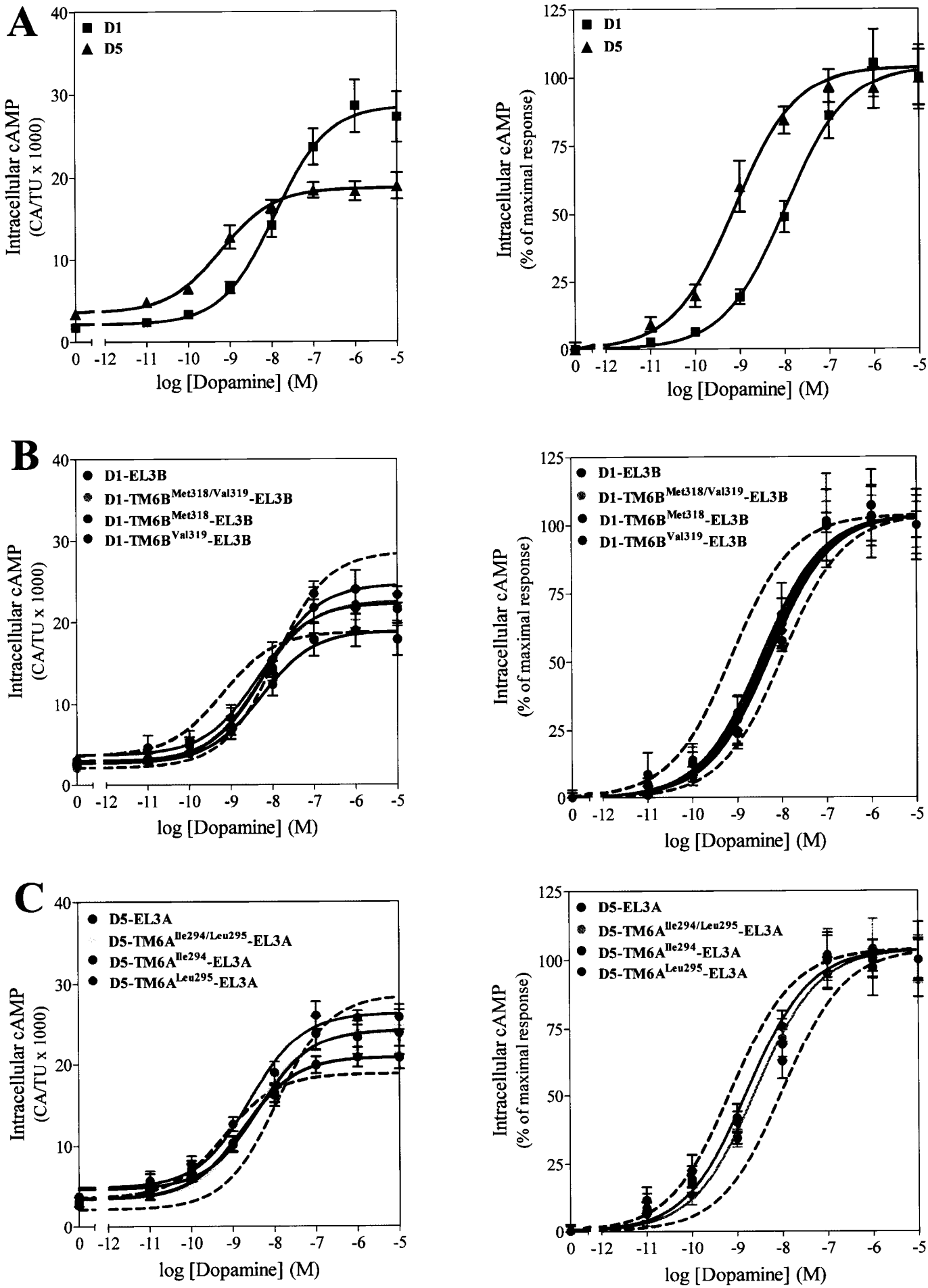

A
D1
D5
Intracellular cAMP (CA/TU x 1000)
log [Dopamine] (M)
Intracellular cAMP (% of maximal response)
B
D1-EL3B
D1-TM6B$^{Met318/Val319}$-EL3B
D1-TM6B^{Met318}-EL3B
D1-TM6B^{Val319}-EL3B
C
D5-EL3A
D5-TM6A$^{Ile294/Leu295}$-EL3A
D5-TM6A^{Ile294}-EL3A
D5-TM6A^{Leu295}-EL3A

Table 6 – DA potency (EC_{50} values) and DA-mediated maximal activation of AC in HEK293 cells expressing wild-type and chimeric D1-like receptors.

Best-fitted parameters (± S.E.) for EC_{50} and DA-mediated maximal stimulation of AC were derived from averaged dose-response curves (n=5) and analyzed with GraphPad Prism (v 4.03) nonlinear curve fitting program using unconstrained and constrained parameters. EC_{50} ratio were obtained from individual experiments and expressed relative to DA potency elicited by D1R. Values are the arithmetic means ± S.E. of five experiments.

*, $p < 0.05$ when compared with D1; #, $p < 0.05$ when compared with D5.

Wild-type and Chimeric D1-like receptors	EC_{50} [95% confidence interval]	EC_{50} Ratio (Relative to D1R)	Maximal Activation	B_{MAX} (pmol/mg)
D1	10.2 [7.36-1.42]	1	28.6 ±0.58	4.70 ±0.54
D1-EL3B	4.51 [2.72-7.48] (* #)	2.34 ±0.39	18.9 ±0.42 (*)	3.42 ±0.57
D1- $TM6B^{Met318/Val319}$-EL3B	3.91 [2.45-6.24] (* #)	3.31 ±1.37	22.5 ±0.64 (* #)	3.45 ±0.17
D1-$TM6B^{Met318}$-EL3B	5.37 [3.02-9.52] (* #)	1.43 ±0.23	24.6 ±0.80 (* #)	3.75 ±0.12
D1-$TM6B^{Val319}$-EL3B	3.45 [2.09-5.69] (* #)	2.53 ±0.47	22.3 ±0.58 (* #)	3.16 ±0.22
D5	0.75 [0.50-1.13] (* #)	10.86 ±1.21	18.7 ±0.23 (*)	4.09 ±0.30
D5-EL3A	1.71 [1.17-2.51] (* #)	5.67 ±0.97	20.8 ±0.40 (* #)	3.27 ±0.24
D5- $TM6A^{Ile294/Leu295}$-EL3A	2.61 [1.76-3.87] (* #)	4.43 ±0.98	24.1 ±0.50 (* #)	2.79 ±0.33
D5-$TM6A^{Ile294}$-EL3A	3.20 [1.62-6.28] (* #)	3.65 ±1.86	24.3 ±0.85 (* #)	2.84 ±0.17
D5-$TM6A^{Leu295}$-EL3A	2.22 [1.37-3.57] (* #)	4.23 ±1.14	26.4 ±0.70 (* #)	3.40 ±0.25

(Figure 11). A similar increase was also observed with D1-ELB chimeras harboring the variant residues of D5-TM6. Notably, TM6 does not change significantly the EL3-induced increase of DA potency observed in cells expressing D1-EL3B chimera. Additionally, the increase of DA potency measured in cells expressing the D1 chimeras was significantly different from DA potency at wild-type D5. Therefore,the DA potency measured at the different D1 chimeras is partially switched in comparison with wild-type receptors.

In a similar fashion to D1 chimeras, D5 chimeras all exhibited a partial switch in DA potency when compared with wild-type receptors (Figure 11 and Table 6). However, in contrast to D1 chimeras, the partial switch mediates a significant decrease of DA potency (i.e. increased EC_{50} value) in comparison to wild-type D5 (Figure 11 and Table 6). Additionally, the partial switch in DA potency observed with D5 chimeras harboring TM6 residues and EL3 region was not altered in comparison with D5-EL3A. Overall, our data suggest that TM6 does not participate in regulating the D1 and D5 coupling to Gs proteins.

Furthermore, we investigated the DA-mediated maximal stimulation of AC in wild-type and chimeric receptors using the best-fitted maximal response values obtained from dose-response curves using the raw data (Figure 11, right panels). Our results show that DA mediate a higher maximal activation of AC in HEK293 cells expressing the wild-type D1 as compared with cell expressing wild-type D5 (Figure 11 and Table 6). These results are in agreement with previous studies from

our group (Tiberi and Caron 1994; Iwasiow et al. 1999; Jackson et al. 2000). Notably, we also confirmed that EL3 is the receptor region that regulates the phenotypic expression of the D1-like subtype-specific DA-mediated maximal activation of AC (Figure 11). Indeed, cells expressing D1-EL3B display a DA-mediated maximal activation of AC that is significantly lower than that measured in cells expressing wild-type D1 (Figure 11 and Table 6). In fact, the DA-mediated maximal activation in cells harboring D1-EL3B is similar to cells expressing wild-type D5. Interestingly, the decrease in DA-mediated maximal activation of AC observed in cells expressing D1-EL3B was partially blocked by the presence of TM6 residues found in D5 (Figure 11 and Table 6). Data obtained with chimeras harboring one of the variant residues of the exofacial end of TM6 indicate that intramolecular interactions underlying DA-mediated maximal activation are potentially regulated in an antagonistic manner by Met^{318} and Val^{319}. Indeed, the increase in DA-mediated maximal activation observed with D1-TM6B^{Met318}-EL3B and D1-TM6B-^{Val319}-EL3B was not augmented in an additive or synergistic manner when compared to cells expressing D1-TM6B$^{Met318/\ Val319}$-EL3B (Table 6). A somewhat different picture was observed with D5 chimeras. Cells expressing D5-EL3A exhibited a DA-mediated maximal activation of AC that significantly increased relative to cells harboring wild-type D5 (Figure 11 and Table 6). Meanwhile, the DA-mediated maximal activation of AC in cells expressing D5-EL3A remained lower in comparison to cells expressing wild-type D1. In contrast to D1-TM6 chimeras, D5-EL3A chimeras harboring the variant residues of the

exofacial end of the TM6 of D1, display somehow an increase in the DA-mediated maximal activation of AC. On one hand, these results suggest that TM6 may be involved in shaping the optimal D1 conformation used in DA-mediated maximal activation of AC. One the other hand, our studies also indicate that the two variants residues of the exofacial end of TM6 region of D1 are exerting on each other interfering or antagonistic effects.

Overall, these studies suggest that EL3 plays a role in shaping the intramolecular interactions required for G protein coupling and AC activation. Moreover, we show that a potential interplay may exist between the exofacial end of TM6 and EL3 in modulating the G protein coupling properties of D1-like receptors. However, this interplay appears to play a less significant role in D1-like receptor/G protein coupling than D1-like receptor binding. Notably, we cannot rule out that cAMP results obtained in intact cells may also be explained by differences in the desensitization and internalization mechanisms of wild-type and chimeric receptors.

8. Studies with D1-like chimeras underscore a role of the exofacial end of TM6 region and EL3 in controlling the activation of rhodopsin-like receptors

Classically, structure-activity relationships have supported the notion that extracellular domains of GPCRs are involved only in the binding to peptides (e.g. opioids, neurokinins) and hormones (e.g. angiotensin, FSH, LH) while having no major role in the binding to small and diffusible ligands such as catecholamines (Gether 2000). However, besides its obvious function in the architecture of GPCRs, recent studies clearly indicate that EL3 is not just an inert peptide linker between the TM6 and TM7 regions but it also regulates agonist binding and selectivity as well as receptor activation (Lawson and Wheatley 2004). In fact, mutations near the junction of the TM6 and EL3 resulted in the increased agonist affinity and constitutive activation of all five muscarinic acetylcholine receptor subtypes (Spalding et al. 1995; Ford et al. 2002). Additionally, studies using cysteine modification approaches and fluorophore-labeled purified wild-type and constitutively active mutant (CAM) β_2AR have shown that conformational changes involving a motion of the cytoplasmic side of TM6 is shared by CAM-β_2AR and agonist-bound β_2AR and CAM (Gether et al. 1997a; Javitch et al. 1997; Rasmussen et al. 1999; Ghanouni et al. 2001; Jensen et al. 2001). Mutagenesis studies using rhodopsin and β_2AR have shown that proline residues of TM6 play a key role in the TM6 movement and molecular rearrangements following activation

of these GPCRs (Farrens et al. 1996; Gether et al. 1997a). Meanwhile, the role of the exofacial end of TM6 and EL3 in the motion of the cytoplasmic end of TM6 during receptor activation has yet to be investigated using fluorophore-labeled purified receptors. Data obtained with our chimeric D1-like receptors potentially support a coordinated role of the exofacial end of TM6 and EL3 in the regulation of the movement of the cytoplasmic side of TM6 following constitutive activation and agonist-induced activation of rhodopsin-like GPCRs such as the D1 and D5 dopaminergic receptors. Further studies using fluorophore-labeled purified wild-type and chimeric D1 and D5 receptors may help addressing this issue. However, these experimental approaches are not currently used in our lab.

9. Role of the exofacial end of TM6 and EL3 in the regulating the formation of the agonist and inverse agonist binding pocket regions of D1 and D5 receptors?

Interestingly, the mapping of receptor domains underlying the ligand binding selectivity of delta-opioid receptor indicated that a region containing TM6 and EL3 is absolutely critical for this selectivity (Meng et al. 1996). Moreover, site-directed mutagenesis studies investigating the molecular mechanisms regulating ligand recognition of the human delta-opioid receptor showed that an exchange of the EL3 of this receptor with that of the human mu-opioid receptor caused a dramatic decrease in the affinity of some of the delta-selective agonists.

These data indicate that EL3 is an important domain in the selectivity of delta-opioid agonists (Varga et al. 1996). Likewise, a study investigating the structural requirements of receptor-ligand interactions of the human neuropeptide Y receptor Y1 (NPY1) has demonstrated that a residue located at the junction between TM6 and EL3, controls peptide binding (Sjodin et al. 2006). Most importantly, our work shows for the first time that a similar interplay between the exofacial end of TM6 and EL3 may also play a role in the binding and selectivity of agonists and inverse agonists of non-peptidergic receptors. However, while residues within the extracellular domains have been shown to participate directly in the binding to peptides and hormones (Gether 2000), it is unclear whether residues found in the exofacial end of TM6 and/or EL3 serve similar function in the binding of D1 and D5 to agonists and inverse agonists. Earlier studies have shown that mutations within the TM core regions altered the binding of ligands to D1 receptors (Pollock et al. 1992; Tomic et al. 1993). In fact, Ser^{198} and Ser^{199} located in TM5 region of D1 (also conserved in D5) lead to a loss of affinity for agonists and inverse agonists/antagonists (Pollock et al. 1992; Tomic et al. 1993). In addition, mutations of residues found in TM2 (Asp^{70} and Lys^{81}), TM3 (Cys^{106} and Ser^{107}), TM6 (Cys^{283}) and TM7 (Phe^{319} and Phe^{321}) of the D1 receptor (also conserved in D5) have been shown to decrease affinity of DA (TM2, TM3, TM6) and SCH (TM2, TM3, TM6 and $TM7^{Tyr321}$) (Tomic et al. 1993). It remains to be clearly established whether these residues are part of the binding pocket for dopaminergic compounds. If so, because of the location of these residues within the TM core

regions, it is difficult to imagine that residues found in the exofacial end of TM6 and EL3 would also participate directly in a binding pocket arising from TM core residues. However, it possible that the TM core residues regulate the overall conformation of the exofacial end of TM6 and EL3 thereby exposing within these regions the residues leading to the formation of a binding pocket for dopaminergic ligands. In a similar fashion, the exofacial end of TM6 and EL3 regions may shape the orientation of the TM core residues involved in the optimal binding to dopaminergic ligands.

Recent studies investigating the molecular mechanisms of agonist binding to the β2AR and neurokinin A receptor suggest that agonist binding is a sequential or multistep process (Palanche et al. 2001; Kobilka 2004; Liapakis et al. 2004; Swaminath et al. 2004). In fact, the sequential agonist binding model proposes that the agonist binds to and stabilize a series of intermediate conformational states of the GPCR, which display distinct cellular signaling properties (Palanche et al. 2001; Kobilka 2004; Liapakis et al. 2004; Swaminath et al. 2004). Perhaps, in their unliganded states, the exofacial end of TM6 and EL3 of D1-like receptors allow the formation of a minimal low-affinity binding site, facilitating interactions between the receptor and a few structural characteristics of DA (e.g. aromatic ring or catechol hydroxyls). The interaction of DA with the low-affinity binding site shaped by the TM6 and EL3 of the D1 and D5 may then promote further conformational transitions of D1 and D5. The new D1-like conformational transitions may then allow more structural characteristics of DA to bind to

different molecular determinants within the D1 and D5 receptors (e.g. TM core residues), thereby stabilizing another D1-like receptor conformational change culminating in the activation of G proteins. Meanwhile, it is currently unknown whether a similar multistep process takes place in the inverse agonist binding. Further studies using purified fluorophore-labeled receptor and cysteine modifications may help addressing this issue.

Conclusion

The lack of drugs displaying DA receptor-subtype selectivity has made it difficult to investigate the physiological relevance of the two D1-like receptor subtypes, as well as to understand the underlying basis of therapeutic efficacy and side effects of dopaminergic drugs. As mentioned before, the primary sequence of the D1R and D5R share ~60% identity overall and a high degree of amino acid similarity (~82%) in their transmembrane spanning domains (Jarvie and Caron 1993). Both D1R and D5R have been shown to activate AC, although, D5R has a higher constitutive activity (agonist-independent activity) but a lower DA-mediated maximal activation of AC (agonist-dependent activity) in comparison with D1R. Other significant differences between these two D1-like subtypes include the higher affinity for agonists exhibited by the D5R, and the higher affinity for inverse agonists exhibited by the D1R (Sunahara et al. 1991; Tiberi et al. 1991; Tiberi and Caron 1994). Therefore, one can take advantage of these structural similarities to develop dopaminergic compounds displaying a more D1-like pharmacology while differences in the amino acid sequence found within the two D1-like receptors may shed light on the structural determinants that could potentially help designing drugs binding selectivity to D1R or D5R or modulating in a subtype-specific fashion their agonist-independent and agonist-dependent G protein coupling properties.

In an attempt to identify the structural determinants responsible for the subtype-specific binding affinity and activation properties of D1-like receptors, studies in our lab have demonstrated that distinct domains/residues can regulate

the unique and distinguishable pharmacological and functional properties of the D1R and D5R. Through the use of chimera/mutations approaches, our lab has shown that the terminal receptor locus (TRL); a region including TM6, EL3, TM7 and CT; plays an important role in receptor expression (Bmax) as well as the phenotypic expression of agonist affinity and potency, constitutive activity, DA-mediated G protein activation and AC stimulation (Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al. 2003; Tumova et al. 2004).

In the present study, I have used a chimeric approach to investigate further the structural relationships that potentially exist between the exofacial end of TM6 region and EL3 in regulating ligand binding and G protein coupling properties of the human D1 and D5 receptors. Specifically, I studied whether one or two of the variants residues found in the exofacial end of TM6 residues (Ile^{294}/Leu^{295} in D1R and Met^{318}/Val^{319} in D5R) play a role in the EL3-mediated regulation of D1 and D5 functionality. Importantly, my work has elucidated one of the potential structural mechanisms explaining why our group had not previously detected any effect on the antipsychotic drug affinity and selectivity using TRL chimeras (Iwasiow et al. 1999). My results clearly show that the two variant residues of the exofacial end of TM6 (with a more predominant role of $D1^{Ile294}$ and $D5^{Met318}$), in the context of a full TRL swap, blocked the EL3-mediated effect on inverse agonist affinity and selectivity. Similar effects of TM6 were also detected on EL3-mediated regulation of agonist affinity.

Additionally, my studies suggest that the EL3 of D5 allows this receptor to be expressed at a level similar to that found for D1 in the same cellular environment. This is an important issue since D5 displays a significantly higher constitutive activity. GPCR constitutive activity is often associated with structural instability (lower Bmax values) in comparison with GPCRs exhibiting little or no constitutive activity (e.g. D1). Therefore, EL3 may contain the molecular determinants that impart greater structural stability to D5. Importantly, the exofacial end of TM6 may modulate the extent of EL3-mediated structural stability of D5. Meanwhile, my work has also demonstrated that a molecular interplay between the exofacial end of TM6 and EL3 regions potentially regulate the agonist-independent and agonist-dependent G protein coupling properties of D1 and D5 receptors.

Finally, I believe that studies investigating the contribution of TM6/EL3 structure-activity relationships in the phenotypic expression of D1-like subtype-specific ligand binding and G protein coupling properties may prove useful in the design of novel D1 and D5-selective dopaminergic drugs for the treatment of pathologies displaying compromised D1-like receptor functionality.

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