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Uncovering the Molecular Interplays of Dopamine D1-like Receptor Activation

by

Katerina Tumova

This thesis is submitted as a partial fulfillment of the M.Sc. program in Neuroscience

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Abstract

D1-like dopaminergic receptor family consists of two heptahelical G protein-coupled receptors (GPCRs), termed D1A (or D1) and D1B (or D5). In this study, we have used a functional complementation of chimeric D1-like receptors to address the molecular interplay between the third extracellular loop (EL3) and the cytoplasmic tail (CT) in coordinating the functional properties of D1-like receptors expressed in transfected human embryonic kidney 293 (HEK 293) cells. Our results indicate that EL3 and CT participate in interfering intramolecular interactions that regulate the total functional receptor number and the agonist-mediated G protein coupling properties of the D1-like receptors in HEK 293 cells. In contrast, the agonist-independent activity of the D1-like receptors appears to be regulated solely by the sequences of the CT. In addition, we have identified the proximal region of CT, known as the fourth intracellular loop (IL4), as the pivotal domain underlying the CT-induced properties of the D1-like receptors. Overall, our study provides an insight into the molecular basis of D1-like receptor activation with possible implications for entire field of GPCR signaling.

Table of Contents

Abstract ii

Table of Contents iii

List of Tables vi

List of Figures vii

List of Abbreviations viii

Acknowledgements xi

Thesis Format xii

CHAPTER 1: GENERAL INTRODUCTION 1

1.1 G Protein-Coupled Receptors (GPCRs) 2

1.1.1 Functional Features of GPCRs 2

1.1.2 Signaling via Heterotrimeric G Proteins 4

1.1.3 Molecular Mechanism of GPCR Activation and G Protein Coupling 6

1.1.4 GPCR Desensitization and Post-Translational Modification 7

1.2 Models of GPCR Activation 9

1.2.1 The Ternary Complex Model (TCM) 9

1.2.2 The Allosteric (Extended) TCM 9

1.2.3 The Multistate Model 10

1.3 Constitutively Active Receptors 12

1.3.1 General Features of Constitutively Active Receptors 12

1.3.2 Constitutively Active Receptors in Disease and Health 13

1.4 The Dopaminergic System 15

1.4.1 Function and Innervation of the Dopaminergic System 15

1.4.2 Classification of Dopamine Receptors as GPCRs 16

1.4.3 Dopamine Receptor Subtypes and Signal Transduction Mechanisms 17

1.4.4 Pharmacology and Amino Acid/ Genomic Organization of Dopamine Receptor Subtypes 19

1.4.5 Distribution of Dopamine Receptors 22

1.4.6 Genetic Approaches to the Study of Dopamine Receptor Subtypes ... 23

1.4.7 Structure-Function Analysis of Dopamine Receptor Signaling 28

1.5 Focus on D1-like Receptors 31

1.5.1 Pathophysiological relevance of D1-like Receptors 31

1.5.2 Dopamine D1B Receptor – A Naturally Occurring Constitutively Active Receptor? 32

1.6 Molecular Analysis of D1-like Receptor Activation 33

1.7 Objectives of Study and Hypotheses 40

1.7.1 EL3CT Chimeras 40

1.7.2 IL4 Chimeras 42

CHAPTER 2: INSIGHT INTO THE MECHANISM OF G PROTEIN-COUPLED RECEPTOR ACTIVATION. EVIDENCE FOR A MOLECULAR INTERPLAY BETWEEN THE THIRD EXTRACELLULAR LOOP AND CYTOPLASMIC TAIL OF D1-LIKE DOPAMINERGIC SUBTYPES 44

Summary 46

Introduction 47

Experimental Procedures 50

Results....56

Discussion....63

Acknowledgements....68

References....69

CHAPTER 3: ROLE OF THE CYTOPLASMIC HELIX-8 REGION OF D1-LIKE DOPAMINERGIC RECEPTORS IN CONFERRING SUBTYPE SPECIFIC LIGAND BINDING AND G PROTEIN COUPLING PROPERTIES....79

Abstract....81

Introduction....82

Materials and Methods....86

Results....92

Discussion....98

Acknowledgements....102

References....103

CHAPTER 4: GENERAL DISCUSSION....111

CHAPTER 5: REFERENCES....121

APPENDIX 1....156

APPENDIX 2....157

List of Tables

CHAPTER 1

Table 1 Characteristics of the DA receptor subtypes defined from molecular, physiological, pharmacological, and biochemical studies 21

CHAPTER 2

Table I Dissociation constants (K_d) for wild-type and chimeric D1-like receptors .. 74

CHAPTER 3

Table I Dissociation constants (K_d) and binding capacity (R) values for wild-type and chimeric D1-like receptors 108

List of Figures

CHAPTER 1

Figure 1 General structure of G protein-coupled receptors.....3

Figure 2 Structure of the human D1-like receptors.....39

CHAPTER 2

Figure 1 Net changes in standard free energy values for DA affinity of EL3CT chimeras relative to wild-type receptors.....75

Figure 2 Total functional receptor number for wild-type and chimeric D1-like subtypes expressed in HEK 293 cells.....76

Figure 3 Constitutive activity and DA-mediated maximal stimulation of AC by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.....77

Figure 4 Dose-response curves of DA for AC stimulation by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.....78

CHAPTER 3

Figure 1 Constitutive activity and DA-mediated maximal stimulation of AC by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.....109

Figure 2 Dose-response curve of DA for AC stimulation by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.....110

List of Abbreviations

AA	Arachidonic Acid
α_2-AR	α_2-Adrenergic Receptor
AC	Adenylyl Cyclase
AMP	3',5'-adenosine monophosphate
ANOVA	Analysis of Variance
AT	Angiotensin
β_2-AR	β_2-Adrenergic Receptor
CA	[^{3}H]cAMP formed
CAM	Constitutively Active Mutant
cAMP	cyclic AMP
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
CT	Cytoplasmic Tail
DA	Dopamine
DAT	Dopamine Transporter
DNA	Deoxyribonucleic Acid
EC_{50}	Half-maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
EL	Extracellular Loop
GAP	GTPase-Activating Protein
GEF	GDP/ GTP Exchange Factors
G_i	Inhibitory G protein

GnRH	Gonadotropin-Releasing Hormone
G_{olf}	Olfactory G protein
G_s	Stimulatory G protein
G protein	GTP-binding protein
GPCR	G Protein-Coupled Receptor
GRK	G Protein-Coupled Receptor Kinase
HBS	HEPES-buffered Saline
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
5-HT	5-hydroxytryptophan
IBMX	1-methyl-3-isobutylxanthine
IL	Intracellular Loop
IP	Inositol Phosphate
K_d	Equilibrium dissociation constant for N-[methyl-^{3}H]SCH23390
KSHV	Kaposi's Sarcoma-associated Herpes Virus
LH	Luteinizing Hormone
MDCK	Madin-Darby Canine Kidney
MEM	Minimal Essential Medium
mRNA	Messenger Ribonucleic Acid
NMR	Nuclear Magnetic Resonance
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography

PLA_2	Phospholipase A_2
PLC	Phospholipase C
PKA	Protein Kinase A
PKC	Protein Kinase C
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone related Peptide
R	Receptor number
RGS	Regulators of G protein Signaling
SCH23390	R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
TCM	Ternary Complex Model
TM	Transmembrane
TRL	Terminal Receptor Locus
TSH	Thyroid Stimulating Hormone
TU	Total Uptake
$V_{1a}R$	Vasopressin V_{1a} Receptor
VIP-1	Vasoactive Intestinal Peptide

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Thesis format

This thesis is written in the collection of manuscripts format. Chapter 1 is a general introduction to the work where theoretical background is discussed. Chapter 2 is a manuscript titled "Insight into the mechanism of G protein-coupled receptor activation. Evidence for a molecular interplay between the third extracellular loop and cytoplasmic tail of D1-like dopaminergic subtypes." This manuscript has been submitted to the Journal of Biological Chemistry. Chapter 3 consists of a second manuscript titled "Role of the cytoplasmic helix-8 region of D1-like dopaminergic receptors in conferring subtype-specific ligand binding and G protein coupling properties." to be submitted to Molecular Endocrinology. Chapter 4 is an overall discussion of the work.

CHAPTER 1

General Introduction

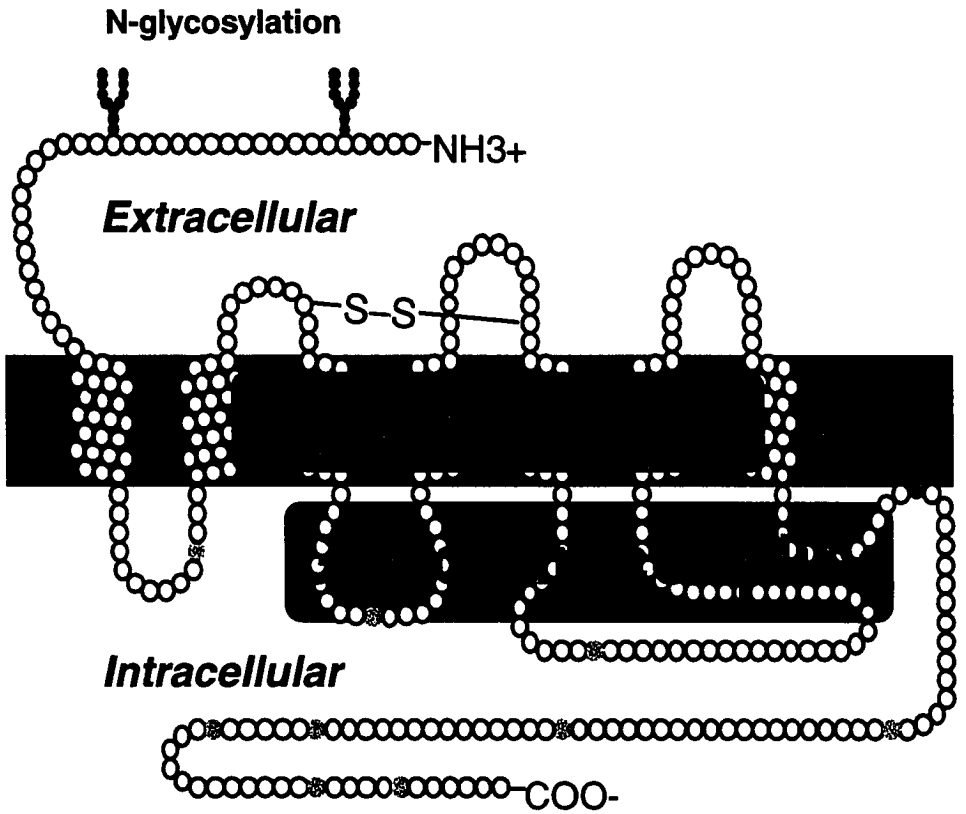
1.1 G Protein-Coupled Receptors (GPCRs)

1.1.1 Functional Features of GPCRs

Given their constantly changing environment, cells depend on rapid communication and responses for their survival. Cellular responses to extracellular stimuli are often initiated at the plasma membrane by integral membrane proteins called receptors. A large number of these receptors belong to the superfamily of G protein-coupled receptors (GPCRs). Originally identified in visual and hormonal signaling, they have since been implicated in a wide array of functions, including synaptic neurotransmission, sensory perception and mitogenesis (Lefkowitz, 2000). As such, GPCRs comprise the largest family of proteins found in nature. They are encoded by more than 1% of the genes present in the human genome (Venter *et al.*, 2001) and are the target of ~60% of drugs currently available (Leurs *et al.*, 1998).

Despite their multitude and functional diversity, members of the GPCR superfamily share conserved tertiary structure. Also known as 'heptahelical' or seven-transmembrane '7TM' receptors, GPCRs are characterized by the presence of seven α-helical membrane-spanning domains connected by three extracellular and three intracellular loops (Fig.1). The N-terminus of the receptors is located extracellularly while the C-terminus is located in the cytoplasm of the cell. Although they contain certain highly conserved residues, GPCRs do not share high degree of identity in their primary structure. Significant sequence homology, however, exists among subfamilies of GPCRs when grouped by amino acid similarity (Wess, 1998; Gether, 2000). Alternatively, GPCRs can be subdivided based on ligand structure and G protein coupling properties.

Figure 1. General structure of G protein-coupled receptors.


N-glycosylation
NH3+
Extracellular
S–S
Intracellular
COO-

1.1.2 Signaling via Heterotrimeric G Proteins

As their name suggests, GPCRs act primarily through their interaction with heterotrimeric G proteins (GTP-binding proteins), which in turn regulate a variety of enzymes and ion channels. G proteins consist of three subunits: an α subunit that binds and hydrolyzes GTP and a βγ complex that serves to anchor the G protein to the plasma membrane. In the resting state, the G protein exists as a heterotrimer with GDP attached to the α subunit. When activated, GPCR associates with the G protein causing an exchange of GDP for GTP on the α subunit, followed by dissociation of α-GTP from the βγ complex. At the same time, α-GTP and the βγ complex are released from the GPCR and are free to interact with effector enzymes and ion channels. Signaling persists until α-GTP is hydrolyzed to α-GDP by the intrinsic GTPase activity of the α subunit. This reaction returns the system to the resting state and reunites the α subunit with the βγ complex (Neer, 1995). Some of the effector enzymes regulated by G protein signaling include adenylyl cyclase (AC), phospholipase C (PLC), and phospholipase A_2 (PLA_2) whose activation and/or inhibition results in rapid changes in the concentration of intracellular signaling molecules such as cAMP, inositol phosphates, diacylglycerol, and arachidonic acid.

The activity of the G protein and thus, the pathway it regulates, depends largely on the nature of the α subunit. To date, twenty-three different α subunits encoded by seventeen different genes have been identified. They can be divided into four major classes (G_s, G_i, G_q, and G_{12}) based on their amino acid similarity. Furthermore, five β and twelve γ subunits add to the molecular heterogeneity of the G proteins (Schwindinger and Robishaw, 2001). Distinguishing between the various classes of G proteins has been

made possible by the use of two bacterial toxins, cholera toxin and pertussis toxin. As enzymes, these toxins catalyze a conjugation reaction (ADP-ribosylation) on the α subunit of the G proteins. Cholera toxin permanently activates the G_s protein by blocking the GTPase activity of the α subunit, whereas the pertussis toxin inhibits activation of the G_i protein by preventing exchange of GDP for GTP on the α subunit. The discovery of intracellular molecules capable of altering the rates of GTP hydrolysis and GDP/GTP exchange introduced an additional level of complexity to the regulation of G protein signaling (Sprang, 1997; Sprang, 2001). Studies have shown that the balance between the active and inactive G protein states is modulated by two families of proteins: the GDP/GTP exchange factors (GEFs), which promote the formation of the active state by stimulating the GDP/GTP exchange on the α subunit and the GTPase-activating proteins (GAPs), which suppress G protein activity by accelerating the rate of GTP hydrolysis. The GAPs have recently been included in a large family of proteins called regulators of G protein signaling (RGS). The RGS are responsible for feedback regulation of G protein function as well as integration of G protein signaling pathways with other cellular systems (Burchett, 2000; Ross and Wilkie, 2000).

The complexity of the G protein signaling network is further augmented by the fact that some GPCRs can interact with more than one G protein and certain G proteins couple with more than one effector. On the other hand, recent studies indicate that some receptors discriminate even between related G proteins of the same family (Gudermann *et al.*, 1996). In turn, type-specific activation of effector isoforms by various G protein subunits has also been demonstrated (Tang and Gilman, 1991). In short, the intricacy of

G protein signaling and its regulation is immense and provides a constant source of inquiry for intensive ongoing research.

1.1.3 Molecular Mechanism of GPCR Activation and G Protein Coupling

G protein activation typically occurs as a result of GPCR activation in response to agonist binding. Multiple methods have been used to map the ligand binding sites of the various GPCRs, including substituted cysteine-scanning mutagenesis, site-directed mutagenesis, and receptor chimeras. Ligand binding of neurotransmitter GPCRs takes place in a binding pocket formed by the hydrophobic transmembrane (TM) domains of the receptor (Tota *et al.*, 1991). Agonist binding leads to receptor activation through a presumed change in receptor conformation. However, the exact mechanism of GPCR activation is still poorly understood. Evidence suggests that one of the key elements in activation of neurotransmitter GPCRs involves protonation of the aspartic acid in the highly conserved DRY motif (Asp-Arg-Tyr) located at the cytoplasmic side of the third transmembrane domain (TM3) (Scheer *et al.*, 1996). It was proposed that protonation of the aspartic acid facilitates conformational rearrangement of transmembrane domains 3 and 6 (TM3 and TM6), thus leading to the formation of receptor active state (Rasmussen *et al.*, 1999). Indeed, precise movements of TM3 and TM6 have been shown to occur during GPCR activation (Gether *et al.*, 1997b). Agonist binding and GPCR activation are followed by G protein coupling and activation of effector systems. Studies utilizing chimeric substitutions and short synthetic peptides have mapped the G protein binding regions of GPCRs to the intracellular loops and in some cases to the proximal part of the carboxy terminus of the receptors. The third intracellular loop (IL3) is considered the

principal determinant of G protein coupling specificity (O'Dowd *et al.*, 1988; Eason and Liggett, 1995), while the second intracellular loop (IL2) is believed to contribute to specificity and efficiency of G protein activation (Nasman *et al.*, 1997; Burstein *et al.*, 1998).

1.1.4 GPCR Desensitization and Post-Translational Modification

Agonist activation of GPCRs also initiates the process of receptor desensitization, an event described as a tendency of receptor responsiveness to wane over time despite continued receptor stimulation (Hausdorff *et al.*, 1990). Desensitization occurs as a consequence of G protein uncoupling due to receptor phosphorylation of serine/ threonine residues of the cytoplasmic tail (CT) and the intracellular loops of GPCRs by both second messenger-dependent protein kinases (PKA, PKC) and G protein-coupled receptor kinases (GRKs) (Ferguson, 2001). GRK-mediated receptor phosporylation triggers GPCRs binding to a family of regulatory proteins called arrestins. Arrestins uncouple GPCRs from their cognate G proteins, thus terminating receptor signaling. It is now well established that in addition to playing a key role in GPCR desensitization, arrestins regulate GPCR internalization via clathrin-coated vesicles and serve as adaptor proteins that link GPCRs to alternative signaling pathways (Pierce and Lefkowitz, 2001).

Alongside phosphorylation, other types of post-translational modification most GPCRs have been shown to undergo are N-glycosylation and palmitoylation. Although precise role of GPCR glycosylation has not been conclusively determined, it has been linked to various aspects of receptor signaling. Namely, glycosylation of receptors for luteinizing hormone (LH), thyroid-stimulating hormone (TSH), follicle-stimulating

hormone (FSH), gonadotropin-releasing hormone (GnRH), vasoactive intestinal peptide (VIP-1), and vasopressin ($V_{1a}R$) was shown to be important for receptor expression and stability (Russo *et al.*, 1991; Davidson *et al.*, 1995; Davis *et al.*, 1995; Couvineau *et al.*, 1996; Davis *et al.*, 1997; Hawtin *et al.*, 2001), while glycosylation of rhodopsin, β_2-adrenergic receptor (β_2-AR), somatostatin receptor, and the gastrin-releasing peptide receptor (bombesin BB_2) was implicated in high-affinity ligand binding and G protein coupling properties (Rands *et al.*, 1990; Rens-Domiano and Reisine, 1991; Kaushal *et al.*, 1994; Kusui *et al.*, 1994). Putative N-glycosylation sites are present in the N-termini of most GPCRs (Raymond *et al.*, 1990). In contrast, palmitoylation of GPCRs occurs on cysteine residue(s) in the CT of the receptors and serves to anchor the CT to the membrane (Morello and Bouvier, 1996). A type of covalent lipid modification, palmitoylation mediates numerous protein-membrane and protein-protein interactions involved in cellular signaling. Due to its reversible nature, palmitoylation has been suggested to play a role in regulating protein function during cycles of activation and deactivation (Dunphy and Linder, 1998). Indeed, the palmitoylation state of the β_2-AR has been shown to mediate an interaction between PKA- and GRK-mediated receptor phosphorylation and desensitization. More specifically, ligand-mediated depalmitoylation of the β_2-AR promotes PKA-mediated phosphorylation of the receptor, which in turn favors subsequent phosphorylation by GRK, resulting in a synergistic action of the two kineses leading to rapid β_2-AR desensitization (Moffett *et al.*, 2001).

1.2 Models of GPCR Activation

1.2.1 The Ternary Complex Model (TCM)

The function of G protein-coupled receptors lies in their ability to transduce external signals across cell membrane. A typical GPCR binds an extracellular signaling molecule, which leads to receptor activation followed by an interaction with a G protein and initiation of a variety of intracellular events via an effector cascade. The classical paradigm of receptor activation, termed 'the ternary complex model' (TCM), describes the binding of an agonist to an inactive receptor state (R) leading to the formation of an active receptor state (R*) and subsequent coupling to a G protein (De Lean *et al.*, 1980). The conversion of R to R* is achieved through a conformational change of the receptor brought forth by the binding of an agonist. Based on the model, antagonists do not alter the conformational state of the receptor and elicit their effects solely by competing with agonists for shared binding sites.

1.2.2 The Allosteric (Extended) TCM

When it was discovered that mutations in the C terminal portion of the third cytoplasmic loop of adrenergic receptors caused constitutively active receptors (Cotecchia *et al.*, 1990), the initial model of receptor activation had to be readdressed. The observation that GPCRs can be activated in the absence of an agonist and subsequent finding that certain antagonists are capable of diminishing this activation lead to the development of a new model, termed 'the allosteric TCM'. The new model, also known as 'the extended TCM', predicted the existence of an equilibrium between two interchangeable conformational states, inactive (R) and active (R*). The transition

between R and R* states is regarded as an isomerization reaction governed by an equilibrium constant J (Samama *et al.*, 1993). In the absence of an agonist, GPCRs are maintained predominantly in an inactive (R) state by constraining intramolecular interactions that prevent effective coupling with G proteins. Agonist binding or mutations relieve these intramolecular constraints and stabilize the GPCR in an active (R*) conformation. A certain fraction of GPCRs assumes the R* state spontaneously, due to sufficiently low energy barrier between the R and R* conformations. The observed suppression of constitutive activity by certain antagonists has been termed 'inverse agonism'. In fact, many compounds previously identified as antagonists display inverse agonism. It is widely assumed that inverse agonists reduce the agonist-independent activity of GPCRs by stabilizing the receptor in an inactive state. Neutral antagonists, according to the model, do not shift the position of the equilibrium between the R and R* states, but can competitively antagonize the effects of both agonists and inverse agonists. However, recent studies indicate that even the extended ternary complex model is too simple to explain the complex behaviour of GPCRs. Latest evidence suggests that GPCRs may exist in possibly multiple conformational states (Iwasiow *et al.*, 1999; Gether, 2000; Jackson *et al.*, 2000).

1.2.3 The Multistate Model

In contrast to two-state models, which present the receptor in either one of two possible conformations (R or R*), the multistate models of receptor activation suggest that GPCRs alternate between multiple active and inactive receptor states (Schwartz *et al.*, 1995). This notion stems from experimental findings indicating that not all agonists

produce the same active state of GPCRs. In fact, latest evidence suggests that agonists are capable of producing ligand-specific receptor conformations leading to distinct downstream effects (Kenakin, 1995). For example, some agonists acting at the serotonergic 5-HT_{2C} receptors expressed in CHO cells preferentially activate PLC-mediated inositol phosphate (IP) accumulation, whereas others favor PLA_2-mediated arachidonic acid (AA) release (Berg *et al.*, 1998). These results suggest that the receptor forms multiple conformations for G protein interactions, which are specific for a particular effector pathway, and agonists can distinguish among these conformations and/or induce them. Aside from differential activation of effectors, the effects of agonists on receptor internalization have also provided valuable evidence in support of ligand-specific receptor conformations. For example, while many agonists of the μ and δ opioid receptors display similar effects on receptor-mediated signaling, they exhibit drastically different effects on intracellular trafficking of the receptors (Keith *et al.*, 1996). This may indicate that the receptor conformations leading to cellular response are not necessarily the same as those leading to receptor internalization. The above data support the hypothesis that receptors can assume multiple active conformations and a given agonist can select and stabilize a unique set of conformations based on its structure (Perez *et al.*, 1996; Krumins and Barber, 1997; Wiens *et al.*, 1998). Although the evidence for multiple receptor conformations is growing, the multistate model of receptor activation remains, for the time being, theoretical.

1.3 Constitutively Active Receptors

1.3.1 General Features of Constitutively Active Receptors

Constitutive GPCR activity was initially described in 1989 for the δ-opioid receptor endogenously expressed in the membranes of NG108-15 neuroblastoma-glioma cells (Costa and Herz, 1989). However, wide recognition of constitutively active GPCRs was not achieved until reports of constitutive activity of overexpressed, mutant α_{1B} and β_2 adrenergic receptors emerged in early 90's (Cotecchia *et al.*, 1990; Kjelsberg *et al.*, 1992; Samama *et al.*, 1993). Observations of constitutive activation of the adrenergic receptors following mutations in the C-terminus of their third intracellular loop (IL3) led to the notion that this region may play a role in the process of receptor activation. More specifically, this region likely functions to constrain the G protein coupling of the receptor, a constraint normally released upon agonist binding. Since their discovery, constitutively active GPCRs have been extensively studied and classified. Computer simulations and experimental data have helped to define several general features of constitutively active G protein-coupled receptors: (i) generation of a signal in the absence of agonist occupancy; (ii) increased agonist affinity for receptor binding; (iii) increased potency of agonists for induction of effector response; and (iv) increased intrinsic activity of partial agonists (Lefkowitz *et al.*, 1993; Tiberi and Caron, 1994; Tiberi and Caron, 1995; Scheer and Cotecchia, 1997). Intrinsic activity describes the degree of physiological response a ligand produces in a biological system per amount of receptor occupancy (Kenakin, 2002). The values of intrinsic activity can reach positive (agonists) and negative (inverse agonists) values and are often evaluated relative to the intrinsic activity of the natural ligand.

1.3.2 Constitutively Active Receptors in Disease and Health

Although constitutive activity among mutant GPCRs was initially demonstrated after mutational substitutions in the C-terminal part of IL3 of adrenergic receptors, constitutively activating mutations have since been identified in various receptor domains of a growing number of receptors (Spiegel, 1996). Furthermore, some constitutively activating mutations have been shown to arise naturally and are linked to a variety of human diseases, including congenital night blindness and retinitis pigmentosa (rhodopsin mutants), familial male precocious puberty (LH receptor mutants), hyperfunctional thyroid adenomas and familial hyperthyroidism (TSH receptor mutants), autosomal-dominant hypoparathyroidism (Ca^{2+}-receptor mutants), and Jansen-type metaphyseal chondrodysplasia (PTH/PTHrP-receptor mutants) (Parma *et al.*, 1993; Shenker *et al.*, 1993; Duprez *et al.*, 1994; Spiegel, 1996). Moreover, constitutively active GPCRs have been shown to transform cells (Allen *et al.*, 1991; Arvanitakis *et al.*, 1997) and can act as oncogenes in human cancers (Spiegel, 1996). As previously stated, somatic mutations activating the TSH receptor are the main cause of thyroid adenomas, a condition characterized by formation of sporadic benign tumors (Parma *et al.*, 1993; Parma *et al.*, 1995). Additionally, constitutively active GPCRs encoded by the Kaposi's sarcoma-associated herpesvirus (KSHV) have been shown to induce oncogenic cellular transformation and tumor formation in mice (Arvanitakis *et al.*, 1997; Bais *et al.*, 1998). Despite the irrefutable involvement of constitutively active GPCRs in the aforementioned disorders, the exact role of constitutive GPCR activity in pathology is unclear. It has been suggested that the true cause of retinitis pigmentosa in humans may not be the constitutive signaling of rhodopsin *per se*, but rather its elevated phosphorylation and

arrestin binding in response to increased signaling (van Soest *et al.*, 1999). Constitutive desensitisation has previously been demonstrated for constitutively active mutant (CAM) β_2-AR (Pei *et al.*, 1994).

Equally uncertain is the role of agonist-independent GPCR activation in normal physiology. Several native systems exhibiting constitutive activity have recently been identified (de Ligt *et al.*, 2000). While certain *in vitro* systems allowed for observations of constitutive activity at endogenous receptors expressed at physiological levels, similar observations in intact animals were not easily attainable. The first indication that constitutive GPCR activation might cause changes in cell function *in vivo* came from a study utilizing transgenic animals. Transgenic mice with cardiac-specific overexpression of wild-type human β_2-AR displayed enhanced myocardial function resulting from constitutive activity at these receptors (Bond *et al.*, 1995). Recently, constitutive activation has been found in animals expressing normal levels of receptor. Specifically, constitutive activity of histamine H_3 autoreceptor was demonstrated by comparing the actions of inverse agonists in the presence or absence of neutral antagonists on H_3-mediated inhibition of histamine release in rat and mouse brain synaptosomes. Thus, constitutive activity of native H_3 receptors has been shown to regulate histamine neurons in rodent brain, suggesting a physiological function for agonist-independent GPCR activation (Morisset *et al.*, 2000).

As our knowledge of constitutive signaling increases, it is likely that an additional number of native GPCRs exhibiting constitutive activity will be discovered. One of the neuronal systems likely to display such activity is the dopaminergic system, whose receptors have demonstrated basal activity in heterologous expression systems (Tiberi

and Caron, 1994; Griffon *et al.*, 1996). Indeed, constitutive activity of the D1B receptor has recently been proposed as one of the mechanisms by which the ovarian steroid estrogen exerts its neurobiological effects in the brain (Lee *et al.*, 1999) (discussed later).

1.4 The Dopaminergic System

1.4.1 Function and Innervation of the Dopaminergic System

Dopamine (DA), a bioactive catecholamine originally described as a precursor to norepinephrine, has been identified as a neurotransmitter in its own right in mid 1960's (Carlsson *et al.*, 1958; Hornykiewicz, 1966). It was shown to play a crucial role in physiological effects on both the peripheral and central nervous system (CNS) in a variety of organisms from simple invertebrates to humans. In the more complex networks of the mammalian brain, DA is involved in the control of learning, perception, emotion, and locomotion as well as neuroendocrine function, such as the release of prolactin and growth hormone from the pituitary. Dysregulation of the dopaminergic transmission has been implicated in drug addiction and the etiology of several pathological conditions such as schizophrenia, Parkinson's disease and hyperprolactinemia. Many drugs used in the treatment of psychiatric, neurological, and endocrinological disorders modify the activity of the dopaminergic system (Missale *et al.*, 1998).

The central dopaminergic system comprises three principal neuronal pathways: the nigrostriatal, the mesolimbocortical, and the tuberoinfundibular. The nigrostriatal pathway originates in the substantia nigra and projects to the striatum. It is involved in the control of locomotion and its degeneration is the main cause of Parkinson's disease. The mesolimbocortical pathway extends from the ventral tegmental area to the limbic

forebrain. It controls a variety of functions including emotion, cognition and reward, and its impairment is thought to underlie the occurrence of drug addiction and the etiology of psychoses, such as schizophrenia. The tuberoinfundibular pathway projects from the hypothalamus to the pituitary and is involved in the regulation of neuroendocrine secretion (Emilien *et al.*, 1999).

1.4.2 Classification of Dopamine Receptors as GPCRs

Actions of DA are mediated by the activation of transmembrane receptors belonging to the superfamily of G protein-coupled receptors. DA receptors are members of the largest and most studied subfamily of GPCRs, the rhodopsin/ β_2-AR - like receptor family, which spans receptors as diverse as the light-detecting opsins, the biogenic amine receptors, and the various peptide receptors (Gether, 2000). In addition to their predicted heptahelical topography, members of the rhodopsin/ β_2-AR - like receptor family are characterized by the presence of a so-called 'fourth intracellular loop' (IL4). This loop is believed to be formed by palmitoylation of one or more conserved cysteine residue(s) in the CT of the rhodopsin/ β_2-AR - like receptors. Many GPCRs have been shown to be palmitoylated, including rhodopsin (Ovchinnikov *et al.*, 1988), the β_2-AR (O'Dowd *et al.*, 1989), and D1 dopaminergic receptor (Ng *et al.*, 1994a; Jin *et al.*, 1999). Surprisingly, the recently obtained crystal structure of rhodopsin revealed the IL4 as helical in structure, thus denoting it as 'α-helix 8' (Palczewski *et al.*, 2000). Using NMR studies, the presence of a helix in this region was likewise demonstrated for the IL4 peptide of the β-adrenergic receptor (Jung *et al.*, 1996) and the angiotensin II (AT_{1A}) receptor (Franzoni *et al.*, 1997).

1.4.3 Dopamine Receptor Subtypes and Signal Transduction Mechanisms

The first evidence for the existence of dopaminergic receptors in the CNS came from a 1971 study linking DA to stimulation of AC (Kebabian and Greengard, 1971). However, not all dopaminergic receptors appeared to mediate their effects through accumulation of cAMP and a new scheme for DA receptor classification emerged in 1979 (Kebabian and Calne, 1979). Based on their distinct pharmacological profiles, tissue distribution, and signal transduction mechanisms, DA receptors were divided into two discrete populations. D1 receptors were defined as coupled to stimulation of AC and cAMP formation, whereas D2 receptors were described as not coupled to this effector. In fact, subsequent studies have linked the D2 receptors to inhibition of AC and activation of K^+ channels (De Camilli *et al.*, 1979; Freedman and Weight, 1988). Concurrently, the existence of vascular DA receptors, believed distinct from their central counterparts, was demonstrated in the periphery. These receptors, termed DA1 and DA2, were later found to resemble strongly their corresponding central subtypes, and the DA1/ DA2 classification was abandoned (Goldberg *et al.*, 1978). Introduction of gene cloning procedures revolutionized the field of molecular biology and allowed for cloning of the first DA receptor, the D2 receptor, in 1988 (Bunzow *et al.*, 1988). Subsequent studies revealed the existence of two splice variants of the D2 receptor, named $D2_{short}$ and $D2_{long}$ (Grandy *et al.*, 1989). The D1 receptor (herein referred to as D1A receptor) was next to be cloned (Dearry *et al.*, 1990; Monsma *et al.*, 1990; Sunahara *et al.*, 1990; Zhou *et al.*, 1990), followed by cloning of three novel receptor subtypes termed D3, D4, and D5 (D5 herein referred to as D1B receptor) (Sokoloff *et al.*, 1990; Grandy *et al.*, 1991; Sunahara *et al.*, 1991; Tiberi *et al.*, 1991; Van Tol *et al.*, 1991; Weinshank *et al.*, 1991; O'Malley *et*

al., 1992). Owing to sequence homology, the D3 and D4 receptors were cloned by means of low stringency hybridization using the D2 receptor as a probe and were eventually classified as members of the expanded D2-like receptor family. The D1B receptor was cloned under similar conditions using the D1A receptor gene as a hybridization probe and was ultimately included in the D1-like receptor family. The pharmacological profiles of the newly identified receptor subtypes were distinct, yet reminiscent of their respective parent prototypes. Analysis of gene structure revealed a plethora of splice variants, polymorphic variants, and pseudogenes among the novel receptor subtypes. Splice variants of the D3 receptor encoding functional (Fishburn *et al.*, 1993) and nonfunctional proteins have been identified (Giros *et al.*, 1991; Snyder *et al.*, 1991). Extensive polymorphic variation was found within the coding sequence of the D4 receptor (Van Tol *et al.*, 1992). Lastly, the existence of two related pseudogenes of the D1B receptor coding for truncated, nonfunctional receptor has also been demonstrated (Grandy *et al.*, 1991; Weinshank *et al.*, 1991). In short, mammalian dopaminergic receptors can be divided into two groups: the D1-like (D1A/ D1 and D1B/ D5) and the D2-like ($D2_{short}$ & $D2_{long}$, D3 and D4). Members of the D1-like family bind prototypical D1 ligands and couple to the activation of AC via the stimulatory G protein ($G_{s/olf}$). Members of the D2-like family bind prototypical D2 ligands and couple to the inhibition of AC via the inhibitory G protein ($G_{i/o}$). It is of note that coupling of D1-like receptors to the phospholipase C (PLC)-mediated phosphoinositide hydrolysis in brain (Undie and Friedman, 1990; Undie *et al.*, 1994; Friedman *et al.*, 1997) and renal cortex (Felder *et al.*, 1989; Vyas *et al.*, 1992) has also been reported. However, as none of the cloned D1-like receptors can stimulate phosphoinositide metabolism robustly (Liu *et al.*, 1992) and efforts at cloning

novel D1-like receptors not coupled to AC have failed, the most likely explanation is that the functional effects caused by D1 agonists are mediated by alternate signaling pathways rather than novel gene products (Montague *et al.*, 2001). Interestingly, additional nonmammalian members of the D1-like receptor family, termed D1C and D1D, coupled to stimulation of AC, have been identified in frog and chicken (Sugamori *et al.*, 1994; Demchyshyn *et al.*, 1995)

1.4.4 Pharmacology and Amino Acid/ Genomic Organization of Dopamine Receptor Subtypes

Within families, the receptor subtypes are, at times, difficult to distinguish. To date, pharmacological separation of the D1A and D1B receptors has not been achieved. Both D1-like receptor subtypes preferentially bind compounds containing the 1-phenyl-3-tetrahydrobenzazepene nucleus, e.g. the antagonist SCH23390 (Billard *et al.*, 1984). The prototypical antagonist of the D2-like receptor family is spiperone (also known as spiroperidol), the heterocyclic-substituted phenylbutylpiperidine. Subtype-preferential compounds (antagonists) have recently been synthesized for the D3 and D4 receptors (Merchant *et al.*, 1996; Patel *et al.*, 1997; Millan *et al.*, 2000). At present time, there are no compounds that discriminate between the short and long variants of the D2 receptor.

Analysis of the primary structure of the cloned DA receptors revealed considerable amino acid conservation within the TM domains of the receptors. The D1-like receptors display the highest degree of sequence identity (~ 82% within the TM domains of D1A and D1B), while the D2-like receptors share approximately 40% amino acid homology (79% identity in TM domains of D2 and D3, 55% identity in TM domains of D3 and D4,

and 54% identity in TM domains of D2 and D4) (Jarvie and Caron, 1993). The D1-like receptors can be differentiated from their D2-like counterparts by a shorter third intracellular loop, a characteristic of G_s-coupled receptors, and by a longer CT (Missale *et al.*, 1998). A curious exception to this rule is illustrated by the goldfish D1A receptor, which features a naturally truncated CT, yet displays binding and coupling properties reminiscent of its mammalian counterpart (Frail *et al.*, 1993).

The primary difference in genomic organization of the DA receptors lies in the presence or absence of non-coding intervening sequences (introns) in their coding sequences (exons). Members of the D1-like receptor family do not contain introns in their coding regions, while the D2-like receptor genes are interrupted by introns. Chromosomal localization likewise differs among the five genes encoding DA receptors and has been examined for evidence of genetic linkage to various pathophysiologies (Gingrich and Caron, 1993; Wong *et al.*, 2000). For summary of molecular and pharmacological characteristics of the DA receptors, including their specific chromosomal localization, please see Table 1.

Table 1. Characteristics of the DA receptor subtypes defined from molecular, physiological, pharmacological, and biochemical studies.

		D1-like		D2-like		
		D1A	D1B	$D2_{(short/\ long)}$	D3	D4
Chromosomal localization		5q 35.1	4p 15.1-16.1	11q 22-23	3q 13.3	11p 15.5
Number of Coding Exons		1	1	7	6	5
Number of Amino Acids		446	477	414/ 443	400	387-515*
Receptor Localization	Brain	Striatum, olfactory tubercle, nucleus accumbens, hypothalamus, thalamus, frontal cortex	Hippocampus, mammilary nuclei and parafascicular nuclei of the thalamus, cortex, striatum	Striatum, olfactory tubercle, nucleus accumbens, septum, hypothalamus, cortex	Nucleus accumbens, olfactory tubercle, islands of Calleja	Frontal cortex, thalamus, hypothalamus, hippocampus, amygdala
	Periphery	Parathyroid gland, heart, gastrointestinal tract, kidney, adrenal cortex, ovaries, retina	Kidney, retina, platelets, blood lymphocytes	Adrenal gland, pituitary, kidney, retina	Kidney, platelets, blood lymphocytes	Heart, kidney, retina, blood lymphocytes
Pharmacological Characteristics	Example Agonists	SKF 38393 Fenoldopam Dopamine	SKF 38393 Fenoldopam Dopamine	Apomorphine Bromocriptine Fenoldopam Dopamine	Apomorphine Quinpirole Dopamine Bromocriptine	Quinpirole Dopamine
	Example Antagonists	SCH 23390 (+)-Butaclamol Cis-Flupentixol	SCH 23390 (+)-Butaclamol Cis-Flupentixol	Spiperone Eticlopride Cis-Flupentixol (+)-Butaclamol Haloperidol Chlorpromazine	Eticlopride Spiperone Cis-Flupentixol (+)-Butaclamol Haloperidol Chlorpromazine	Spiperone Haloperidol Eticlopride Chlorpromazine Clozapine
Biochemical Response		Adenylyl cyclase ↑	Adenylyl cyclase ↑	Adenylyl cyclase ↓	Adenylyl cyclase ↓	Adenylyl cyclase ↓
Physiological Function		Motor function, Cardiovascular function		Motor function, Behavior, Neuroendocrine function, Cardiovascular function		

* Number of amino acids in human D4 receptor depends on number of repeats in 3rd intracellular loop.

1.4.5 Distribution of Dopamine Receptors

DA receptors can be differentiated not only by pharmacological and biochemical criteria, but also based on tissue distribution (Table 1). In the brain, the D1A and D2 receptors are the most widely expressed DA receptor subtypes. They exhibit distinct, yet overlapping tissue distribution. The D1A receptor is present in brain at the highest level, largely in areas associated with motor control. Its mRNA has been localized chiefly to the basal ganglia; namely the striatum, the olfactory tubercle, and the nucleus accumbens. Lesser amounts of the D1A receptor mRNA were found in the cortex, the hypothalamus and the thalamus (Weiner *et al.*, 1991). At the outset, parathyroid gland has been identified as the archetypal peripheral tissue expressing the D1A receptor subtype, which is believed to regulate the release of parathyroid hormone (PTH) (Niznik *et al.*, 1988), however, peripheral localization of the D1A receptor has since been reported for the heart (Ozono *et al.*, 1996), the gastrointestinal tract (Vaughan *et al.*, 2000), the kidney (Nash *et al.*, 1993; O'Connell *et al.*, 1998a), the adrenal cortex (Aherne *et al.*, 1997), ovaries (Mayerhofer *et al.*, 1999), and the retina (Soares *et al.*, 2000). The D2 receptor mRNA has been found mainly in the striatum, the olfactory tubercle, and the nucleus accumbens, with lesser amounts seen in the septum, the hypothalamus and the cortex (Bouthenet *et al.*, 1991; Weiner *et al.*, 1991). Its peripheral distribution has originally been mapped to the adrenal gland (Wu *et al.*, 2001) and the lactotroph cells of the pituitary (Kukstas *et al.*, 1991). Subsequently, peripheral localization of the D2 receptor has been extended to the kidney (Gao *et al.*, 1994) and the retina (Stormann *et al.*, 1990; Dearry *et al.*, 1991). All of the remaining DA receptor subtypes are expressed in the body at much lower levels. In striking contrast to the D1A receptor, the mRNA for the D1B subtype is

virtually absent in the basal ganglia, but is highly associated with the limbic regions. Areas of the highest expression of the D1B receptor mRNA in the brain include the hippocampus, mammilary nuclei, and the parafascicular nuclei of the thalamus (Tiberi *et al.*, 1991; Meador-Woodruff *et al.*, 1992). Low levels of mRNA for this receptor were also found in the cerebral cortex and the striatum (Huntley *et al.*, 1992). In the periphery, the D1B receptor mRNA is expressed in the kidney (Nash *et al.*, 1993), the retina (Soares *et al.*, 2000), platelets (Ricci *et al.*, 2001), and blood lymphocytes (Takahashi *et al.*, 1992; Ricci *et al.*, 1999). The D3 receptor has a specific distribution to limbic brain regions such as the nucleus accumbens, the olfactory tubercle, and the islands of Calleja (Bouthenet *et al.*, 1991; Landwehrmeyer *et al.*, 1993). Its peripheral localization extends to the kidney (Gao *et al.*, 1994; O'Connell *et al.*, 1998b), platelets (Ricci *et al.*, 2001), and blood lymphocytes (Nagai *et al.*, 1993). Similar to the D3 subtype, the D4 receptor exhibits localization specific to the limbic and cortical areas of the brain regulating emotion and cognition. Its central mRNA distribution has been mapped to the frontal cortex, amygdala, thalamus, hypothalamus, and hippocampus (Van Tol *et al.*, 1991; O'Malley *et al.*, 1992). Peripheral localization of the D4 receptor includes the heart (O'Malley *et al.*, 1992), the kidney (Sun *et al.*, 1998), the retina (Cohen *et al.*, 1992), and blood lymphocytes (Bondy *et al.*, 1996).

1.4.6 Genetic Approaches to the Study of Dopamine Receptor Subtypes

Thorough classification of the diverse properties of the various DA receptor subtypes by pharmacological approaches is hindered by the lack of subtype selective drugs, especially within the subfamilies of DA receptors. Correct assignment of various

physiological and behavioral responses to stimulation or blockade of particular receptor subtype is inherently problematic. Similarly, reliable allocation of a given DA receptor subtype to a particular neuronal system is complicated by the existing overlap of certain receptor subtypes in specific brain regions, as well as actual co-expression of receptor subtypes within individual cells (Aizman *et al.*, 2000). Introduction of genetic approaches, such as transgenic techniques, antisense technology, and gene targeting (knockout) have offered new insight into dopaminergic signaling and the functions of DA receptors *in vivo*. Knockout mice exhibiting complete and highly selective loss of all identified DA receptor subtypes have been created and were instrumental in assessing the contribution of each receptor subtype to mammalian development, physiology, behavior and responses to pharmacological agents. In addition, mice deficient in DA and the DA transporter (DAT) were also generated and displayed the most dramatic phenotypes of all the knockouts affecting dopaminergic neurotransmission (Zhou and Palmiter, 1995; Giros *et al.*, 1996). Namely, mice unable to synthesize DA became severely hypoactive and failed to feed, while mice lacking the DAT displayed extreme locomotor hyperactivity to the point of death-promoting exhaustion. Although studies of DA receptor knockout mice report less dramatic phenotypes, they display a considerable heterogeneity of findings pointing to the existence of methodological differences.

Disruption of the D1A receptor gene has initially been reported by two independent groups (Drago *et al.*, 1994; Xu *et al.*, 1994b). The D1A knockout mice generated by both groups exhibited growth retardation, reduced brain size, and a failure to thrive. In terms of locomotor activity, the two groups reported either unchanged or increased locomotor activity in D1A deficient mice compared with wild-type littermates. Similar findings (of

increased locomotion) were later observed by a detailed study applying ethologically-based procedures to resolve more naturalistically the full range of behaviors displayed by the mice (Clifford *et al.*, 1998). In contrast, decrease in initiation of movement was also reported for the D1A knockout mice (Smith *et al.*, 1998). Taken together, the above findings define a crucial role for the dopaminergic D1A receptors in normal development and modulation of locomotor activity. Surprisingly, the effect of D1A receptors on motor behavior is most often described as facilitative by nature (Sibley, 1999). The lack of strong motor deficits displayed by the D1A knockout mice is perhaps due to compensatory mechanisms activated by the lack of D1A receptors. Indeed, compensatory processes and mixed genetic background are the two factors most often cited as confounding variables when interpreting phenotypic changes in knockout mice (Phillips *et al.*, 1999; Gingrich and Hen, 2000). Other features not related to spontaneous locomotor activity displayed by the D1A knockout mice include absence of locomotor response to psychostimulants (Xu *et al.*, 1994a) and impairment of learning and memory (Smith *et al.*, 1998; El-Ghundi *et al.*, 1999). These findings imply the necessity of D1A receptors for the manifestation of locomotor-activating effects of psychostimulants and the involvement of D1A receptors in learning and memory.

Characterization of D2 receptor knockout mice has also been described by two separate groups (Baik *et al.*, 1995; Kelly *et al.*, 1998). One group described the D2 deficient mice as exhibiting severe neurological impairments (abnormal posture and gait, cataleptic-like behavior) and an inability to breed, whereas the other group did not observe such severity of neurological dysfunction. Both groups reported impairment of locomotor function associated with varying degrees of motor deficit. In fact, the

displayed phenotype has been suggested to resemble the extrapyramidal symptoms of Parkinson's disease in humans. Other features of the D2 knockout mice include the absence of rewarding properties of opiates (Maldonado *et al.*, 1997) and the loss of autoreceptor function (Mercuri *et al.*, 1997; L'Hirondel *et al.*, 1998). A follow-up study investigating the loss of autoreceptor function in D2 deficient mice has attributed the observed effect to the short isoform of the D2 receptor. Knockout mice exhibiting selective loss of the long isoform of the D2 receptor retain their ability to regulate DA release presumably through presynaptic $D2_{short}$ receptors (Usiello *et al.*, 2000; Wang *et al.*, 2000). Taken together, the D2 receptor knockout studies have confirmed the role of D2 receptors in positive control of locomotor function and inhibitory autoreceptor regulation of dopaminergic neuronal activity.

Lesions in the gene for the D3 receptor have been studied by multiple groups (Accili *et al.*, 1996; Xu *et al.*, 1997; Jung and Schmauss, 1999). In contrast to mice deficient in D2 receptors, D3 knockout mice were normal in appearance and grew and reproduced normally. Moreover, they exhibited increased levels of locomotor activity. The most salient finding with respect to the D3 receptor knockout mice, however, was the lack of direct evidence for any inhibitory autoreceptor role previously attributed to the D3 receptor by pharmacological studies (Koeltzow *et al.*, 1998).

At present, only one group has reported the generation of D4 receptor deficient mice (Rubinstein *et al.*, 1997). The D4 knockout mice developed and reproduced normally. They exhibited decreased locomotion despite observed augmentation of motor coordination compared with wild-type controls. The above findings indicate a role for the D4 receptors in regulating both spontaneous and coordinated motor functions. In

addition, the D4 knockout mice have been shown to exhibit a decrease in novelty-related exploration (Dulawa *et al.*, 1999). Such a finding gives premise to further examination of the role of the D4 receptor in novelty-seeking behavior previously linked to D4 receptor polymorphism in humans (Benjamin *et al.*, 1996; Ebstein *et al.*, 1996).

The D1B receptor has been the last DA receptor subtype to be studied by gene targeting using homologous recombination. Initial results characterized the D1B receptor deficient mice as viable, fertile, and normal in appearance. In terms of locomotor activity, they were reported to outperform their wild-type littermates in horizontal and vertical levels of activity, total distance traveled, and tests of motor coordination (Sibley, 1999). Upon more detailed examination, the D1B knockout mice were declared to exhibit normal levels of locomotor activity, motor coordination, and general health (Holmes *et al.*, 2001). Such findings do not support a strong role for the D1B receptors in many DA-mediated behaviors, however, their discrete pharmacological profile and anatomical distribution distinct from those of D1A receptors hint at their unique place within the D1-like receptor family. In addition, the possibility of developmental adaptations to the loss of the gene must be born in mind, as increased locomotor activity was reported in earlier studies utilizing D1B antisense (Dziewczapolski *et al.*, 1998). Overall, the characterization of phenotypes associated with selective loss of distinct DA receptor subtypes in mice is expected to elucidate the role of DA receptor subtypes *in vivo* and may provide new clues about the nature of the involvement of dopaminergic signaling in human disorders.

1.4.7 Structure-Function Analysis of Dopamine Receptor Signaling

In order to understand the complex functioning of a receptor, its molecular architecture must be considered first. GPCR signaling is a multifaceted process governed by intramolecular and intermolecular interactions. Some of the functional properties modulating the magnitude of GPCR signaling include ligand binding, G protein coupling, post-translational modifications (e.g. phosphorylation and palmitoylation), desensitization, intracellular trafficking, protein-protein interactions, and receptor oligomerization (Morello and Bouvier, 1996; Hebert and Bouvier, 1998; Zhang *et al.*, 1999; Edwards *et al.*, 2000; Gether, 2000; Ferguson, 2001). Ligand binding of neurotransmitter GPCRs takes place in a binding pocket formed by the hydrophobic TM domains of the receptor. Thanks to structural similarities, analysis of molecular requirements for ligand binding to DA receptors has been based on earlier studies of the β_2-AR function. Indeed, several conserved amino acid residues presumed critical for ligand binding have been identified in the core of the catecholamine receptors. Evidence suggests that three important residues within the TM domains of the catecholamine receptors contribute greatly to the binding of catecholamines (Strader *et al.*, 1989b). In particular, negatively charged aspartate residue in TM3 is believed to participate in an ionic interaction with the positively charged amine group of the catecholamine ligands. Mutation of the aspartate residue reduced the binding affinity of agonists and antagonists at both adrenergic and dopaminergic receptors (Strader *et al.*, 1988; Mansour *et al.*, 1992). Furthermore, two conserved serine residues in TM5 appear to form hydrogen bonds with hydroxyl groups of the catecholamines. In addition to a loss of agonist affinity at the β_2-AR (Strader *et al.*, 1989a), mutations of the serine residues negatively

affected the binding of DA and elicited varied results on binding of other dopaminergic agonists and antagonists at the DA receptors (Pollock *et al.*, 1992). Overall, the above results indicate an important role for the aforementioned residues in ligand binding of the catecholamine receptors, while highlighting differences among the receptor types, the active states they form, and the drug interactions they participate in.

Alongside ligand binding, determination of structural basis of receptor – G protein coupling is paramount to understanding GPCR activation and signal transduction. In agreement with other GPCRs, DA receptor regions involved in G protein coupling have been mapped to the IL2 and IL3 regions of the various receptor subtypes. Through the use of D1A/D2 receptor chimeras, the IL3 was shown to be sufficient for D1A receptor coupling to G_s and stimulation of AC (MacKenzie *et al.*, 1993; Kozell *et al.*, 1994), while both IL2 and IL3 were shown to be required for D2 coupling to G_i and inhibition of AC (Kozell *et al.*, 1994).

In recent years, the notion of receptor dimerization has reached the field of GPCR signaling. Indeed, GPCR dimers were not only shown to exist, but also to regulate GPCR activation and signal transduction (Hebert and Bouvier, 1998). With respect to DA receptors, D2 receptor dimers were detected in whole cell lysate, crude membranes prepared from human brain (Ng *et al.*, 1996). Moreover, incubation of D2 dimers with peptides derived from their putative TM domains led to dissociation of the dimers to monomers, suggesting that receptor dimers are formed by specific intermolecular noncovalent interactions involving TM regions. Although the existence of DA receptor dimers has now been established, the exact role of dimerization in the coupling properties of dopaminergic receptors remains to be investigated.

After initiation of cellular signaling, GPCRs undergo a process of receptor desensitization. As mentioned previously, desensitization occurs as a consequence of G protein uncoupling due to receptor phosphorylation by both second messenger-dependent kinases (PKA, PKC) and GRKs. Putative phosphorylation sites believed to participate in receptor desensitization have been identified in many GPCRs, including the DA receptors (Gingrich and Caron, 1993). Interestingly, the number of these sites and their placement within the intracellular loops and the CT vary among the DA receptor subtypes and may suggest differential pattern of regulation. Further research is required to determine the specific involvement of these sites in DA receptor phosphorylation and desensitization. Thus far, the D1A receptor has generated the greatest amount of knowledge regarding the regulation of dopaminergic signaling. Studies involving mutagenesis of PKA sites of the D1A receptor have identified Thr^{268} in the carboxyl end of IL3 as a key determinant of the rate of agonist-induced desensitization and intracellular trafficking (Jiang and Sibley, 1999; Mason *et al.*, 2002). On the other hand, distinct residues within the CT of the D1A receptor were shown to regulate agonist-induced GRK-mediated desensitization and internalization (Jackson *et al.*, 2002; Lamey *et al.*, 2002). More specifically, mutation of Thr^{360} in the proximal part of the CT abolished agonist-induced phosphorylation and desensitization, while mutations of residues in the distal part of the CT (Thr^{446}, Thr^{439}, and Ser^{431}) caused a disruption of receptor internalization (Lamey *et al.*, 2002). Taken together, the present findings indicate a role for PKA in agonist-induced desensitization and strengthen the belief that desensitization and internalization of the D1A receptor are biochemically distinct mechanisms (Ng *et al.*, 1995).

In terms of post-translational modifications, DA receptors have been shown to be both phosphorylated and palmitoylated. Palmitoylation of conserved Cys residues has been demonstrated for D1A and D2 receptors (Ng *et al.*, 1994a; Ng *et al.*, 1994b). With respect to D1A receptors, palmitoylation has been found to occur independently at Cys^{347} and Cys^{351} of the CT (Jin *et al.*, 1999). Corresponding residues are present in the D1B subtype at Cys^{375} and Cys^{379}. Their contribution to the G protein coupling properties of the D1B receptor, however, remains to be investigated.

1.5 Focus on D1-like Receptors

1.5.1 Pathophysiological Relevance of D1-like Receptors

To date, the involvement of D1-like receptor-mediated signaling has been investigated in human and rat models of schizophrenia and hypertension. Two studies employing positron emission tomography (PET) have identified abnormal levels of D1-like receptors in the prefrontal cortex of schizophrenic patients (Okubo *et al.*, 1997; Abi-Dargham *et al.*, 2002). This alteration of D1-like receptor expression is believed to be related to negative symptoms and cognitive deficits seen in schizophrenia. Among these deficits, the impairment of working memory is one of the most enduring features of the disease. In fact, the connection between prefrontal D1-like receptor signaling and working memory has previously been established in studies of nonhuman primates (Brozoski *et al.*, 1979; Sawaguchi and Goldman-Rakic, 1991; Sawaguchi and Goldman-Rakic, 1994). Similarly, there is evidence that defects in the renal dopaminergic system may underlie some forms of essential (idiopathic) hypertension. Aberrant renal DA production and/or DA receptor function have been reported in human primary

hypertension as well as in genetic models of animal hypertension (Hussain and Lokhandwala, 1998; O'Connell and Aherne, 2000; Amenta *et al.*, 2001). Dysfunction of D1-like receptor signaling has been implicated in two rat models of genetic hypertension (Felder *et al.*, 1990). Moreover, mice exhibiting selective loss of D1A receptor gene (knockout) have been shown to develop hypertension (Albrecht *et al.*, 1996). Identification of D1-like receptor coupling defects in animal models of genetic hypertension has instigated the search for similar defects of dopaminergic signaling in human hypertension. In fact, recent study has identified constitutive desensitization of the D1A receptor in renal proximal tubule cells as a possible factor in the pathogenesis of essential hypertension (Sanada *et al.*, 1999).

Clearly, further research of DA receptors is of crucial importance as it holds the key to a better understanding of dopaminergic signal transduction (and its aberration) in both the CNS and the periphery.

1.5.2 Dopamine D1B Receptor – A Naturally Occurring Constitutively Active Receptor?

In addition to being the focus of intensive research into etiology and pathophysiology of schizophrenia and hypertension, the D1-like receptors provide a useful model for the study of constitutively active GPCRs. The position of the equilibrium between R and R* varies for individual receptors and may be responsible for different levels of constitutive activity of the different GPCRs. This was demonstrated, among others, for the two D1-like DA receptor subtypes, where the D1B displays a higher level of constitutive activity compared to the D1A, as indicated by increased basal

cAMP formation and its susceptibility to inverse agonist treatment in HEK 293 cells (Tiberi and Caron, 1994). In fact, the D1B receptor may represent the first reported, naturally occurring receptor subtype that shares biochemical properties with CAM GPCRs. As such, the D1-like receptors may serve as a model for the study of constitutively active GPCRs and may help elucidate the mechanism of action of inverse agonists, agents that can reduce constitutive activation and be of physiological and clinical relevance.

Moreover, constitutive activity of the D1B receptor has been proposed to participate in neurobiological effects exerted by estrogen in the brain (Lee *et al.*, 1999). More specifically, the D1B receptor has been shown to mediate estrogen-induced stimulation of atrial natriuretic factor (ANF) neurons of the hypothalamus in a cAMP-dependent mechanism. As shown *in vitro*, estrogen augments function of hypothalamic ANF neurons by increasing expression of D1B receptors that generate cAMP in a ligand-independent manner. The estrogen-induced increase in cellular cAMP can be blocked by antipsychotic drugs displaying inverse agonism at the D1B receptor. Overall, these findings may provide the first indication for the role of constitutive activity of dopaminergic receptors *in vivo*.

1.6 Molecular Analysis of D1-Like Receptor Activation

Despite the mapping of certain general features of dopaminergic receptor signaling, the molecular basis for the existence of multiple DA receptor subtypes is ill defined. Focusing on the D1-like dopaminergic receptor family, studies suggest that distinct binding and G protein coupling properties may underlie the basis for D1-like receptor

multiplicity. In general agreement with properties of CAM GPCRs, the D1B receptor distinguishes itself from the D1A subtype by a higher constitutive activity (agonist-independent activity), increased affinity and potency for agonists, decreased affinity for antagonists and lower agonist-mediated maximal activation (Tiberi and Caron, 1994). However, the study of the activation properties of the D1-like receptors is complicated by the lack of subtype-selective ligands. Furthermore, an x-ray crystal structure of the D1A and D1B receptors has not yet been obtained (the same is true for all GPCRs with the recent exception of rhodopsin (Palczewski *et al.*, 2000)) and inferences of GPCR binding and activation properties rely mostly on approaches using chimeric receptors and single amino acid substitutions.

Following the reports of agonist-independent activation, a number of studies have focused on delineating the structural basis underlying constitutive activity profile of the D1B receptor. In agreement with studies of CAM GPCRs, the C-terminal portion of IL3, and in particular residue Ile^{288}, was found to be a major determinant of the constitutive activity of the D1B receptor (Charpentier *et al.*, 1996). However, additional receptor domains were suggested to be required for the full expression of the constitutive activity phenotype of the D1B receptor. These may include regions encoded by the transmembrane domains, the extracellular loops, and the CT. Indeed, studies have shown that mutations occurring in these regions can lead to constitutive activation of various GPCRs (Parker and Ross, 1991; Lefkowitz *et al.*, 1993; Cho *et al.*, 1996; Hasegawa *et al.*, 1996; Nanevicz *et al.*, 1996; Scheer *et al.*, 1996; Scheer and Cotecchia, 1997).

Mutagenesis studies done in our lab indicate that a receptor region referred to as the terminal receptor locus (TRL) encompassing the sixth and seventh transmembrane

domains (TM6 and TM7), the third extracellular loop (EL3), and the CT plays a crucial role in the binding and activation properties of the human D1A and D1B receptors (Iwasiow *et al.*, 1999). Furthermore, studies have shown that mutations in the TRL can dissociate the agonist affinity, agonist-independent and dependent G protein coupling properties of the D1-like receptors. Chimeric receptors in which the TRL region was swapped between the wild-type human D1A and D1B receptors displayed a complete switch in DA affinity, DA potency, and agonist-independent activity, whereas agonist-mediated maximal activation of AC remained unchanged in cells expressing the chimeric receptors. In other words, a chimeric D1A receptor harboring the TRL region of the D1B subtype displayed DA affinity, DA potency, and agonist-independent activity reminiscent of the wild-type D1B receptor, while the agonist-mediated maximal activation of AC remained true to the D1A phenotype. Likewise, a chimeric D1B receptor harboring the TRL region of the D1A subtype displayed DA affinity, DA potency, and agonist-independent activity reminiscent of the wild-type D1A receptor, while the agonist-mediated maximal activation of AC retained the expression of the D1B phenotype. Given the observed results, it appears that the receptor's ability to maximally activate the G protein after agonist stimulation is a property that eludes the spatial relationships induced by the TRL region, suggesting the involvement of additional structural determinants. Alternatively, swapping of large receptor domains, such as the TRL, may obscure the delineation of specific regions involved in the regulation of activation properties of the D1-like receptors. One such region may be the third extracellular loop (EL3), which displays low degree of identity (38%) between the D1A and D1B receptor subtypes (Figure 2) and has been implicated in the regulation of agonist binding and constitutive

activity of the β_2-AR (Zhao *et al.*, 1998). Therefore, in an attempt to narrow down the structural determinants of the TRL involved in the activation properties of the D1-like receptors (especially the agonist-mediated maximal activation of AC), additional chimeric receptors were constructed in which the EL3 was swapped between the D1A and D1B receptors. The resulting chimeras harboring the EL3 of their respective wild-type counterparts exhibited a complete reversal of agonist-mediated maximal activation of AC (Iwasiow *et al.*, 1999). In addition, swap of the EL3 between the D1A and D1B subtypes produced a switch in the binding affinity of inverse agonists (R.I., M.T., unpublished data). The exchange of the EL3, however, failed to reproduce the complete switch in DA affinity and agonist-independent activity observed upon the swap of the TRL. It is therefore likely that the TRL-mediated switch in DA affinity and agonist-independent activity was induced by the swap of the CT. Indeed, the human D1A and D1B receptors share only about 30% identity in their CTs (Figure 2). To test the hypothesis that the CT underlies the degree of DA affinity and agonist-independent activity displayed by the D1-like receptor subtypes, chimeric receptors were generated in which the CT was swapped between the D1A and D1B receptors (Jackson *et al.*, 2000). As predicted, the exchange of the CT resulted in a complete switch in DA affinity and agonist-independent activity, thus recapitulating the results obtained with the swap of the TRL. In addition, DA potency decreased and agonist-mediated maximal activation of AC increased for both chimeras. The decrease in DA potency is particularly intriguing for the D1A-CTB chimera, in light of its increased DA binding affinity. Such dissociation of DA affinity and potency for stimulating AC illustrates that binding and coupling properties do not always correspond and can be separated by mutagenesis. Overall, the above findings

hint at the complexity of intramolecular relationships within the TRL and indicate that discrete receptor domains are responsible for regulating specific GPCR activation properties, which can be dissociated by mutations.

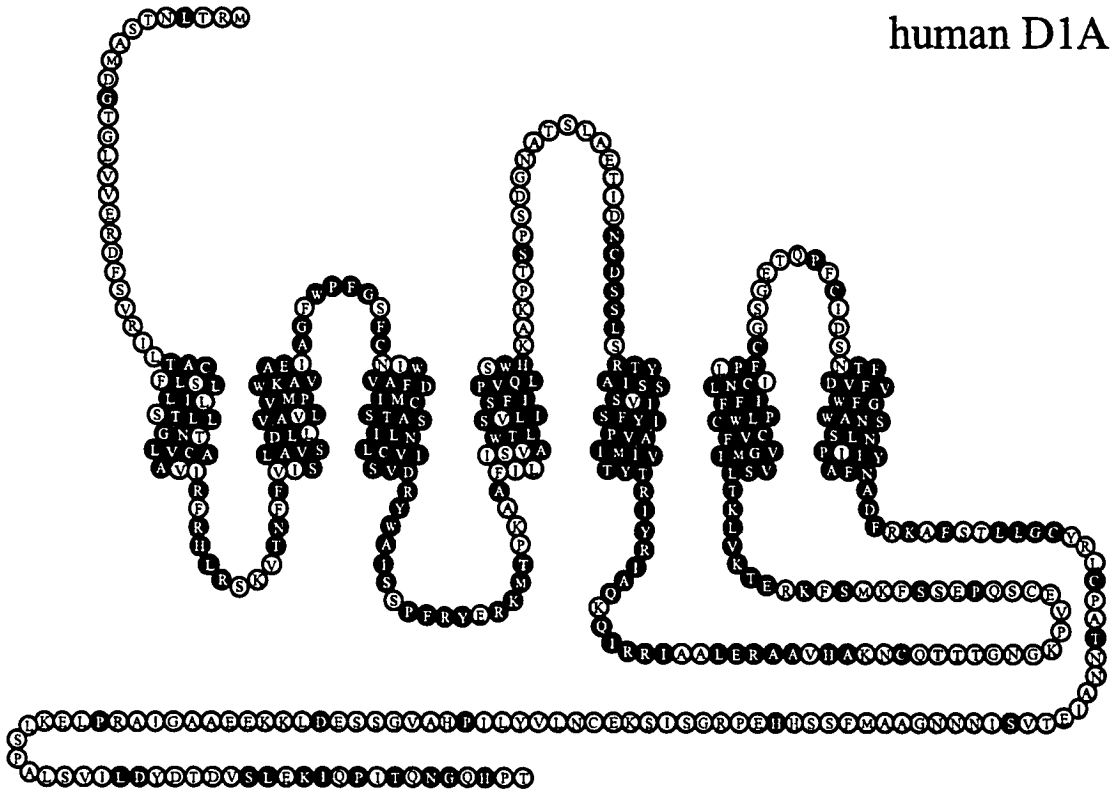
In addition to delineating the structural basis of the D1-like receptor activation properties, the mutagenesis studies uncovered previously unappreciated roles of the given domains in determining the total functional receptor number. More specifically, D1A receptor harboring EL3 of D1B expressed at considerably higher receptor number (approximately 4 fold), while the expression of the human D1A receptor harboring the D1B tail expressed at a dramatically lower receptor number (approximately 15 fold) than wild-type D1A receptor. This loss of total functional receptor number may be related to the receptor's high agonist-independent activity, as structural instability has been reported for CAM GPCRs (Gether *et al.*, 1997a). It is important to bear in mind that receptor number controls the extent of DA cellular responsiveness and has been linked to human disease, such as schizophrenia and Tourette's syndrome (Singer *et al.*, 1992; Okubo *et al.*, 1997; Wong *et al.*, 1997; Muller-Vahl *et al.*, 2000; Abi-Dargham *et al.*, 2002).

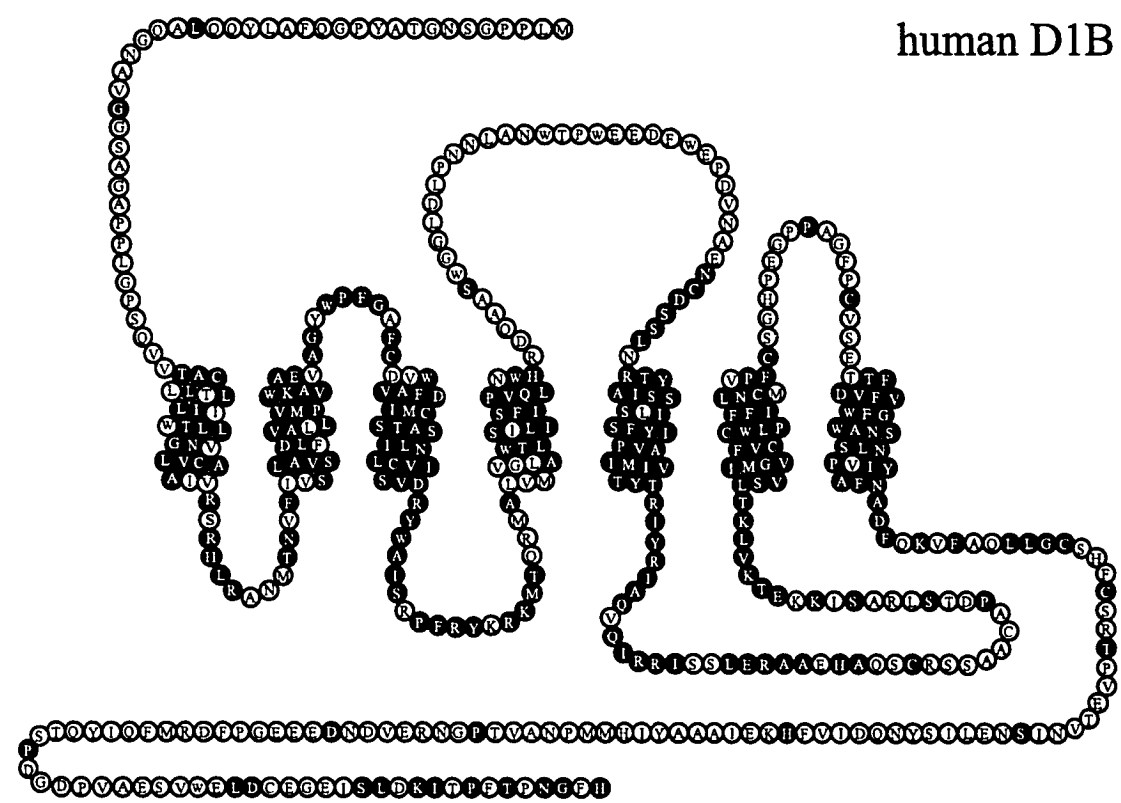
In light of the recent findings linking the CT to the subtype-specific phenotypes of the D1-like receptors (Demchyshyn *et al.*, 2000; Jackson *et al.*, 2000), efforts have been directed at defining the structural domains of the CT responsible for the distinct activation properties of the D1A and D1B receptors. A study utilizing serially truncated D1A receptors has mapped CT residues downstream of Cys^{351} as negative regulators of G protein coupling efficiency and AC activation (Chaar *et al.*, 2001). On the other hand, a series of D1B truncation/deletion mutants has identified a CT region encoded by amino

acids 438-448 as a structural determinant regulating the high-affinity agonist binding profile of the D1B receptor. Moreover, mutation of Gln^{439}, an absolutely conserved amino acid among all cloned members of the vertebrate D1B subclass, has significantly increased the agonist affinity of the D1B receptor (Demchyshyn *et al.*, 2000). Taken together, the two studies have highlighted the role of specific CT regions in the activation properties of the D1-like receptor subtypes and have underscored the complexity of D1-like receptor signaling.

Figure 2. Structure of the human D1-like receptors.

Identical residues between the D1A and D1B subtypes are represented in black.


human D1A


human D1B

1.7 Objectives of Study and Hypotheses

1.7.1 EL3CT Chimeras

As mentioned previously, chimeric D1A receptor harboring the CT of the D1B subtype behaves functionally as a constitutively active GPCR, displaying increased DA binding affinity and constitutive activity. However, the observed increase in DA affinity is not accompanied by a concomitant increase in DA potency, as predicted by the model of receptor activation. Furthermore, the receptor number of D1A-CTB chimera is drastically reduced compared to the wild-type D1A receptor. These findings bring to attention the need for additional D1B receptor domains required for the full expression of DA potency and receptor number exhibited by the D1A-CTB chimera. Given that the D1A receptor harboring the TRL region of the D1B subtype displays high DA potency and unaltered total functional receptor number (reminiscent of the wild-type D1B receptor), the EL3 region of the D1B receptor is a likely candidate for such a role. As part of my Master's thesis, I aimed to test whether the addition of the third extracellular loop (EL3) of D1B receptor would result in rescue of DA potency and receptor number of D1A-CTB chimera.

Presently, little is known about the contribution of EL3 to the formation of active and inactive receptor states as well as overall receptor structure and stability. For peptide receptors, residues within the extracellular domains have long been known to participate in ligand recognition (Berthold and Bartfai, 1997). However, recent studies have suggested that EL residues might also be important to GPCRs that bind 'small molecule' non-peptide ligands. In adenosine receptors, amino acids in EL2 were found to contribute to agonist and antagonist binding (Kim *et al.*, 1996). Similarly, three amino acids at the

C-terminal end of EL2 of the α_1-adrenergic receptor were shown to be responsible for subtype-specific antagonist binding (Zhao *et al.*, 1996). However, evidence pertaining to the role of EL3 in GPCR function of non-peptide receptors remains minimal. To date, a study using chimeric receptors between α_{1A}-AR (a G_q-coupled receptor) and β_2-AR (a G_s-coupled receptor) has suggested that EL3 of the β_2-AR modulates receptor/ G protein affinity (Zhao *et al.*, 1998).

To investigate whether EL3 cooperates with the CT to regulate functional properties of the D1-like receptors, chimeric receptors were generated in which both the EL3 and the CT were exchanged between the human D1A and D1B receptors. In particular, I examined the ability of EL3 and the CT to regulate in a coordinated manner the total functional receptor number, ligand binding, and G protein coupling properties of the D1-like receptors.

Hypothesis:

- EL3 of D1B rescues DA potency and total functional receptor number of D1A-CTB chimera.

1.7.2 IL4 Chimeras

An additional part of my Master's project consisted of narrowing down the specific regions of the CT involved in activation properties of the D1-like receptor subtypes. Although recent work has begun to delineate the sequence-specific motifs within the CT responsible for the distinct binding and coupling properties of the D1A and D1B receptor subtypes (Demchyshyn *et al.*, 2000; Chaar *et al.*, 2001), no study has looked for a region common to both receptor subtypes that may regulate in a reciprocal fashion the CT-conferred properties of the D1-like receptors. To investigate whether IL4 underlies the CT-conferred properties of the D1-like receptors, chimeric receptors were generated in which only the IL4 portion of the CT was exchanged between the D1A and D1B receptors.

IL4 is formed by the palmitoylation of conserved cysteine residue(s) present in the CT of rhodopsin/β_2-AR - like family of GPCRs. Palmitoylation of Cys^{341} of β_2-AR has been shown to play an important role in receptor – G protein coupling and stimulation of AC. Mutation of the palmitoylation site of β_2-AR abolished the formation of IL4 and caused enhanced receptor desensitization as a result of an exposure of a previously buried phosphorylation site to the regulatory activity of PKA (Moffett *et al.*, 1996). Although the elimination of palmitoylation sites of the D1A dopaminergic receptor did not result in alteration of receptor – G protein coupling (Jin *et al.*, 1997), the contribution of IL4 to the overall topology of GPCRs remains valid. Indeed, recent studies have proposed a role for the IL4 of rhodopsin in docking and binding of the G protein γ and α subunits, respectively (Ernst *et al.*, 2000). Assuming a functional similarity of G protein activation between rhodopsin and D1-like receptors, the interaction between IL4 and the γ subunit

of the G protein could prove significant, given that D1A and D1B receptors display differential dependence on the G protein γ subunit for activation of AC in HEK 293 cells (Wang *et al.*, 2001).

To study the involvement of IL4 in ligand binding and G protein coupling properties of the D1-like receptor subtypes, chimeric receptors were generated in which only the IL4 segment of the CT was exchanged between the human D1A and D1B receptors.

Hypothesis:

- IL4 underlies the CT-conferred properties of the D1-like receptors.

CHAPTER 2

Manuscript

Supplemental information regarding the construction of chimeric receptors is provided in Appendix 1.

Insight into the mechanism of G protein-coupled receptor activation. Evidence for a molecular interplay between the third extracellular loop and cytoplasmic tail of D1-like dopaminergic subtypes.

Running Title: Multiple active conformations of dopamine D1-like receptors

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SUMMARY

The D1B/D5 dopaminergic receptor has the phenotypic properties of constitutively active mutant G protein-coupled receptors (GPCR), namely a higher basal activity, increased agonist affinity and potency. Previously, a chimeric D1A receptor harboring the cytoplasmic tail (CT) of the D1B subtype (D1A-CTB) has been used to show that CT imparts the D1B receptor's high dopamine (DA) affinity and constitutive activity (Jackson, A., Iwasiow, R. M., and Tiberi, M (2000) *FEBS Lett.* 470, 183-188). However, the D1A-CTB chimera exhibits a significantly lower DA potency for stimulating adenylyl cyclase (AC) and a drastically lower structural stability when compared to its wild-type D1-like counterparts. In the present study, we aim to pinpoint the human D1B receptor regions regulating the intramolecular relationships that lead to an increased DA potency and structural stability using a functional complementation of chimeric D1-like receptors. Our results unequivocally show that addition of variant residues of the third extracellular loop (EL3) of the human D1B receptor into D1A-CTB chimera leads to a constitutively active mutant GPCR displaying an increased DA affinity, potency and structural stability. These results strongly suggest that constitutively active D1-like receptors can adopt multiple active conformations, notably one conferring increased DA affinity with decreased DA potency and structural stability, while another imparting increased DA affinity with a strikingly increased DA potency and structural stability. Overall, we show for the first time that a novel molecular interplay between EL3 and CT may regulate multiple active conformations of D1-like receptors and possibly other biogenic GPCRs.

INTRODUCTION

Dopamine (DA), a catecholamine neurotransmitter, elicits its diverse physiological and endocrine effects through the interaction with D1-like and D2-like G protein-coupled receptor (GPCR) subfamilies (1). D1-like receptors couple to the activation of adenylyl cyclase (AC) and in mammals are further divided into D1A (or D1) and D1B (or D5) subtypes. The D1B receptor distinguishes itself from the D1A subtype by a higher constitutive activity (agonist-independent activity), increased affinity and potency for agonists, decreased affinity for antagonists and a lower agonist-mediated maximal activation (2). Interestingly, a recent study highlighted the potential physiological relevance of the D1B receptor constitutive activity (3). Indeed, the extent of the D1B receptor constitutive activity controls the estrogen-induced mRNA expression of atrial natriuretic factor in primary hypothalamic neurons, a neuroendocrine process that potentially plays an important role in D1B receptor-mediated facilitation of female sexual behavior (3). Moreover, the D1-like subtype mRNA expression and/or activity in human breast and neuroendocrine gastrointestinal tumor cells (4,5) underscore the importance of these receptors, and potentially their levels of constitutive activation in regulating DA autocrine and paracrine function in pathophysiological conditions as shown previously with other constitutively active mutant GPCRs (6). Thus, D1A and/or D1B subtype-specific ligands may help in the treatment of pathologies for which compromised D1-like receptor responsiveness is purported (7-9). However, the structure-function relationships that shape the functional properties of the D1-like receptor subtypes remain unclear.

Previously, we have shown that a structural domain (referred to as the terminal receptor locus or TRL) encompassing the sixth and seventh transmembrane regions (TM6 and TM7), third extracellular loop (EL3) and cytoplasmic tail (CT) plays an important role in the phenotypic expression of binding and G protein coupling properties of D1-like receptors (10). To narrow down the structural determinants within TRL involved in subtype-specific ligand binding and G protein activation properties of the D1-like receptors, additional mutant receptors were constructed in which either the EL3 (10) or CT sequences (11) were swapped between the D1A and D1B receptors. Chimeric receptors harboring EL3 of their respective wild-type counterparts exhibited a complete reversal of agonist-mediated maximal activation of AC, whereas DA affinity and agonist-independent activity of the chimeras were only partially modulated by the exchange (10). In contrast, chimeric receptors harboring the CT of their respective wild-type counterparts displayed a full switch in DA affinity and agonist-independent activity, whereas DA potency decreased and agonist-mediated maximal activation of AC increased for both chimeras (11). The decrease in DA potency is particularly intriguing for the constitutively active D1A-CTB chimera in light of its ability to bind DA with high affinity, reminiscent of the DA affinity for wild-type D1B receptors (11). Moreover, mutagenesis studies revealed a previously unappreciated role of these receptor regions in determining the fate of functional D1-like subtype number and/or structural stability (10-13); a facet of the D1-like receptor function that may play an important role in cognitive and working memory deficits observed in schizophrenia (14,15). Overall, these previous findings hint at the complexity of intramolecular relationships within the TRL and indicate that discrete receptor domains responsible for regulating specific GPCR

activation properties can be dissociated by mutations. Furthermore, our previous chimera studies suggest the existence of multiple active conformations of constitutively active D1-like receptors, notably an active conformation imparting increased constitutive activity, DA affinity and potency with unchanged structural stability (e.g. wild-type D1B *versus* wild-type D1A receptors) or another conferring increased constitutive activity and DA affinity but decreased DA potency and structural stability (e.g. D1A-CTB chimera *versus* wild-type D1-like receptors).

In the present study, we test whether a molecular interplay between EL3 and CT regions controls the phenotypic expression of D1-like subtype-specific ligand binding and G protein coupling properties. To do so, we used a mutagenesis approach and functional complementation of different chimeric D1-like receptors expressed in human embryonic kidney 293 (HEK 293) cells to probe the potential molecular interplay between EL3 and CT regions. Here we report that a functional complementation of the constitutively active D1A-CTB chimera with the EL3 region of the D1B receptor leads to slight increase in DA affinity while causing a drastic increase in DA potency and total functional receptor number. Thus, these results indicate the existence of distinct molecular interplays between the CT and EL3 involved in the regulation of discrete aspects of D1-like receptor signaling (e.g. multiple active conformations and structural stability).

EXPERIMENTAL PROCEDURES

Drugs

N-[methyl-^{3}H]SCH23390 (82 Ci/mmol), [^{3}H]adenine (27 Ci/mmol), and [^{14}C]cAMP (275 mCi/mmol) were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Dopamine (DA), cis-flupentixol, and 1-methyl-3-isobutylxanthine (IBMX) were from Sigma/Research Biochemicals International (Oakville, Ontario, Canada).

Construction of chimeric human D1A and D1B receptors

To construct chimeric receptors harboring only the EL3 and CT regions of their wild-type counterparts, we took advantage of existing chimeras in which the EL3 region was swapped between the D1A and D1B subtypes (10). We have utilized these chimeras (D1A-EL3B and D1B-EL3A) and wild-type receptors as templates to engineer two additional chimeric receptors (D1A-EL3CTB and D1B-EL3CTA), in which the CT regions were exchanged by gene splicing using a PCR-based overlap extension approach. The receptor sequences were swapped at the junction between the TM7 and CT regions. The junction corresponds to the amino acid 334 and 362 in the D1A and D1B receptor, respectively. The D1A-CTB and D1B-CTA chimeras have been described previously (11).

In the case of D1A-EL3CTB chimera, the first round of PCR generated two fragments: fragment AB1 encoding the third intracellular loop, TM6, EL3 and TM7 of D1A-EL3B receptor and fragment B2 coding for the CT of D1B receptor. The fragment AB1 was amplified using primers 5'-CACCACAGGTAATGGAAA-3' (forward primer)

and 5'-ATTAAACGCGTAAAT-3' (reverse primer). The fragment B2 was generated using primers 5'-ATTTACGCGTTTAATGCCGACTTTCAGAAGGTGTTTG-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3' (reverse primer). Likewise, in the case of D1B-EL3CTA chimera, the first round of PCR generated two fragments: fragment BA1 coding for the N- terminal region of the D1B-EL3A receptor up to TM7 and fragment A2 encoding the CT of D1A receptor. The fragment BA1 was amplified using primers 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GTTGAAGGCATAGAT-3' (reverse primer). The fragment A2 was amplified using primers 5'-ATCTATGCCTTCAACGCTGATTTTCGGAAAGCTTTTTCAACCCTC-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3' (reverse primer). To facilitate the construction and identification of the chimeric receptors, a silent mutation was introduced in each construct to create a unique restriction endonuclease site. For the D1A-EL3CTB chimera, a *Mlu*I site was introduced at a nucleotide sequence corresponding to amino acids 335 and 336 (5'-TA**T**GC**C**-3'→5'-TA**C**GC**G**-3') near the 3' end of the D1A receptor TM7 region, immediately upstream of the D1B receptor CT sequence. For D1B-EL3CTA chimera, a *Hind*III restriction site was introduced at a nucleotide sequence encompassing the residues 363 and 364 (5'-AA**G**GC**A**-3'→5'-AA**A**GC**T**-3'), located 3' of the junction between the D1B receptor TM7 region and D1A receptor CT sequence. Amplified fragments were separated on a 1% agarose gel and appropriate bands were excised and purified by QIAEX II gel extraction method (Qiagen, Valencia, CA, USA). Diluted aliquots of the paired fragments were combined (AB1+B2 or BA1+A2) and subjected to overlap PCR using appropriate 5' and 3' flanking primers. The resulting PCR products were cut with *Bcl*I and *Xba*I and subcloned into pBluescript

II SK^+ (Stratagene) containing *Hind*III/*Xba*I fragment of the wild-type D1A receptor (for PCR product AB1/B2) or the full-length coding sequence of D1B receptor (for PCR product BA1/A2). The full-length expression constructs for wild-type and chimeric receptors were ultimately engineered into the pCMV5 expression vector. The D1A-EL3CTB chimera was subcloned into pCMV5 together with the N-terminal portion (*EcoR*I/*Hind*III fragment) of wild-type D1A receptor in a 3-piece ligation reaction. The *EcoR*I/*Hind*III D1A fragment was ligated to *Hind*III fragment of D1A-EL3CTB and linearized pCMV5 (*EcoR*I/*Hind*III). The D1B-EL3CTA chimera was subcloned into empty pCMV5 linearized with *Sal*I and *Xba*I. The nucleotide sequence of PCR products (encompassed by *Bcl*I and *Xba*I) and cloning sites was confirmed by dideoxy sequencing using Sequenase version 2.0 from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada.).

Cell Culture and Transfection

HEK 293 cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C and 5% CO_2 in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (10 μg/ml) (Invitrogen, Burlington, Ontario, Canada.). Cells were seeded into 100-mm dishes (2.5 x 10^6 cells/dish) and transiently transfected with 5 μg of DNA/dish using a modified calcium phosphate precipitation procedure as described (16). When less than 5 μg DNA was used in transfections, empty pCMV5 vector was added to normalize the total amount of DNA. All experiments were performed with cells from 38 to 52 passages.

Membrane Preparation

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK 293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Transfected HEK 293 cells were then washed with cold PBS, scraped from the dish in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5mM EDTA) and centrifuged at 40 000 x g for 20 min at 4°C. The crude membrane pellet was resuspended in lysis buffer using a Brinkmann Polytron (17 000 rpm for 15 s) and centrifuged at 40 000 x g for 20 min at 4°C. The final pellet was resuspended in lysis buffer and membranes either used immediately (saturation studies) or frozen in liquid nitrogen and stored at –80°C until required (competition studies).

Radioligand Binding Assays

Fresh or frozen membranes were diluted in binding buffer (final in binding assays: 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) and briefly mixed using a Brinkmann Polytron. Binding assays were performed with 100 µl of membranes in a total volume of 500 µl using N-[methyl-^{3}H]SCH23390 as radioligand. Saturation studies were done using fresh membranes and concentrations of N-[methyl-^{3}H]SCH23390 ranging from 0.01 to 6 nM. Non-specific binding was assessed in the presence of a final concentration of 10 µM cis-flupentixol (dissolved in milli-Q-water). For competition studies, frozen membranes were thawed on ice and incubated with a constant concentration of N-[methyl-^{3}H]SCH23390 (~0.6 nM) and increasing concentrations of competing ligand. Competition studies using DA were done in the presence of 0.1 mM ascorbic acid (dissolved in milli-Q-water). Binding

assays were incubated for 90 min at room temperature and terminated using rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed four times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS 6500). Protein concentrations were measured using the Bio-Rad assay kit with bovine serum albumin as standard. To determine the equilibrium dissociation constant (K_d) and binding capacity values (herein referred to as the total functional receptor number), binding isotherms were analyzed using the non-linear curve-fitting program LIGAND (17).

Whole Cell cAMP Assay

Regulation of AC activity by wild-type and chimeric receptors was assessed using a whole cell cAMP assay as described previously (10,11). Following overnight incubation with the DNA-calcium phosphate precipitate, HEK 293 cells were reseeded in 6- or 12-well dishes. The next day, cells were labeled with [^{3}H]adenine (2 μCi/ml) in fresh MEM containing 5% (v/v) FBS and gentamicin (10 μg/ml) for 18 h at 37°C and 5% CO_2. The labeling medium was then removed and HEK 293 cells were incubated in 20 mM HEPES-buffered medium containing 1 mM IBMX in the presence or absence of DA for 30 min at 37 °C. At the end of incubation period, the medium was aspirated and 1 ml lysis solution (2.5% (v/v) perchloric acid, 0.1mM cAMP, and [^{14}C]cAMP (2.5-5 nCi, ~5 000-10 000 cpm)) was added to each well and cells 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 (1 500 rpm) at 4 °C. The

amount of intracellular [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex (AG 50W-X4) and alumina columns as described previously (18). The amount of [^{3}H]cAMP (CA) over the total amount of [^{3}H]adenine uptake (TU) was calculated to determine the relative intracellular cAMP level (CA/TU). Dose-response curves to DA were analyzed by a four-parameter logistic equation using ALLFIT (19). The total functional receptor number was determined using a saturating concentration (~6 nM) of N-[methyl-^{3}H]SCH23390.

Statistics

Equilibrium dissociation constants (K_d) are expressed using the geometric mean ± S.E. as described (20). All other data are reported as arithmetic means ± S.E. unless stated otherwise. All statistical tests used in this study have been described elsewhere (21). Homoscedasticity of variances was assessed using Bartlett or F_{max} tests prior to statistical analyses. One sample *t*-test, Student's *t*-test and one-way ANOVA (with Newman-Keuls multiple comparison test) were performed using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. The level of significance was established at $p < 0.05$.

RESULTS

EL3 and CT regions coordinate the D1-like subtype-specific DA affinity.

Table I shows the dissociation constants (K_d values) for N-[methyl-^{3}H]SCH23390 binding to wild-type and chimeric human D1-like receptors obtained with saturation studies. As indicated by the K_d values, the chimeric receptors maintained their ability to bind this D1-like antagonist with high affinity suggesting that the protein folding required for ligand binding is functional. Specifically, D1A-EL3CTB chimera displayed an increase in binding affinity for N-[methyl-^{3}H]SCH23390 compared with the wild-type D1A receptor. These results are consistent with studies showing that benzazepine-like compounds can behave as partial agonists in cells expressing D1-like receptors (2,22,23). Meanwhile, the binding affinity of D1B-EL3CTA chimera remains unchanged in comparison with wild-type D1B receptor.

Furthermore, competition studies were performed to examine the changes in DA affinity for wild-type and chimeric receptors. As shown in Table I and in agreement with previous studies (1,10-12), DA exhibited a higher affinity for the D1B receptor compared with the D1A receptor. Furthermore, swapping the EL3 region between the D1A and D1B subtypes resulted in partial modulation of DA affinity, while swapping the CT resulted in a nearly complete switch in DA affinity to that of cognate wild-type counterparts. Exchange of both EL3 and CT regions resulted in a further pronounced switch in the DA affinity for chimeric receptors. Namely, the D1A-EL3CTB chimera exhibited an increased DA affinity extending beyond that of chimeric D1A-CTB receptors. Meanwhile, the chimeric D1B-EL3CTA receptor displayed a decreased DA

affinity falling below that of the D1B-CTA chimera. Importantly, the DA affinity values for EL3CT chimeras are essentially indistinguishable from their respective cognate wild-type receptors (Table I). However, based upon the EL3B- and CTB-induced effects individually exerted on the D1A subtype for DA affinity, it would be expected that a chimeric D1A receptor harboring the EL3B and CTB regions displays an even further increased DA affinity beyond that of the wild-type D1B subtype and the one measured experimentally for D1A-EL3CTB chimera. To address this issue, we have used the standard free energy (ΔG_o) values derived from DA affinity constants ($\Delta G_o = -RT\ln(1/K_d)$) to calculate the sum of net change induced separately by EL3 and CT regions (EL3+CT) and compare that with the net change caused by the addition of both regions (Fig. 1). The net change calculated for the D1B-EL3CTA chimera is not statistically different from zero suggesting that a full switch has been implemented (Fig. 1, right panel). Interestingly, similar results were obtained using the sum of net changes produced individually by EL3A and CTA regions, which was not statistically different from zero or changes observed with the D1B-EL3CTA chimera. These results are suggestive of an additive effect of EL3 and CT in regulating the DA affinity of D1B-EL3CTA chimera. Likewise, the net change measured for the D1A-EL3CTB was not statistically different from zero. However, in stark contrast, the sum of net changes elicited independently by EL3B and CTB regions were statistically different from zero and D1A-EL3CTB value, indicative of an interfering effect exerted by these regions in the D1A-EL3CTB chimera (Fig. 1, left panel).

Subtype-specific regulation of the total functional receptor number by EL3 and CT regions of human D1A and D1B dopaminergic receptors expressed in HEK 293 cells.

One interesting finding stemming from our saturation studies is the potential role of distinct molecular interplays between EL3 and CT regions of D1A and D1B receptors in regulating their structural stability as indexed by the total functional receptor number. Indeed, our saturation studies reveal stark differences in the total functional receptor number among the various chimeric receptors (Fig. 2). As reported before (11), there was a significant reduction in the total functional receptor number of D1A-CTB chimera (Fig 2A). Conversely, there was a large increase in the total functional receptor number of D1A-EL3B chimera (Fig. 2A). Importantly, inserting the EL3 of the D1B subtype into the D1A receptor harboring the D1B tail (D1A-CTB) led to a complete rescue of the total functional receptor number of D1A-CTB chimera. In fact, D1A-EL3CTB chimera displayed a total functional receptor number that is indistinguishable from the wild-type D1A receptor (Fig. 2A). The reverse was somewhat true for the chimeric D1B receptors, where insertion of EL3 of the D1A subtype decreased the total functional receptor number as we reported previously (10). Meanwhile, insertion of the CT of the D1A subtype increased significantly the total functional receptor number compared with wild-type D1B receptor (Fig 2B). In a previous study, a similar trend was detected but not established as statistically significant (11). Interestingly, however, a chimeric D1B receptor harboring the EL3 and CT regions of the D1A subtype (D1B-EL3CTA) displayed a total functional receptor number that is significantly increased from those measured with wild-type and chimeric D1B receptors (Fig. 2B).

To assess whether the molecular interplay between EL3 and CT regions exert additive, synergistic or interfering effects on the spatial relationships controlling the total functional receptor number, the binding capacities of chimeric D1A-like (EL3B, CTB and EL3CTB chimeras) and D1B-like receptors (EL3A, CTA and EL3CTA) were expressed as net changes relative to the wild-type D1A and D1B subtype, respectively (Fig. 2C & D). Data depicted in Fig. 2C indicate that the net change put forth by insertion of both EL3B and CTB (black bar) is significantly lower than the sum of net changes induced individually by EL3B and CTB (white bar) suggestive of a non-additive effect or interference. In contrast, the sum of the net change obtained by introducing both EL3A and CTA (black bar) is significantly higher than the sum of net changes produced separately by EL3A and CTA (white bar) indicative of a synergism (Fig. 2D). These results indicate that EL3 and CT regions exert in the overall receptor conformation not only unique but also opposing and synergistic effects on the intramolecular interactions regulating the total functional number of D1A and D1B receptors. These results suggest a potential role of these two regions in regulating the structural stability and/or receptor turnover. In a series of preliminary experiments, we have tested the potential role of EL3 in regulating the D1-like receptor structural stability using a membrane assay as previously described (24). Membrane preparations from HEK 293 cells expressing D1A-EL3B or D1B-EL3A chimeras were incubated at 37°C for 24 hours and tested for N-[methyl-^{3}H]SCH23390 functional binding to assess the role of EL3 region in regulating the structural stability of D1-like receptors. In agreement with an EL3-mediated regulation of structural stability, membranes expressing D1A-EL3B or D1B-EL3A

chimeras display in comparison with wild-type receptors a higher and lower total functional receptor number, respectively (data not shown).

Opposing EL3- and CT-induced intramolecular interactions confer D1-like receptor-specific G protein coupling properties.

To determine the combined effect of EL3 and the CT on agonist-independent AC activation, whole cell cAMP assays were performed in HEK 293 cells expressing wild-type or chimeric D1-like receptors (Fig. 3A). Reminiscent of constitutively active mutant (CAM) GPCRs, the D1B receptor displayed higher agonist-independent activity compared with the D1A subtype. In agreement with previously published results (10), the exchange of the EL3 region caused a partial modulation in agonist-independent activity (data not shown). Furthermore, the swap of the CT further substantiated the functional role of this cytoplasmic region in subtype-specific agonist-independent activity of the D1A and D1B receptors (data not shown), likewise supported by our previous findings (11). The exchange of both the EL3 and the CT resulted in chimeras displaying a full switch in agonist-independent activation of AC as compared with parent receptors (Fig. 3A). Indeed, the constitutive activation of D1A-EL3CTB chimera was not statistically different from the wild-type D1B receptor. In this particular series of experiments, the slightly lower degree of constitutive activation of D1A-EL3CTB displayed in HEK 293 cells when compared with wild-type D1B receptors (~24%) is in agreement with the smaller total functional receptor number of D1A-EL3CTB (~20%). Thus, our results strongly suggest that EL3B and CTB regions in the D1A-EL3CTB chimera partake in the full switch of agonist-independent activity of wild-type D1A receptors. Similarly, an

exchange of both EL3 and CT regions of D1B receptors with those of the D1A subtype resulted in a chimeric D1B receptor displaying a statistically significant diminution of its constitutive activity to a level akin to that of wild-type D1A receptors (Fig. 3A).

Some of the molecular determinants underlying D1-like subtype-specific agonist-mediated G protein coupling properties have been identified previously (2,10,11). Nonetheless, the molecular interplay between the extracellular and intracellular domains that may impart the D1-like subtype-specific G protein coupling properties remain unclear. As shown in Fig. 3B, the DA-mediated maximal activation of AC activity was unchanged in cells expressing D1A-EL3CTB and D1B-EL3CTA chimeras compared with their parent receptors, i.e. wild-type and chimeric D1A receptors display a higher DA-mediated maximal activation of AC as compared with their respective counterparts. To examine further the role of EL3 and CT in shaping the intramolecular interactions governing subtype-specific G protein-coupling properties, dose-response curves were performed in HEK 293 cells expressing similar levels of total functional number of wild-type or chimeric receptors (~1 pmol/mg protein). Under these experimental conditions, we observed that the subtype-specific DA potency in HEK 293 cells was modulated differentially by EL3 and CT regions (Fig. 4). As depicted in Fig. 4, DA potency (as indexed by EC_{50} values) is ~10-fold higher in HEK 293 cells expressing the wild-type D1B receptor in comparison with the D1A subtype. In agreement with binding results, EL3 region exerts a partial modulation of DA potency in cells expressing D1A-EL3B or D1B-EL3A chimeras (Fig. 4). In striking contrast to what would be expected from constitutively active GPCRs, cells expressing D1A-CTB chimera displayed a decreased DA potency in comparison with cells expressing wild-type D1B receptors but also

significantly lower than the potency value for wild-type D1A receptors (Fig. 4A & B). Interestingly, insertion of EL3B region into the D1A-CTB chimera rescued the DA potency for the chimeric receptor, i.e. by bringing the EC_{50} value closer to that of wild-type D1B receptor (Fig. 4A & B). Indeed, the D1A-EL3CTB chimera displayed ~6-fold increase in DA potency compared with wild-type D1A receptor. As reported previously (11), D1B-CTA chimera displayed a statistically significant decrease in DA potency compared with wild-type D1B receptors (Fig. 4C & D). These results are consistent with a role of the CTA region in imparting to heptahelical D1-like receptors a constrained conformation. This assertion is further supported by our results showing that HEK 293 cells expressing wild-type D1A receptors display higher DA potency in comparison to those transfected with the D1B-CTA chimera (Fig. 4C & D). Importantly, CTA-induced constrained conformation was attenuated by the insertion of the EL3A into D1B-CTA chimera, which brought the EC_{50} to a value indistinguishable from cells expressing wild-type D1A receptors (Fig. 4C & D). Overall, these results suggest that interfering intramolecular interactions between EL3 and CT regions also control DA potency in HEK 293 cells.

DISCUSSION

In the present study, we have used a functional complementation of chimeric D1-like receptors to address the molecular interplay between an extracellular region, EL3, and an intracellular region, CT, in coordinating the functional properties (ligand binding, G protein coupling, and receptor number) and multiple active conformations of GPCRs. By constructing chimeric receptors in which EL3 and/or CT were exchanged between the D1A and D1B receptors, we were able to establish unequivocally that interfering, synergistic and additive intramolecular interactions put forth by these regions coordinate D1-like subtype-specific functional properties and multiple active GPCR conformations.

The prime objective of our study was to explore the underlying molecular basis for constitutively active wild-type and chimeric forms of D1-like receptors displaying similar increased agonist affinity but unexpectedly divergent agonist potency. Such dissociation of DA potency and affinity deviates from one of the established paradigms for CAM GPCRs, i.e. increased agonist affinity translates into increased agonist potency (2,25,26). An interesting finding from our studies is the potential role EL3 plays in controlling the CT-mediated regulation of D1-like subtype-specific DA potency in HEK 293 cells. Evidence pertaining to the role of EL3 in regulating GPCR coupling function remains minimal (27). Interestingly, a study using EL3 chimeras made between the hamster β_2-adrenergic receptor (G_s-coupled GPCR) and rat α_{1A}-adrenergic receptor (G_q-coupled GPCR) has serendipitously found that EL3 can modulate β_2-adrenergic receptor/G protein affinity (28). In agreement with the latter study, we show that the partial modulation of DA potency induced by the different EL3 regions of D1-like receptors

(increase for D1A-EL3B and decrease for D1B-EL3A) is consistent with the partial modulation of DA affinity observed herein and previously (10). Thus, we believe that the EL3 primary structure plays a key role in shaping the CT-induced intramolecular interactions regulating the D1-like subtype-specific G protein coupling properties. Most importantly, this issue is best highlighted by the insertion of EL3 of the D1B subtype into the D1A-CTB chimera, which imparts to the constitutively active D1A-EL3CTB chimera an increased DA affinity and potency.

Meanwhile, the CT region has also been shown to regulate agonist-induced phosphorylation, desensitization and internalization of several GPCR types including D1A receptors (29-34). Therefore, the observed loss of agonist potency of D1A-CTB chimera in HEK 293 cells could be potentially explained by a constitutive desensitization of the receptor. Indeed, there is evidence that CAM GPCRs are subjected to desensitization in the absence of agonist stimulation (35-37). If constitutive desensitization plays a significant role in regulating DA potency in HEK 293 cells expressing D1A-CTB, this process is attenuated by the insertion of EL3 region of the D1B receptor into the D1A-CTB chimera as indexed by DA potency. At present time, the issue of constitutive desensitization remains to be resolved fully. However, we believe that the loss of DA potency exhibited by the constitutively active D1A-CTB chimera is likely to be explained for the most part by CT-specific intramolecular interactions that underlie the agonist-induced receptor conformation for G protein coupling rather than constitutive or tonic desensitization. This idea is supported by findings showing that DA-induced maximal stimulation of AC in HEK 293 cells expressing D1A-CTB is

significantly increased instead of being decreased when compared with wild-type D1-like receptors (11) or D1A-EL3CTB (data not shown).

In fact, results pertaining to agonist-mediated maximal activation of AC address an important issue raised by our previous study showing that chimeric D1-like receptors harboring the EL3 region from their wild-type counterparts exhibited a full switch of DA-mediated maximal activation of AC in HEK 293 cells (10). In striking contrast, the extent of DA-mediated maximal activation of AC (higher for D1A and lower for D1B) was not significantly changed using chimeric receptors in which sequences composed of TM6, TM7, EL3 and the CT (TRL region) were switched between the D1A and D1B subtypes (10). In the present study, we demonstrate that in a similar fashion to TRL chimeras (10), the constitutive activity was switched whereas the extent of agonist-mediated maximal activation of AC was not affected by the exchange of both EL3 and CT regions. Previously, we showed that EL3 controls the extent of agonist-mediated maximal stimulation of AC displayed by D1-like receptor subtypes in HEK 293 cells (10). Thus, other structural determinants within the TRL of D1A and D1B receptors must prevent EL3 from regulating DA-mediated maximal activation of AC. Based on results obtained with EL3CT chimeras, we propose that these structural determinants are located within the CT. Importantly, our data suggest that TM6 and TM7 residues do not prevent EL3 from exerting its effects on agonist-mediated maximal activation (10). This idea is further supported by our studies using D1A and D1B receptors harboring single point mutations of their variant TM6 and TM7 residues. These TM6 and TM7 single-point mutant receptors do not exhibit any major changes in their agonist-independent and dependent activation properties (R. M. Iwasiow and M. Tiberi, unpublished data). Thus, EL3 and

CT appear to impose on the overall receptor conformation interfering intramolecular interactions in controlling the extent of DA-mediated maximal activation of AC in HEK 293 cells.

Furthermore, we have also observed that EL3 and CT regions play an important role in controlling the functional number of D1A and D1B receptors in HEK 293 cells. For instance, the chimeric D1A-CTB receptor exhibited a drastically reduced total functional receptor number, possibly reflective of its high constitutive activity profile, although wild-type D1B receptors did not show lower amount of total functional receptor number in comparison to HEK 293 cells expressing the wild-type D1A subtype. Meanwhile, marked structural instability has been reported as a result of constitutive activation of mutant GPCRs (24,38,39). Thus, one likely possibility is that swapping of CT region of the D1A subtype with that of the D1B receptor imparts to the D1A-CTB chimera a decreased structural stability, which happens to be functionally rescued by an insertion of the EL3B region (D1A-EL3CTB chimera). It is worth mentioning that the extent of the total functional D1-like receptor number may also be explained in part by a role of EL3 and/or CT regions in controlling the D1-like chimera turnover mediated by internalization-independent and dependent degradation processes (29,40,41).

Taken together, the findings of our study suggest that distinct molecular interplays between EL3- and CT-regions regulate the D1-like subtype specific ligand binding and G protein coupling properties. However, these molecular interplays may also require residues located outside the TRL, notably for the agonist-mediated maximal activation of AC. Furthermore, the distinct molecular interplays between EL3 and CT may also underlie the multiple active conformations adopted by constitutively active D1-like

subtypes, which can be grouped into receptors displaying increased DA affinity with decreased DA potency and structural stability (D1A-CTB chimera), increased DA affinity with increased DA potency and structural stability (D1A-EL3B chimera), or increased DA affinity with increased DA potency and unchanged structural stability (wild-type D1B receptors and D1A-EL3CTB chimera). The topological locations of EL3 and CT suggest that they may serve as conformational switches in α-helical packing and/or movement/tilting of the TM6 and TM7 of D1A and D1B receptors, a biophysical process involved in rhodopsin and β_2-adrenergic receptor activation (27,42-46). Importantly, regulation of TM6 and TM7 intramolecular bonds has also been implicated in agonist-independent and dependent activation of GPCRs (27,42-52).

In conclusion, our study has clearly demonstrated that a set of molecular interplays between EL3 and CT of the D1A and D1B receptors controls the underlying spatial relationships of D1-like subtype-specific active conformations. Whether acting together or against each other, these interactions define the spatial relationships underlying DA binding, as well as agonist-dependent and independent G protein coupling properties of D1-like receptors. Further studies are underway in our laboratory to identify specific residues of the CT and EL3 that participate in the formation of these intramolecular bonds. Finally, our results may be of potential physiological relevance for the treatment of pathological conditions caused by a compromised D1-like receptor function (9). This is further underscored by the identification of missense and nonsense mutations in the EL3 region of the D1B receptor that may play a role in the phenotypic expression of neuropsychiatric disorders (53,54).

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Table I. Dissociation constants (K_d) for wild-type and chimeric D1-like receptors.

K_d values (nM) are expressed as geometric means ± S.E. of five experiments done in duplicate determinations. [^{3}H]SCH, N-[methyl-^{3}H]SCH23390; DA, dopamine;
*, $p < 0.05$ when compared with D1A; Ψ, $p < 0.05$ when compared with D1B; #, $p < 0.05$ when compared with D1A-EL3CTB; †, $p < 0.05$ when compared with D1B-EL3CTA.

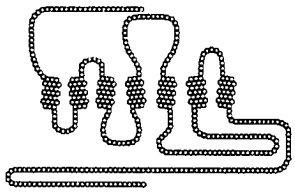
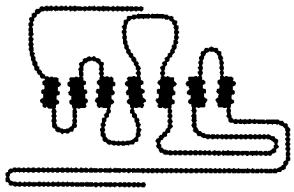
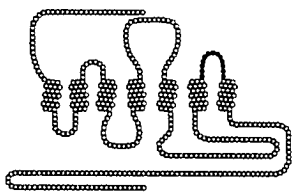
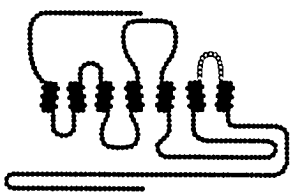
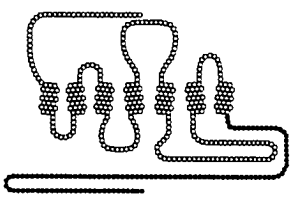
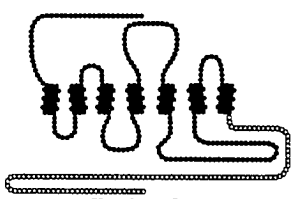
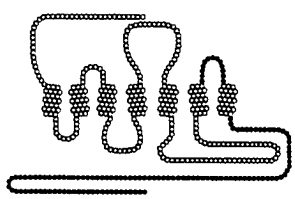
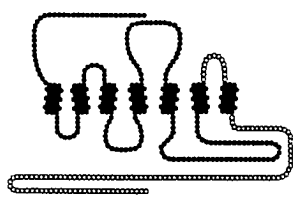
	[^{3}H]SCH	DA
 D1A	**0.39** ± 0.02 (#)	**10010** ± 511 (#Ψ)
 D1B	**0.52** ± 0.02 (#)	**741** ± 25 (†*)
 D1A-EL3B	**0.45** ± 0.03 (#)	**3659** ± 115 (*#†Ψ)
 D1B-EL3A	**0.45** ± 0.02 (#)	**1219** ± 79 (*#†Ψ)
 D1A-CTB	**0.30** ± 0.02 (†Ψ)	**1035** ± 80 (*#†)
 D1B-CTA	**0.51** ± 0.01 (#)	**7047** ± 232 (*#†Ψ)
 D1A-EL3CTB	**0.26** ± 0.01 (*†Ψ)	**680** ± 46 (*†)
 D1B-EL3CTA	**0.54** ± 0.02 (#)	**11232** ± 120 (#Ψ)

Figure 1. Net changes in standard free energy values for DA affinity of EL3CT chimeras relative to wild-type receptors.

Standard free energy values (ΔG_o) of DA affinities for wild-type and chimeric D1-like receptors were computed as $-RT\ln(1/K_d)$. Variations in standard free energy ($\Delta\Delta G_o$) values between wild-type D1A and D1B receptors ($\Delta\Delta G_o^{D1A \rightarrow D1B}$), wild-type and chimeric D1A receptors ($\Delta\Delta G_o^{D1A \rightarrow D1A\ chimera}$), wild-type D1B and D1A receptors ($\Delta\Delta G_o^{D1B \rightarrow D1A}$), and wild-type and chimeric D1B receptors ($\Delta\Delta G_o^{D1B \rightarrow D1B\ chimera}$) were also calculated. The sum of individual $\Delta\Delta G_o$ values of EL3A and CTA, or EL3B and CTB were used to obtain the $\Delta\Delta G_o$ values for EL3A+CTA ($\Delta\Delta G_o^{D1B \rightarrow EL3A+CTA}$) and EL3B+CTB ($\Delta\Delta G_o^{D1A \rightarrow EL3B+CTB}$), respectively. Net changes were computed using $\Delta\Delta G_o$ values to determine whether differences between D1A-EL3CTB and D1A-EL3B+D1A-CTB (left panel) or D1B-EL3CTA and D1B-EL3A+D1B-CTA (right panel) were statistically different.

*, $p < 0.05$ when compared to a value of zero; #, $p < 0.05$ when compared with EL3CTB.

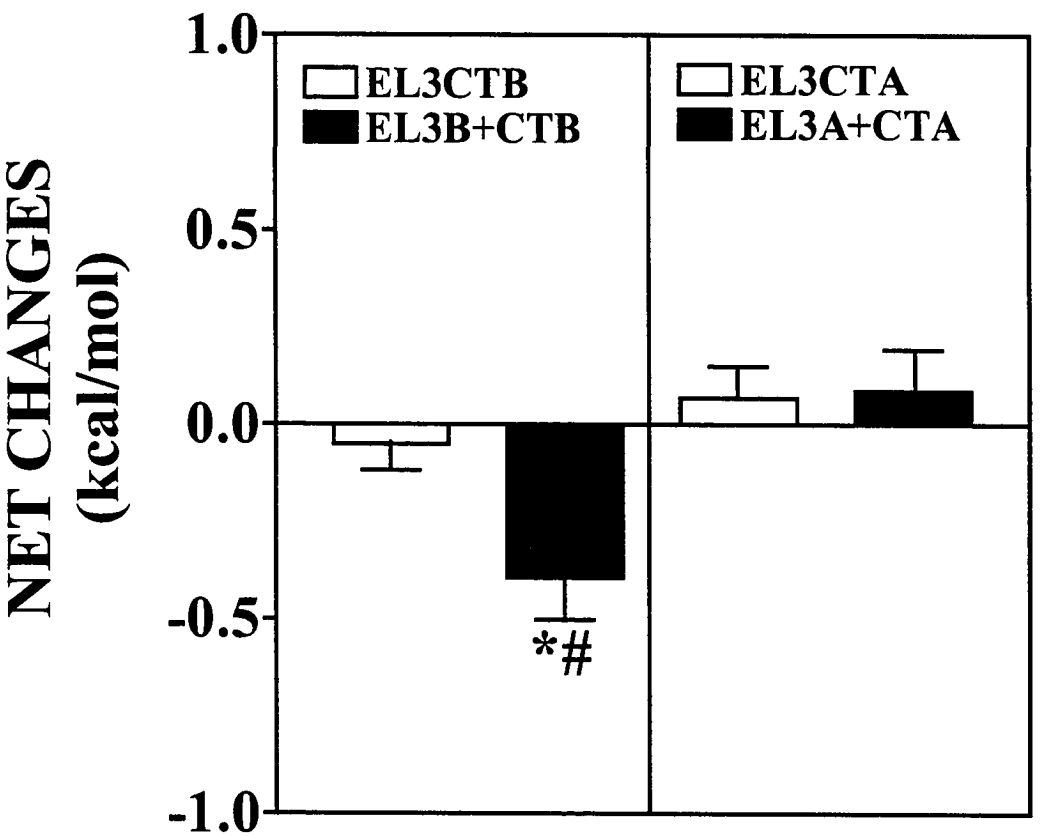

NET CHANGES
(kcal/mol)
1.0
0.5
0.0
-0.5
-1.0
EL3CTB
EL3B+CTB
EL3CTA
EL3A+CTA
*#

Figure 2. Total functional receptor number for wild-type and chimeric D1-like subtypes expressed in HEK 293 cells.

Values are expressed as arithmetic means ± S.E. of five experiments done in duplicate determinations of wild-type and chimeric D1A receptors (**A**), and wild-type and chimeric D1B receptors (**B**). Net changes of total functional receptor number of chimeric D1A receptors relative to the wild-type D1A receptor (**C**), and of chimeric D1B receptors relative to the wild-type D1B receptor (**D**) are shown.

A, *, $p < 0.05$ when compared with D1A; #, $p < 0.05$ when compared with D1A-EL3B.

B,Ψ, $p < 0.05$ when compared with D1B; †, $p < 0.05$ when compared with D1B-EL3A.

C,*, $p < 0.05$ when compared with a value of zero; #, $p < 0.05$ when compared with EL3B+CTB.

D,Ψ, $p < 0.05$ when compared with a value of zero; †, $p < 0.05$ when compared with EL3A+CTA.

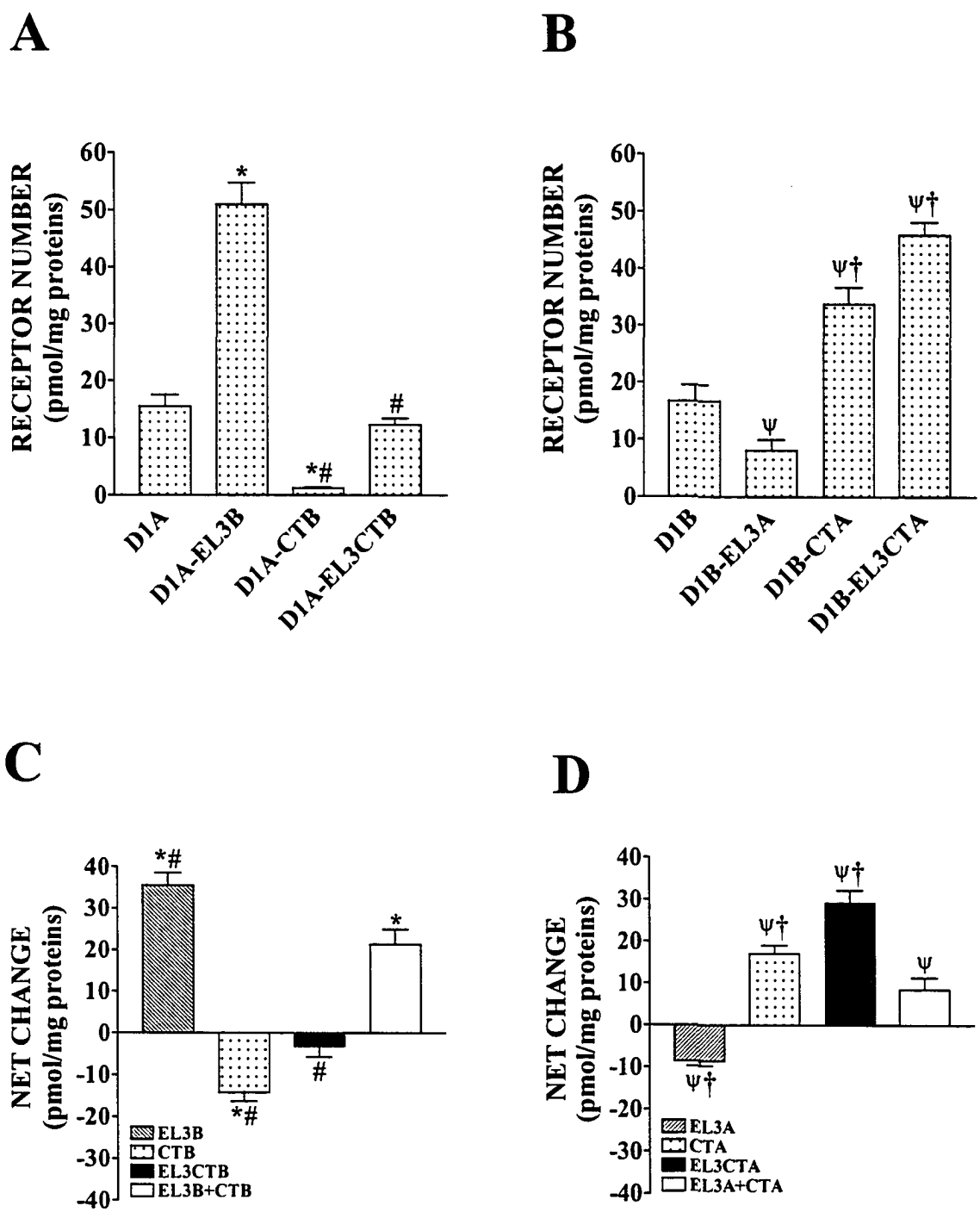
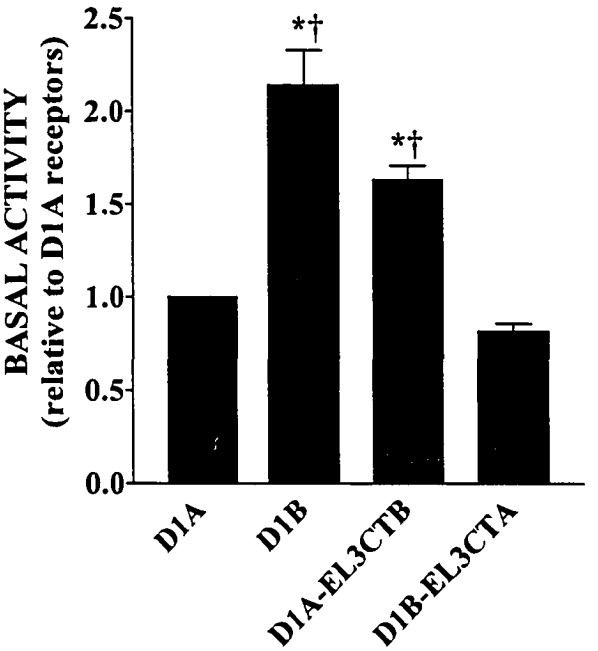

A
RECEPTOR NUMBER (pmol/mg proteins)
60
50
40
30
20
10
0
*
*#
#
D1A
D1A-EL3B
D1A-CTB
D1A-EL3CTB
B
RECEPTOR NUMBER (pmol/mg proteins)
ψ
ψ†
D1B
D1B-EL3A
D1B-CTA
D1B-EL3CTA
C
NET CHANGE (pmol/mg proteins)
40
30
20
10
0
-10
-20
-30
-40
*#
#
EL3B
CTB
EL3CTB
EL3B+CTB
D
NET CHANGE (pmol/mg proteins)
EL3A
CTA
EL3CTA
EL3A+CTA

Figure 3. Constitutive activity and DA-mediated maximal stimulation of AC by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.

Constitutive activation and DA-mediated maximal stimulation of AC are expressed as geometric and arithmetic means ± S.E. of eight experiments done in triplicate determinations, respectively. The total functional receptor number in picomole/mg of membrane proteins (expressed as the arithmetic mean ± S.E.) was 16.2 ± 1.8 (D1A), 14.4 ± 1.0 (D1B), 11.6 ± 0.8 (D1A-EL3CTB) and 24.6 ± 3.0 (D1B-EL3CTA). Basal and maximal levels of AC activity were determined in single wells of a 6-well dish using whole cell cAMP assays in the absence or presence of 10 μM DA.
A, Basal activity of wild-type and chimeric D1-like receptors. **B**, DA-mediated maximal stimulation of AC activity in HEK 293 cells transfected with wild-type and chimeric D1-like receptors.
*, $p < 0.05$ when compared with D1A; #, $p < 0.05$ when compared with D1A-EL3CTB; †, $p < 0.05$ when compared with D1B-EL3CTA.

A


BASAL ACTIVITY
(relative to D1A receptors)
2.5
2.0
1.5
1.0
0.5
0.0
*†
*†
D1A
D1B
D1A-EL3CTB
D1B-EL3CTA

B

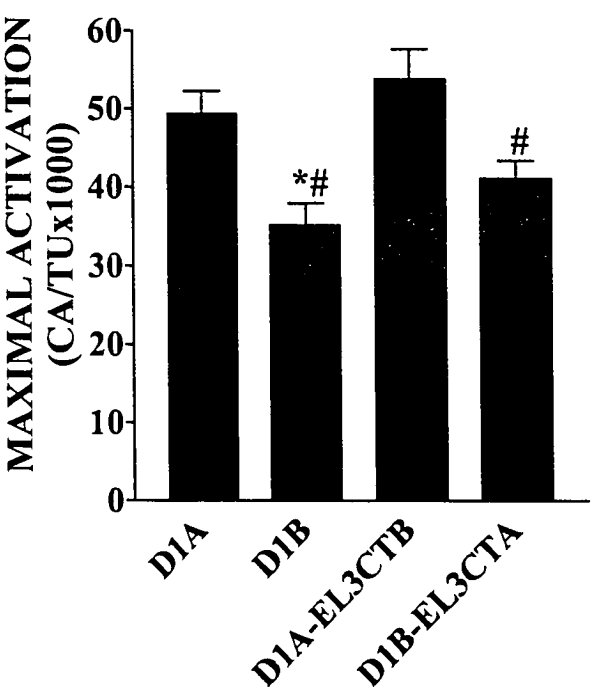
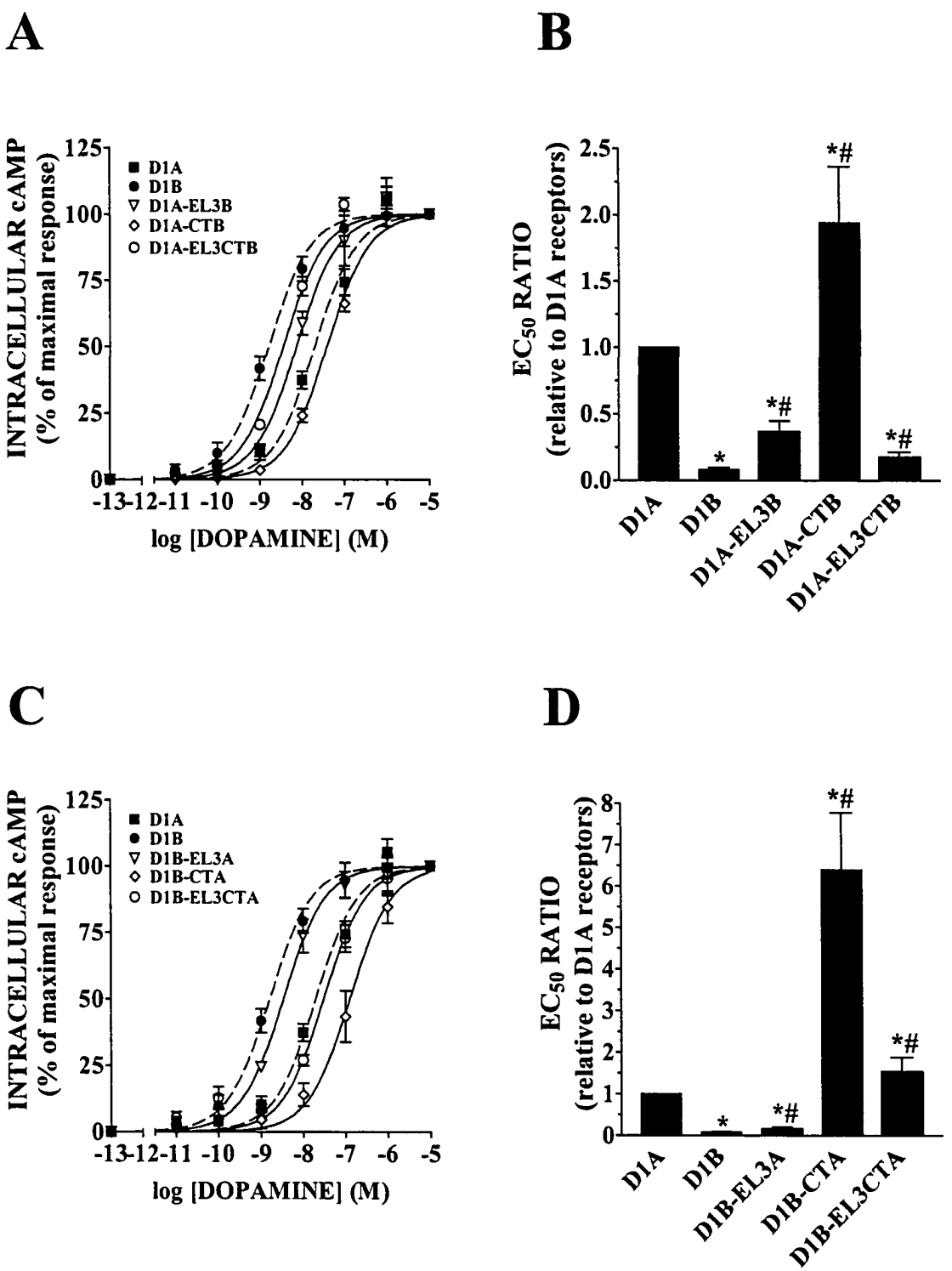

MAXIMAL ACTIVATION
(CA/TUx1000)
60
50
40
30
20
10
0
*#
#
D1A
D1B
D1A-EL3CTB
D1B-EL3CTA

Figure 4. Dose-response curves of DA for AC stimulation by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.

HEK 293 cells transfected with wild-type or chimeric D1-like receptors using the following amounts of DNA per dish: 0.02 μg of D1A, 0.02 μg of D1B, 0.02 μg of D1A-EL3B, 5.0 μg of D1A-CTB, 0.04 μg of D1A-EL3CTB, 0.1 μg of D1B-EL3A, 0.02 μg of D1B-CTA, or 0.02 μg of D1B-EL3CTA. Under these experimental conditions, the total functional receptor numbers were similar. The values in picomole/mg of membrane protein (expressed as the arithmetic mean ± S.E.) were 1.6 ± 0.3 (D1A), 1.4 ± 0.3 (D1B), 0.8 ± 0.3 (D1A-EL3B), 0.8 ± 0.3 (D1B-EL3A), 1.4 ± 0.3 (D1A-CTB), 0.7 ± 0.2 (D1B-CTA), 1.1 ± 0.2 (D1A-EL3CTB) and 0.9 ± 0.2 (D1B-EL3CTA). Intracellular cAMP levels were measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA as described under "Experimental Procedures" and plotted as a function of log of DA concentrations. Each point is the arithmetic mean ± S.E. of eight experiments done in triplicate determinations and expressed as percentage of maximal activation obtained with the respective wild-type or chimeric receptor after subtracting the basal value. Curves were analyzed by simultaneous curve fitting using ALLFIT. Statistical significance was determined using unconstrained and constrained simultaneous curve fitting. For the graphical representation, dose-response curves of D1A and D1B chimeras are shown in **A** and **C**, respectively. The EC_{50} values are as follows (in nM ± approximate S.E. as obtained with ALLFIT): 19.9 ± 3.0 (D1A), 1.6 ± 0.2 (D1B), 7.3 ± 1.1 (D1A-EL3B), 38.6 ± 6.0 (D1A-CTB), and 3.5 ± 0.5 (D1A-EL3CTB). 3.4 ± 0.5 (D1B-EL3A), 127.1 ± 19.3 (D1B-CTA), and 30.7 ± 4.7 (D1B-EL3CTA). The EC_{50} ratios of the wild-type D1B and chimeric receptors relative to the D1A subtype are shown as values ± approximate S.E. (as obtained with ALLFIT) in **B** and **D**. The values are as follows: 0.08 ± 0.02 (D1B), 0.37 ± 0.08 (D1A-EL3B), 0.17 ± 0.04 (D1B-EL3A), 1.94 ± 0.42 (D1A-CTB), 6.4 ± 1.4 (D1B-CTA), 0.18 ± 0.04 (D1A-EL3CTB) and 1.5 ± 0.3 (D1B-EL3CTA). *, $p < 0.05$ when compared with D1A; #, $p < 0.05$ when compared with D1B.


A
B
C
D
INTRACELLULAR cAMP
(% of maximal response)
125
100
75
50
25
0
-13 -12 -11 -10 -9 -8 -7 -6 -5
log [DOPAMINE] (M)
D1A
D1B
D1A-EL3B
D1A-CTB
D1A-EL3CTB
D1B-EL3A
D1B-CTA
D1B-EL3CTA
EC50 RATIO
(relative to D1A receptors)
2.5
2.0
1.5
1.0
0.5
0.0
8
7
6
5
4
3
2
1
0
*
*#

CHAPTER 3

Manuscript

Supplemental information regarding the construction of chimeric receptors is provided in Appendix 2.

Role of the cytoplasmic helix-8 region of D1-like dopaminergic receptors in conferring subtype-specific ligand binding and G protein coupling properties.

Running Title: The fourth intracellular loop of D1-like receptors

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Keywords: dopamine, D1-like receptor, fourth intracellular loop, helix-8, ligand binding, constitutive activation, G proteins.

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ABSTRACT

Recently, we have shown that the cytoplasmic tail (CT) regulates the subtype-specific ligand binding and G protein-coupling properties displayed by the D1-like dopaminergic receptors (Jackson, A., Iwasiow, R. M., and Tiberi, M (2000) *FEBS Lett.* 470, 183-188). In the present study, we investigate whether the putative α-helix region (helix-8) of the CT, also known as the fourth intracellular loop (IL4), underlies the CT-conferred functional properties of dopamine (DA) D1-like receptors. For this purpose, we constructed chimeric receptors in which the IL4 was exchanged between the human D1A and D1B receptors. The resulting chimeras expressed in human embryonic kidney (HEK 293) cells bind to DA with an affinity reminiscent of their respective wild-type counterparts while the antagonist binding profile remains unchanged. These chimeras also displayed a full switch in the agonist-independent/ constitutive activity. Moreover, DA potency in cells expressing chimeric D1B receptors harboring the IL4 region of the D1A subtype (D1B-IL4A) was significantly reduced in comparison with cells expressing wild-type D1B receptors. Interestingly, chimeric D1A receptors harboring the IL4 region of the D1B subtype did not display a reciprocal increase in DA potency. Taken together, our results show for the first time that differences in the primary sequence of the helix-8 region of G protein-coupled receptors (GPCRs) play a crucial role in imparting subtype-specific ligand binding and G protein coupling properties. Additionally, our findings suggest the need for additional domains located outside of the IL4 region for the full expression of the CT-conferred functional properties of the D1A dopaminergic subtype.

INTRODUCTION

Dopamine (DA), a catecholamine neurotransmitter, mediates a variety of physiological effects in the brain and periphery through the activation of transmembrane receptors belonging to the superfamily of G protein-coupled receptors (GPCRs). Five distinct genes encoding the DA receptors have been isolated and classified into D1-like and D2-like receptors based on their ability to activate or inhibit adenylyl cyclase (AC) activity, respectively. In mammals, two genes encoding D1-like dopaminergic receptors are known and referred to as D1A and D1B subtypes (also called D1 and D5, respectively) (1). Functionally, the D1B receptor distinguishes itself from the D1A subtype by a higher constitutive activity (agonist-independent activity), increased affinity and potency for agonists, decreased affinity for antagonists, and a lower agonist-mediated maximal activation (2). Importantly, a recent study has indicated a potential physiological role for the D1B receptor constitutive activity in mediating estrogen-induced stimulation of hypothalamic atrial natriuretic factor neurons (3). Moreover, recent studies showing D1-like subtype mRNA expression and/or activity in human breast and neuroendocrine gastrointestinal tumor cells (4, 5) bring to attention the possible involvement of constitutive activity of these receptors in pathophysiological conditions, as shown previously with other constitutively active mutant GPCRs. (6) As such, the development of D1A and/or D1B subtype-specific ligands may prove beneficial to the treatment of disorders associated with the dysregulation of D1-like receptor signaling (7-9). However, the structural determinants that underlie the functional properties of D1-like receptors remain to be fully elucidated.

Recently, we have shown that the CT plays a crucial role in conferring the subtype-specific DA binding affinities and G protein coupling properties displayed by the D1-like receptors (10). Although the Gln^{439} residue located on the distal part of the D1B receptor's CT region has been implicated in exclusive regulation of the high DA affinity displayed by this D1-like subtype (11), the precise CT structural determinants imparting the D1-like subtype-specific DA binding and G protein coupling properties remain to be identified. Recently, using serially truncated forms of the rat D1A receptor, we have observed that a D1A receptor truncated at Cys^{351}, and thus harboring only the N-terminal segment of the CT, displayed an increased DA affinity and constitutive activity (12). These results suggest that this CT region plays a key role in regulating the equilibrium between the inactive and active states of the D1A receptor. Interestingly, Cys^{351} of the D1A dopaminergic receptors belongs to a group of conserved cysteine residues shown to be palmitoylated within the CT domains of certain GPCRs (13). As palmitoylation of these residues has been proposed to anchor the CT to the membrane, the encompassed N-terminal segment of the CT has been referred to as the fourth intracellular loop (IL4) (14, 15). Interestingly, the IL4 has recently been suggested to exist independently of palmitoylation (16). Moreover, its unique α-helical conformation, deduced from a crystal structure of rhodopsin, has been postulated to govern the formation of IL4 (17). As a result, the IL4 segment has recently been referred to as α-helix 8 region, whose involvement in GPCR activation is gaining increasing support (18). Indeed, early mutagenesis studies have indicated the importance of IL4 for G protein coupling of rhodopsin and β_2-adrenergic receptors (β_2-AR) (19, 20). Subsequently, using synthetic peptides, the IL4 of rhodopsin was shown to interact directly with α and γ subunits of G

protein transducin (G_t) (21, 22). In addition to its role in binding to heterotrimeric G proteins, the IL4 region has been shown to modulate the regulatory properties of GPCRs. Substitution of a Gly residue for the palmitoylated Cys^{341} of the β_2-AR abolished the formation of IL4 and largely uncoupled the receptor from G_s (23). The absence of IL4 was postulated to alter the structural organization of the CT of the β_2-AR resulting in an exposure of previously buried phosphorylation sites to the regulatory activity of intracellular kinases leading to receptor desensitization (24). Taken together, the results demonstrate that the presence of IL4 modifies the topology of the CT of GPCRs with varied effects on G protein coupling.

To date, the contribution of IL4 to the function of D1-like dopaminergic receptors has not been studied in detail. Nonetheless, the presence of palmitoylated cysteine residues (Cys^{347} and Cys^{351}) has been demonstrated for the CT of the D1A receptor (13). Elimination of Cys palmitoylation sites of the D1A receptor, however, failed to affect receptor–G protein coupling suggesting that dopaminergic and adrenergic receptors are differentially regulated (25). The importance of Cys palmitoylation for the function of the D1B receptor has not been addressed yet. However, conserved Cys residues corresponding to Cys^{347} and Cys^{351} of the D1A receptor are present in the D1B subtype at Cys^{375} and Cys^{379} of the CT. Most importantly, the primary structure of the D1A and D1B subtype extending from the seventh transmembrane region to the two conserved Cys residues (putatively forming helix-8 or IL4), displays striking differences that may impart the CT-conferred D1-like receptor-specific functional features.

To study the potential role of IL4 in the CT-conferred ligand binding and G protein coupling properties of the D1-like receptors, chimeric receptors were generated in which

the variant IL4 residues of the CT were exchanged between the wild-type D1A and D1B receptors. Our results strongly suggest that IL4 is an important structural determinant underlying the CT-conferred D1-like subtype-specific characteristics.

MATERIALS AND METHODS

Drugs

N-[methyl-^{3}H]SCH23390 (82 Ci/mmol), [^{3}H]adenine (27 Ci/mmol), and [^{14}C]cAMP (275 mCi/mmol) were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Dopamine, cis-flupentixol, (+)-butaclamol, and 1-methyl-3-isobutylxanthine (IBMX) were from Sigma/Research Biochemicals International (Oakville, Ontario, Canada).

Construction of chimeric human D1A and D1B receptors

To construct the chimeric receptors, we took advantage of existing chimeras in which the CT was swapped between the human D1A and D1B receptors. All CT residues downstream of the second palmitoylation site were restored to wild-type, leaving only IL4 in a mutated form. In case of the D1A-IL4B chimera, the CT of D1B was terminated at Cys^{379}, followed by D1A residues to complete the tail. In case of the D1B-IL4A chimera, the CT of the D1A receptor was terminated at Cys^{351}, followed by D1B residues for completion of the CT. The swap of the IL4 between the D1A and D1B subtypes was achieved by gene splicing using a PCR-based overlap extension method. With respect to the D1A-IL4B chimera, the first round of PCR generated two fragments: the first fragment coding the C-terminal portion of IL3A, TM6A, EL3A, TM7A, and IL4B and the second fragment coding the CT of the D1A receptor downstream of IL4A. The first fragment was amplified using primers 5'- CACCACAGGTAATGGAAA-3' (forward primer) and 5'-GCAGAAGTGGCTGCACCCCAG-3' (reverse primer). The second

fragment was amplified using primers 5'-TGCAGCCACTTCTGCCCTGCGACGAATAAT-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3'(reverse primer). Likewise, with respect to the D1B-IL4A chimera, the first round of PCR generated two fragments: the first coding the amino terminal of the D1B receptor up to IL4A and the second fragment coding the CT of the D1B receptor downstream of IL4B. The first fragment was amplified using primers 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GCAAAGTCTGTAGCATCCTAAGAGGGT-3' (reverse primer). The second fragment was amplified using primers 5'-TGCTACAGACTTTGCTCCCGCACGCCGGTG-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3'(reverse primer). To facilitate the construction and identification of the chimeric receptors, a silent mutation was introduced in each construct to create a unique restriction endonuclease site. For D1A-IL4B chimera, a *Mlu*I site was introduced at nucleotide sequence corresponding to amino acid residues 331 and 332 (5'-TA**T**GC**C**-3'→5'-TA**C**GC**G**-3') near the 3' end of D1A receptor TM7 region, immediately upstream of the D1B receptor IL4 sequence. For D1B-IL4A chimera, a *Hind*III restriction site was introduced at nucleotide sequence corresponding to residues 367 and 368 (5'-AA**G**GC**A**-3'→5'-AA**A**GC**T**-3'), located 3' of the junction between the D1B receptor TM7 region and D1A receptor IL4 sequence. Amplified fragments were separated on a 1% agarose gel and appropriate bands were excised and purified by QIAEX II gel extraction method (Qiagen, Valencia, CA, USA). Diluted aliquots of the paired fragments were combined and subjected to overlap PCR using appropriate 5' and 3' flanking primers. The resulting PCR products were engineered into the expression vector pCMV5. The D1A-IL4B fragment was subcloned

into D1A-CTB/pCMV5 vector using restriction enzymes *MluI* and *XbaI*. The D1B-IL4A fragment was subcloned into pCMV5 together with the N-terminal portion of D1B-CTA in a 3-piece ligation process. The D1B-CTA fragment was subcloned using restriction enzymes *KpnI* and *HindIII*, while the D1B-IL4 fragment was subcloned using only *HindIII*. The nucleotide sequence of PCR products and cloning sites was confirmed by dideoxy sequencing using Sequenase version 2.0 from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada.).

Cell Culture and Transfection

HEK 293 cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C and 5% CO_2 in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (10μg/ml) (Invitrogen, Burlington, Ontario, Canada.). A modified calcium phosphate-mediated transfection of cells ($2.5x10^6$ cells/dish) was carried out as described (26). Briefly, 100 μl of 2.5 M $CaCl_2$ was added to a plastic tube containing 10 μg of receptor DNA in 900 μl of sterile milli-Q-water and mixed. Then 1000 μl of 2x HEPES-buffered saline (HBS), pH 7.1 (0.28 M NaCl, 0.05 M HEPES, pH 7.0, 1.5 mM Na_3PO_4, pH 7.1) was added dropwise to the DNA-calcium solution, gently mixed by flicking the tube and used to transfect two 100-mm dishes at a time. Typically, 1 ml of transfection mixture was added dropwise to a 100 mm-dish of cells containing 10 ml of complete MEM. When less than 5 μg DNA was used in transfections, empty pCMV5 vector was added to normalize the total amount of DNA. All experiments were performed with cells from 38 to 52 passages.

Membrane Preparation

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK 293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Transfected HEK 293 cells were then washed with cold PBS, scraped from the dish in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5mM EDTA) and centrifuged twice at 40 000x g for 20 min at 4°C. The crude membrane pellet was resuspended in lysis buffer using a Brinkmann Polytron (17 000 rpm for 15 s) and either used immediately (saturation studies) or frozen in liquid nitrogen and stored at –80°C until required (competition studies).

Radioligand Binding Assays

Fresh or frozen membranes were resuspended in binding buffer (final concentrations in the binding 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 performed with 100 µl of membranes in a total volume of 500 µl using N-[methyl-^{3}H]SCH23390 as radioligand. Saturation studies were done on fresh membranes using concentrations of N-[methyl-^{3}H]SCH23390 ranging from 0.01 to 6 nM. Non-specific binding was determined by the addition of 10µM cis-flupentixol (dissolved in milli-Q-water). For competition studies, frozen membranes were thawed on ice and incubated with a constant concentration of N-[methyl-^{3}H]SCH23390 (~0.6 nM) and increasing concentrations of competing ligand. Competition studies using DA were done in the presence of 0.1 mM ascorbic acid (dissolved in milli-Q-water). Binding assays were incubated for 90 min at room temperature and terminated using rapid filtration through

glass fiber filters (GF/C, Whatman). The filters were washed four times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS 6500). Protein concentration was measured using the Bio-Rad assay kit with bovine serum albumin as standard. To determine the equilibrium dissociation constant (K_d) and binding capacity (R) values, binding isotherms were analyzed using the non-linear curve-fitting program LIGAND (27).

Whole Cell cAMP Assay

Regulation of AC activity by wild-type and chimeric receptors was assessed using a whole cell cAMP assay as described previously (2). Following overnight incubation with the DNA-calcium phosphate precipitate, HEK 293 cells were reseeded in 6- or 12-well dishes. The next day, cells were labeled with [^{3}H]adenine (2μCi/ml) in fresh MEM containing 5% (v/v) FBS and gentamicin (10μg/ml) for 18h at 37°C and 5% CO_2. The labeling medium was then removed and HEK 293 cells were incubated in 20 mM HEPES-buffered medium containing 1 mM IBMX in the presence or absence of DA for 30 min at 37 °C. At the end of incubation period, the medium was aspirated and each well filled with 1 ml lysis solution containing 2.5% (v/v) perchloric acid, 0.1 mM cAMP, and [^{14}C]cAMP (2.5-5 nCi, ~5 000-10 000 cpm) for 30 min at 4°C. The lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH (neutralizing solution) and precipitates were sedimented by a low-speed centrifugation (1 500 rpm) at 4 °C. The amount of intracellular [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex (AG 50W-X4) and alumina columns as

described previously (28). The amount of [^{3}H]cAMP (CA) over the total amount of intracellular [^{3}H]adenine (TU) was calculated to determine the relative AC activity (CA/TU). Dose-response curves to DA were analyzed by a four-parameter logistic equation using ALLFIT (29). Receptor expression was determined using a saturating concentration (~6 nM) of N-[methyl-^{3}H]SCH23390.

Statistics

Equilibrium dissociation binding constants (K_d) are expressed using the geometric mean ± S.E. as described (30). All other data are reported as arithmetic means ± S.E. unless stated otherwise. All statistical tests used in this study have been described elsewhere (31, 32). Homoscedasticity of variances was assessed using F_{max} or Bartlett tests prior to statistical analyses. One sample *t*-test and analysis of variance (one-way ANOVA) with Newman-Keuls multiple comparison test were performed using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. The level of significance was established at $p < 0.05$.

RESULTS

IL4 modulates receptor number and dopamine binding affinity of D1-like receptors

The binding affinities (K_d values) of the radioligand N-[methyl-^{3}H]SCH23390 for wild-type and chimeric human D1-like receptors obtained during saturation studies are shown in Table I. Results indicate that the chimeric substitution of IL4 does not impair the ability of the mutant receptors to bind N-[methyl-^{3}H]SCH23390 with high affinity. Indeed, the binding affinities of D1A-IL4B and D1B-IL4A chimeras are higher than those of their respective parental wild-type receptors (Table I). However, saturation studies revealed a drastic decrease in the total functional receptor number of the D1A-IL4B chimera, in line with previously reported decrease in the total functional receptor number in HEK 293 cells expressing the D1A-CTB chimera (10). In contrast, the binding capacity of D1B-IL4A chimera was unaffected by the exchange of the IL4 region and reflected the total functional receptor number of the wild-type D1B receptor under the same transfection conditions.

To assess the contribution of IL4 to the pharmacological profile of D1-like receptors, competition studies were performed to examine the changes in agonist and antagonist affinities for wild-type and chimeric receptors. As previously reported (2), the endogenous agonist, DA, exhibited a higher affinity for the D1B receptor compared with the D1A subtype (Table I). Upon the reciprocal exchange of the IL4 region, the D1A-IL4B chimera displayed an increased affinity for DA that was reminiscent of the binding affinity for the wild-type D1B receptor. On the other hand, the D1B-IL4A chimera bound DA with partially reduced affinity that did not reach the levels of the wild-type D1A

receptor, suggesting that IL4 cannot be ascribed as the sole domain underlying this CT-conferred property of the D1A receptors (10). Overall, these findings indicate an important role of IL4 in modulating DA binding to the D1-like receptors.

IL4 does not govern the binding affinity of antipsychotics

Previous studies have demonstrated that antagonists bind with higher affinity to the D1A receptor compared with the D1B subtype (2, 10, 33). To determine whether antagonist binding at the D1-like receptors is dependent on IL4-induced conformational changes, competition studies were performed using cis-flupentixol and (+)-butaclamol, two antipsychotic drugs that display inverse agonism at both D1-like receptor subtypes. In agreement with previous results obtained upon the exchange of the CT (10), the swap of the IL4 segment between the D1A and D1B receptors did not greatly alter the binding affinity of the antipsychotics (Table I). Although slightly increased, the binding affinities of both chimeras for cis-flupentixol were not statistically different from their respective parent receptors. Similar shift in affinity was also observed with (+)-butaclamol, where the increase in affinity was greater and statistically significant for both chimeras compared with their parent receptors. In addition, we carried out competition studies to test the binding affinity of the benzazepine SCH23390, a selective D1-like antagonist that behaves as a partial agonist at both D1A and D1B receptors when expressed in HEK 293 cells (2). Although structurally different from both flupentixol and (+)-butaclamol, SCH23390 also exhibited increased binding affinity for the two chimeras compared with their respective parent receptors. Similar trend was observed during saturation studies using N-[methyl-^{3}H]SCH23390 as radioligand (Table I).

IL4 underlies the CT-conferred constitutive activity phenotype of the D1-like receptors

To assess the role of IL4 in agonist-independent activation of AC, a series of cAMP assays was performed on cells expressing wild-type and chimeric D1-like receptors. Following a transfection with 5 μg DNA for maximum expression of each of the four receptors, agonist-independent activity generated by cells expressing the D1B receptor was approximately 2.5-fold higher than basal cAMP levels generated by cells expressing the D1A subtype (Fig. 1A), as shown previously (2, 10, 33). In addition, substitution of the variant IL4 residues produced a decrease of the agonist-independent activation of D1B-IL4A chimera in comparison with the wild-type D1B receptor. Importantly, the constitutive activation of D1B-IL4A chimera was indistinguishable from that of the D1A subtype. In contrast, a reciprocal increase of the constitutive activity of D1A-IL4B chimera reaching levels of the wild-type D1B receptor was not detected, presumably due to the significantly lower total functional receptor number of D1A-IL4B chimera. Indeed, a linear relationship between receptor number and constitutive activity has been established (2, 34, 35). Concurrently, the investigation of DA-mediated maximal stimulation of AC by the wild-type and chimeric D1-like receptors expressed in HEK 293 cells was carried out. Under the conditions of maximal receptor expression, the DA-mediated maximal activation of AC was higher in cells expressing D1A receptors than D1B receptors (Fig. 1B) as previously reported (2, 10, 33). On the other hand, the exchange of IL4 between the D1A and D1B receptors did not produce statistically significant results relating to DA-mediated maximal activation of AC in cells expressing the D1A-IL4B and D1B-IL4A chimeric receptors. However, our study provided an interesting observation regarding the amount of receptors required to elicit the seemingly

even intracellular accumulation of cAMP in response to maximal DA stimulation. More specifically, the D1A-IL4B chimera displayed a DA-mediated maximal activation of AC akin to that of wild-type and D1B-IL4A receptors, despite a nearly 20-fold decrease in the total functional receptor number. Potentially, these results suggest that the coupling efficiency of the D1A-IL4B chimera to G_s/AC pathways is increased in comparison with D1B-IL4A and wild-type receptors. To address further the agonist-independent and dependent G protein coupling properties of wild-type and chimeric D1-like receptors, dose-response curves were carried out in cells expressing comparable levels of total functional receptor number (~1-2 pmol/mg protein) of wild-type and chimeric receptors. Additional inferences regarding the basal and maximal activation of AC were drawn from ALLFIT best-fitted values obtained for the minimal and maximal response, respectively.

As seen in cells expressing the maximal receptor expression (Fig. 1A), the D1B receptor displayed higher basal activity in comparison with the D1A subtype (Fig. 2A). Similarly, the D1B-IL4A chimera recapitulated its loss of constitutive activity as compared with the wild-type D1B receptor and beyond the levels measured for the D1A subtype. However, in stark contrast to results depicted in Fig. 1A, we were able to show that at a comparable level of total functional receptor number, the D1A-IL4B chimera exhibited a significant increase in constitutive activity when compared with the wild-type D1A receptor. The constitutive activity of D1A-IL4B chimera was indistinguishable from the wild-type D1B receptor (Fig. 2A). Importantly, the exchange of IL4 reproduced the results obtained upon the swap of the entire CT (10), resulting in a full switch of constitutive activity as compared with parent receptors.

To further the underlying role of IL4 in the CT-induced changes of maximal activation of AC elicited by the D1-like receptors, we compared the DA-mediated maximal stimulation of AC in cells expressing comparable levels of wild-type and chimeric receptors using the best-fitted maximal response values obtained by ALLFIT. As shown in Fig. 2B, DA-mediated maximal stimulation of AC was greater in cells expressing the D1A receptor than those expressing the D1B subtype. Interestingly, upon exchange of the IL4 region, the D1A-IL4B chimera exhibited a significant increase in agonist-mediated maximal activation of AC when compared with the wild-type D1A receptor. Conversely, D1B-IL4A chimera displayed a significant decrease of the DA-mediated maximal activation of AC when compared with the wild-type D1B receptor. These results suggest that an exchange of the cytoplasmic helix-8 region regulates in distinct fashion the degree of maximal activation elicited by D1-like receptors, as the swap of the CT led to chimeric receptors that both displayed a significantly higher DA-mediated maximal activation of AC as compared with their parent receptors (10).

The functional role of IL4 in the CT-induced changes in DA potency (EC_{50}) was also assessed using dose-response curves performed in HEK 293 cells expressing similar levels of wild-type and chimeric D1-like receptors (Fig. 2C & D). Under the present experimental conditions and in agreement with previous studies (2, 10, 33), DA potency was ~8-fold greater in HEK 293 cells expressing the wild-type D1B receptor than the wild-type D1A receptor (Fig. 2C & D). Substitution of the variant IL4 residues produced a significant loss of DA potency for both receptors (Fig. 2C). More specifically, D1A-IL4B and D1B-IL4A chimeras displayed a significant 2.5- and 19-fold decrease in DA potency as compared with their respective parent receptors (Fig. 2D). These findings

recapitulate the loss of DA potency displayed by chimeric receptors in which the entire CT was exchanged between the D1A and D1B receptors (10). Such results reinforce the notion that the IL4 segment underlies the CT-induced changes in G protein coupling properties of the D1-like receptors.

DISCUSSION

Recently, the CT has been implicated as a key cytoplasmic region in regulating the distinct DA binding and G protein coupling properties of the human D1-like receptors (10-12). In the study reported here, we have used a mutagenesis approach to narrow down the structural determinant responsible for D1-like subtype-specific DA affinity and G protein coupling properties previously attributed to the CT. Specifically, we have focused our attention on the N-terminal segment of CT also known as IL4. Although initial reports regarding the role of IL4 in GPCR function were contradictory (14, 36-38), current evidence suggests that this domain is important for GPCR binding to and activation of heterotrimeric G proteins. Indeed, studies have shown that IL4 plays an important role in receptor – G protein interactions of rhodopsin and β_2-AR (19-22).

By constructing chimeric receptors in which the IL4 region was exchanged between the human D1A and D1B receptors, we were able to investigate its contribution to the CT-conferred functional properties of D1-like receptors. Analysis of the pharmacological profile has revealed that D1A-IL4B chimera displayed a full switch in DA affinity suggesting that the variant residues found within the IL4 region of D1B receptors lead to a less constrained D1A receptor conformation. In an opposite fashion, the D1B-IL4A chimera exhibited a partial decrease in DA affinity suggesting that IL4 region of the D1A subtype imparts to the D1B receptor a more constrained conformation. However, our findings fall short of the results obtained with the entire CT of the D1A subtype, which produced a full switch in the binding affinity for DA (10). These results suggest that IL4 is not the sole determinant of the CT involved in regulating DA affinity at the D1A

subtype, and thus the apparent effect induced by IL4 of D1A and D1B subtypes is not reciprocally conferred to the chimeric receptors. In contrast, the lack of major changes in the binding affinity of inverse agonists upon the exchange of IL4 mirrors the findings obtained upon the swap of the CT and supports the notion that inverse agonist binding is most likely independent of CT-induced conformational changes (10).

The functional characterization of our chimeras suggests that agonist-independent and -dependent G protein coupling properties of the D1-like subtypes are differentially regulated by IL4. Whole cell cAMP assays demonstrated a reversal of agonist-independent activation of AC upon a swap of IL4 between the D1A and D1B receptors. Moreover, our findings showing that the D1A-IL4B chimera displays constitutive activity reminiscent of the wild-type D1B receptor whereas the D1B-IL4A chimera exhibits constitutive activity comparable to the wild-type D1A receptor, imply a role of IL4 in regulating the thermodynamic equilibrium between the active and inactive conformations of the D1-like receptors. Indeed, critical conformational changes of IL4 believed to represent the transition between inactive and active states of rhodopsin have recently been demonstrated (39). Namely, the IL4 was proposed to function as conformational switch alternating between a helical conformation (inactive state) and a looplike structure (active state) in response to changes in membrane environment. Likewise, a similar concept of a helix to coil transition has been put forward as a possible molecular mechanism underlying the G protein coupling of angiotensin II AT_{1A} receptor (40). Whether a similar role is performed by the IL4 of dopaminergic receptors remains to be investigated further. However, the results of our study clearly support the involvement of

helix-8 of D1-like receptors in determining the extent of agonist-independent activation of AC.

In addition, IL4 appears to exert a distinct influence on the formation of D1-like receptor active states upon DA binding. In agreement with our previous study highlighting the chimeric substitution of the full CT, the exchange of the IL4 segment between the D1A and D1B receptors produced a loss of DA potency in both chimeras. The observed decrease in DA potency is particularly intriguing for the D1A-IL4B chimera, in light of its increased DA binding affinity. Similar uncoupling of DA affinity and potency has been addressed in our earlier studies and suggests the formation of multiple active conformational states (10, 12) (K. Tumova & M. Tiberi, manuscript submitted). This view is further supported by the observed changes in agonist-mediated maximal activation of AC displayed by the chimeric receptors. Despite its loss of DA potency, the D1A-IL4B chimera exhibited a significant increase of the DA-mediated maximal activation of AC when compared with the wild-type D1A receptor. In contrast, the D1B-IL4A chimera displayed a decrease of DA-mediated maximal activation of AC in comparison with the wild-type D1B receptor. It is of note that the third extracellular loop (EL3) has been mapped as the domain underlying the degree of agonist-mediated maximal activation elicited by the D1-like receptors (33). However, new evidence suggests that differences in the primary structure of IL4 of the D1A and D1B subtypes may help explain some of the subtype-specific G protein coupling properties of the D1-like receptors. Above and beyond the role of IL4 in binding of G protein α and γ subunits as illustrated by rhodopsin (21, 22), the D1-like receptors may use IL4 to bind G proteins of distinct subunit composition. Indeed, a differential dependence of the D1A and D1B

receptors on the G protein γ subunit for activation of AC has recently been demonstrated (41).

Taken together, the results of our mutagenesis study support the involvement of IL4 in conferring the distinct DA binding and G protein coupling properties of the D1-like receptors, although some of the functional features displayed by CT chimeras (DA affinity and agonist-mediated maximal activation of AC) were not completely reproduce by the swap of the helix-8 region. These findings indicate that additional cytoplasmic domains located outside of the IL4 are required for the full expression of the CT-conferred properties of the D1-like receptors. Overall, our study shows for the first time that differences in the primary structure of the cytoplasmic helix-8 region underlie the subtype-specific ligand binding and G protein-coupling properties of GPCRs.

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Table I. Dissociation constants (K_d) and binding capacity (R) values for wild-type and chimeric D1-like receptors.

K_d (nM) and R (pmol/mg protein) values are expressed as geometric means ± S.E. of six experiments done in duplicate determinations. [^{3}H]SCH, N-[methyl-^{3}H]SCH23390; DA, dopamine; FLU, flupentixol; BUTA, (+)-butaclamol; SCH, SCH23390.
*, $p < 0.05$ when compared with D1A; Ψ, $p < 0.05$ when compared with D1B; #, $p < 0.05$ when compared with D1A-IL4B; †, $p < 0.05$ when compared with D1B-IL4A.

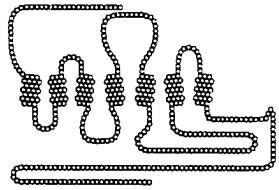
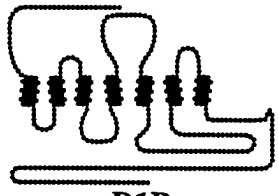
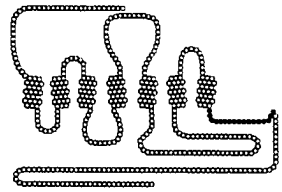
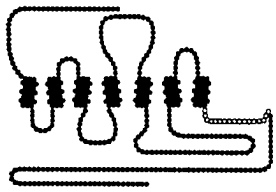
	[^{3}H]SCH	DA	FLU	BUTA	SCH	R
 D1A	**0.44** ± 0.02 (Ψ#†)	**7953** ± 422 (Ψ#†)	**2.51** ± 0.32 (Ψ†)	**3.97** ± 0.29 (Ψ#†)	**0.43** ± 0.03 (#Ψ)	**10.4** ± 1.08 (#)
 D1B	**0.81** ± 0.02 (*#)	**602** ± 7 (*†)	**6.76** ± 0.40 (*#)	**40.9** ± 3.82 (*#†)	**0.86** ± 0.09 (*#)	**16.0** ± 1.10 (#)
 D1A-IL4B	**0.14** ± 0.01 (*Ψ†)	**559** ± 56 (*†)	**1.38** ± 0.08 (Ψ†)	**1.56** ± 0.08 (*Ψ†)	**0.18** ± 0.001 (*Ψ†)	**0.50** ± 0.06 (*Ψ†)
 D1B-IL4A	**0.64** ± 0.03 (*#)	**2488** ± 170 (*Ψ#)	**5.11** ± 0.29 (*#)	**23.8** ± 0.85 (*Ψ#)	**0.59** ± 0.02 (#)	**15.9** ± 0.47 (#)

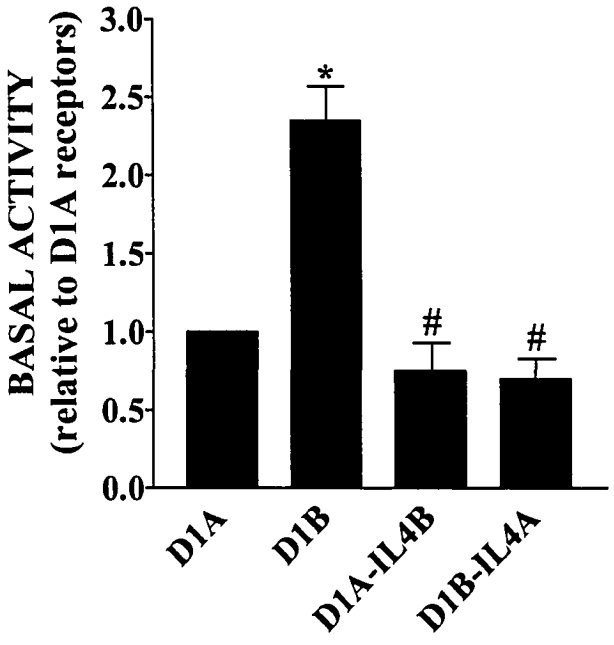
Figure 1. Constitutive activity and DA-mediated maximal stimulation of AC by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.

Constitutive activation and DA-mediated maximal stimulation of AC are expressed as arithmetic means ± S.E. of three experiments done in triplicate determinations. The total functional receptor number in picomole/mg of membrane proteins (expressed as the arithmetic mean ± S.E.) was 12.6 ± 2.1 (D1A), 13.6 ± 0.3 (D1B), 0.6 ± 0.2 (D1A-IL4B), 10.1 ± 1.2 (D1B-IL4A). Basal and maximal levels of AC activity were determined in single wells of a 6-well dish using whole cell cAMP assays in the absence or presence of 10 μM DA.

A, Basal activity of wild-type and chimeric D1-like receptors. **B**, DA-mediated maximal stimulation of AC activity in HEK 293 cells transfected with wild-type and chimeric D1-like receptors.

*, $p < 0.05$ when compared with D1A; #, $p < 0.05$ when compared with D1B.

A


BASAL ACTIVITY
(relative to D1A receptors)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
D1A
D1B
D1A-IL4B
D1B-IL4A

B

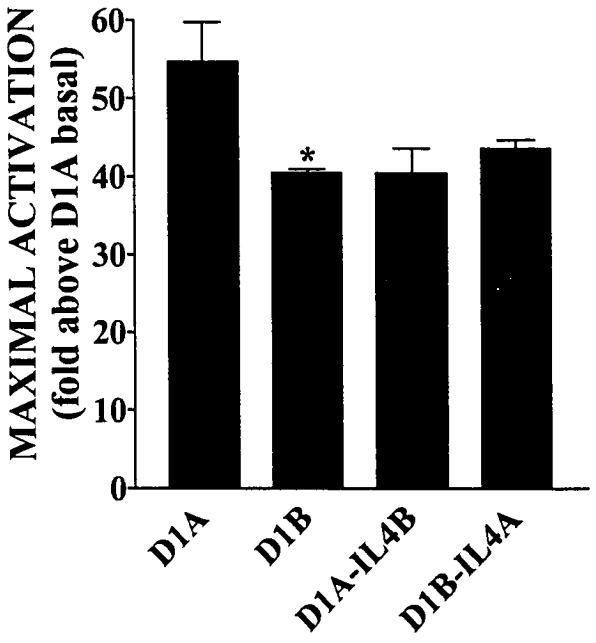
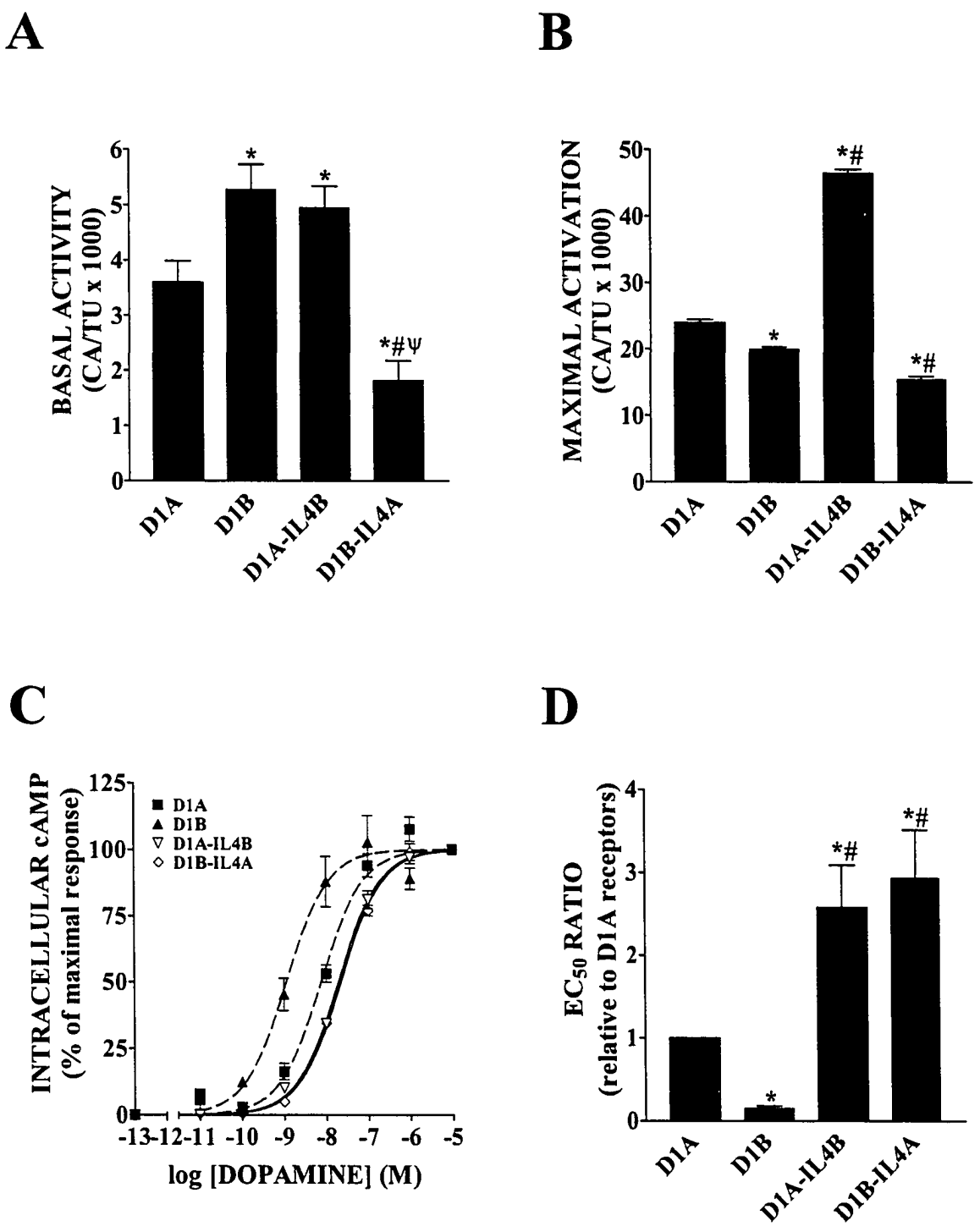

MAXIMAL ACTIVATION
(fold above D1A basal)
60
50
40
30
20
10
0
*
D1A
D1B
D1A-IL4B
D1B-IL4A

Figure 2. Dose-response curve of DA for AC stimulation by wild-type and chimeric D1-like receptors expressed in HEK 293 cells.

HEK 293 cells were transfected with wild-type or chimeric D1-like receptors using the following amounts of DNA per dish: 0.02 μg of D1A, 0.02 μg of D1B, 5.0 μg of D1A-IL4B, 0.02 μg of D1B-IL4A. Under these experimental conditions, the total functional receptor numbers were comparable. The values in picomole/mg of membrane protein (expressed as the arithmetic mean ± S.E.) were 2.8 ± 0.3 (D1A), 2.6 ± 0.4 (D1B), 0.6 ± 0.1 (D1A-IL4B), 1.3 ± 0.2 (D1B-IL4A). Intracellular cAMP levels were measured in single wells of a 12-well dish in the absence or presence of increasing concentrations of DA as described under "Materials and Methods" and plotted as a function of log of DA concentrations. **A**, Constitutive activity of wild-type and chimeric D1-like receptors. Data are expressed as arithmetic mean ± S.E. of four experiments done in triplicate determinations. The basal levels (best-fitted values for minimal response) of AC activity were determined using CA/TU values and then analyzed by simultaneous curve-fitting using ALLFIT. **B**, DA-mediated maximal stimulation of AC by wild-type and chimeric D1-like receptors. Data are expressed as arithmetic mean ± S.E. of four experiments done in triplicate determinations. The maximal activation (best-fitted values for maximal response) of AC activity was determined using CA/TU values and then analyzed by simultaneous curve-fitting using ALLFIT. **C**, Dose-response curve of DA for AC stimulation by wild-type and chimeric D1-like receptors. Each point is the arithmetic mean ± S.E. of four experiments done in triplicate determinations and expressed as percentage of maximal activation obtained with the respective wild-type or chimeric receptor after subtracting the basal value. Curves were analyzed by simultaneous curve fitting using ALLFIT. Statistical significance was determined using unconstrained and constrained simultaneous curve fitting. The EC_{50} values are as follows (in nM): 7.6 ± 1.1 (D1A), 1.2 ± 0.2 (D1B), 19.6 ± 2.8 (D1A-IL4B), 22.3 ± 3.2 (D1B-IL4A). **D**, EC_{50} ratios of wild-type D1B and chimeric receptors relative to the D1A subtype. The values ± approximate S.E. (as obtained with ALLFIT) are as follows: 0.15 ± 0.03 (D1B), 2.58 ± 0.51 (D1A-IL4B), 2.93 ± 0.58 (D1B-IL4A).

*, $p < 0.05$ when compared with D1A; #, $p < 0.05$ when compared with D1B; Ψ, $p < 0.05$ when compared with D1A-IL4B.


A
BASAL ACTIVITY (CA/TU x 1000)
0
1
2
3
4
5
6
*
*
*#ψ
D1A
D1B
D1A-IL4B
D1B-IL4A
B
MAXIMAL ACTIVATION (CA/TU x 1000)
0
10
20
30
40
50
*
*#
*#
D1A
D1B
D1A-IL4B
D1B-IL4A
C
INTRACELLULAR cAMP (% of maximal response)
0
25
50
75
100
125
D1A
D1B
D1A-IL4B
D1B-IL4A
-13 -12 -11 -10 -9 -8 -7 -6 -5
log [DOPAMINE] (M)
D
EC50 RATIO (relative to D1A receptors)
0
1
2
3
4
*
*#
*#
D1A
D1B
D1A-IL4B
D1B-IL4A

CHAPTER 4

General discussion

A full understanding of the molecular basis of DA receptor activation is of crucial importance since impairment in the activity of dopaminergic receptor systems has been implicated in a variety of human disorders. Specifically, the D1-like receptors have been indicated as potential molecular targets for the design of novel drugs used in the treatment of Parkinson's disease, schizophrenia, and hypertension. The rational development of such drugs relies in part on mutagenesis studies for delineation of structural determinants underlying the ligand binding and G protein-coupling properties of the D1-like receptors. At the same time, the molecular basis of D1-like receptor multiplicity can be addressed.

To date, mutagenesis studies of the D1-like dopaminergic receptors have identified several important regions underlying the distinct activation properties of the D1A and D1B receptor subtypes. Specifically, recent work from our lab demonstrated that a receptor region referred to as the terminal receptor locus (TRL) encompassing the TM6 and TM7, EL3, and the CT is a key structural domain regulating ligand binding and G protein coupling properties of the D1-like receptors (Iwasiow *et al.*, 1999). Upon closer examination, the EL3 region has been identified as the domain underlying the degree of agonist-mediated maximal activation elicited by the D1-like receptors (Iwasiow *et al.*, 1999). On the other hand, the CT has been shown to play a crucial role in agonist binding affinity and constitutive activity phenotype of the D1-like receptors, while modulating in distinct fashion the agonist-mediated properties of the D1-like subtypes (Jackson *et al.*, 2000). Despite providing invaluable insight into the structure – function relationships of the D1-like receptors, these studies did not fully resolve certain issues pertaining to our current view of constitutive and agonist-mediated activation of GPCRs, including the D1-

like receptors. Our current work aims to address some of these issues and strives to enhance our understanding of the molecular basis of D1-like receptor activation and GPCR structure – function relationships in general.

In keeping with the established model of GPCR activation, the binding affinity of an agonist predicts its potency for stimulating effector response, i.e. increased agonist affinity translates into increased agonist potency. Indeed, initial studies utilizing CAM GPCRs produced receptors displaying corresponding increases in agonist affinity and potency when expressed in heterologous systems (Samama *et al.*, 1993; Scheer and Cotecchia, 1997). In striking contrast, studies performed in our lab using the D1-like dopaminergic receptors indicate that activation properties of GPCRs can be dissociated by mutations (Iwasiow *et al.*, 1999; Jackson *et al.*, 2000). For instance, a chimeric D1A receptor harboring the CT of the D1B subtype (D1A-CTB) displays reduced potency for stimulating AC despite a large increase in the binding affinity for DA, when compared with the wild-type D1A receptor (Jackson *et al.*, 2000). In the present study, we show that insertion of EL3 of the D1B receptor into the D1A-CTB chimera rescued the DA potency of the chimeric receptor and brought its EC_{50} value closer to that of the wild-type D1B. Based on the observation that the EL3 region induces a partial increase in DA potency in cells expressing the D1A-EL3B chimera, while the CT causes a significant loss of DA potency in cells expressing the D1A-CTB chimera, we propose that the degree of DA potency displayed by the D1-like receptors is controlled by interfering intramolecular interactions between the EL3 and CT regions.

Importantly, the extent of agonist-mediated maximal activation of AC also appears to be affected by these interfering intramolecular interactions. While our previous study

showed that EL3 controlled the extent of agonist-mediated maximal activation of AC by D1-like receptors (Iwasiow *et al.*, 1999), additional substitution of the CT between the D1A and D1B receptors prevented EL3 from imposing upon the receptors the subtype-specific properties pertaining to maximal activation of AC. Indeed, the degree of maximal stimulation was not affected by the exchange of EL3 and CT regions, i.e. wild-type and chimeric D1A receptors displayed higher maximal activation of AC when compared with their respective counterparts. This finding sheds light on an unresolved issue raised by our previous study employing the TRL chimeras, which also reported the silencing of the EL3-induced effect on agonist-mediated maximal activation of AC, but could not rule out the involvement of TM6 and TM7 (Iwasiow *et al.*, 1999). Taken together, these findings support our belief that EL3 and CT participate in interfering intramolecular interactions that regulate the expression of agonist-mediated G protein coupling properties of the D1-like receptors. In contrast, the agonist-independent activity of the D1-like receptors appears to be regulated solely by the sequences of the CT. Indeed, chimeric substitution of the EL3 and CT regions between the D1A and D1B subtypes resulted in a full switch of constitutive activity of the D1-like receptors, thus recapitulating the results obtained upon the swap of the CT (Jackson *et al.*, 2000). Currently, studies are underway in our laboratory to identify the specific residues involved in the CT-induced intramolecular interactions regulating the D1-like subtype-specific G protein coupling properties. Thus far, residues located downstream of Cys^{351} of the D1A receptor have been identified as negative regulators of G protein coupling efficiency and AC activation (Chaar *et al.*, 2001), while CT region encoded by amino acids 438 - 448 has been mapped as a structural determinant underlying the high-affinity

binding profile of the D1B receptor (Demchyshyn *et al.*, 2000). Moreover, a mutagenesis study examining the role of membrane attachment of the CT by palmitoylation (leading to the formation of IL4) has demonstrated that elimination of palmitoylation sites of the D1A receptor does not affect receptor - G protein interaction (Jin *et al.*, 1997). Whether a similar assertion could be made regarding the D1B subtype remains to be investigated. However, considering that IL4 has been suggested to exist independently of palmitoylation (Yeagle *et al.*, 1996), its possible contribution to the D1-like receptor activation properties remains valid.

In an attempt to determine whether the N-terminal segment of the CT, known as IL4, underlies the CT-conferred properties of the D1-like receptors, we created additional chimeras in which the IL4 region was exchanged between the D1A and D1B subtypes. While previous evidence pertaining to the role of IL4 in GPCR function is often contradictory (Takemoto *et al.*, 1986; O'Dowd *et al.*, 1988; Konig *et al.*, 1989; Liggett *et al.*, 1991; Osawa and Weiss, 1994; Weiss *et al.*, 1994; Ernst *et al.*, 2000; Marin *et al.*, 2000), our mutagenesis study clearly supports the involvement of IL4 in ligand binding and G protein coupling properties of the D1-like receptors. Analysis of the pharmacological profile of the chimeric receptors has revealed a significant alteration of the binding affinity of the natural ligand, DA, in comparison with the wild-type receptors. In general, the observed changes recapitulated the results obtained upon the swap of the entire CT, thus implicating the IL4 segment in the CT-mediated regulation of DA binding affinity of the D1-like receptors. Moreover, the lack of major changes in the binding affinity of inverse agonists for the chimeric receptors likewise supports the findings

obtained upon the swap of the CT and strengthens the notion that inverse agonist binding is most likely independent of CT-induced conformational changes (Jackson *et al.*, 2000).

By monitoring the effect of its chimeric substitution on cAMP accumulation in HEK 293 cells expressing the wild-type and chimeric receptors, the strong influence of IL4 on agonist-independent and -dependent G protein coupling properties of the D1-like receptors became apparent. Indeed, the swap of the IL4 segment between the D1A and D1B receptors caused a full switch in constitutive activity, reminiscent of the chimeras in which the entire CT was exchanged. Importantly, the D1A-IL4B chimera exhibited increased constitutive activation similar to the D1A-CTB chimera. Also in agreement with the D1A-CTB chimera, the D1A-IL4B mutant exhibited a loss of DA potency, a phenomenon not generally associated with constitutively active GPCRs. However, despite its loss of DA potency, the D1A-IL4B chimera exhibited a significant increase in maximal activation of AC compared with the wild-type D1A receptor. Similar dissociation of ligand binding, agonist-dependent and -independent G protein coupling properties of the D1-like receptors was addressed in our previous study and points to the existence of multiple active conformational states. It is important to note that the formation of multiple active states is not a property of mutated receptors only, as numerous wild-type GPCRs, including β_2-AR, 5-HT_{2C} serotonergic receptors, and μ and δ opioid receptors have been shown to display multiple, agonist-specific active conformations (Gether *et al.*, 1995; Keith *et al.*, 1996; Berg *et al.*, 1998). For instance, fluorescent labelling of purified β_2-AR revealed the existence of a unique set of conformations in response to binding of various adrenergic agonists (Gether *et al.*, 1995). Alternatively, the observed increase in maximal activation of AC by the D1A-IL4B

chimera may be due to the presence of spare receptors, which have been proposed to contribute to maximal responses of certain drugs at low receptor occupancy (Kenakin, 1993). Moreover, differences in receptor uncoupling or internalization could be responsible for observed changes in maximal activation of AC by the wild-type and chimeric D1-like receptors; a possibility, which, at present time, remains to be investigated. Taken together, the data obtained from the IL4 project complement and extend the findings of the EL3CT study. Indeed, based on our results obtained with the IL4 chimeras, we predict that IL4 underlies the molecular interplays of the CT with the EL3 region.

Furthermore, our study has addressed the role of EL3, CT, and IL4 in controlling the total functional number of the D1A and D1B receptors in HEK 293 cells. As mentioned previously, a chimeric D1A receptor harboring the CT of the D1B subtype (D1A-CTB) exhibited a drastically reduced receptor number, possibly reflective of its high constitutive activity profile. Similar loss of receptor number was later observed with the constitutively active D1A-IL4B chimera. The apparent loss of receptor number is thought to result from enhanced structural instability associated with CAM GPCRs (Gether *et al.*, 1997a; Samama *et al.*, 1997; Rasmussen *et al.*, 1999). Thus, parallel to distinct mutations conferring constitutive activity to the β_2-AR and α_2-AR, the swap of the CT regions between the D1A and D1B subtypes could potentially impart to the D1A-CTB and D1A-IL4B chimeras a decreased structural stability. In the present study, we show that the potential decrease in structural stability displayed by the D1A-CTB chimera is corrected by additional replacement of the EL3 domain of the D1A subtype with that of the D1B receptor. Indeed, insertion of EL3 of the D1B receptor into the D1A-CTB chimera led to

a complete rescue of total functional receptor number of D1A-CTB. Notably, the resulting D1A-EL3CTB chimera expressed at receptor number indistinguishable from the wild-type D1A receptor. Due to their location on opposite sides of the plasma membrane, direct interaction between EL3 and CT cannot be proposed. In fact, our observations are most likely attributable to changes in α-helical packing and/or movement/ tilting of the TM6 and TM7, which have been reported to take place during activation of the β_2-AR and rhodopsin (Altenbach *et al.*, 1996; Farrens *et al.*, 1996; Gether *et al.*, 1997b; Javitch *et al.*, 1997; Altenbach *et al.*, 2001). Despite detailed description of TM movements provided by rhodopsin's high-resolution structure (Palczewski *et al.*, 2000), the current model of rhodopsin does not shed further light on the possible mechanism of the EL3 – CT interplay of the D1-like receptors presented in this study. However, our findings suggest that at least some of the effects exerted by the CT of the D1-like receptors may be attributed to its N-terminal segment, the IL4. Indeed, the involvement of IL4 in the activation process of GPCRs is supported by studies of rhodopsin (Ernst *et al.*, 2000; Marin *et al.*, 2000; Krishna *et al.*, 2002). More specifically, the IL4 of rhodopsin is believed to bind the α and γ subunits of transducin (G_t) by virtue of its α-helical conformation, as revealed by rhodopsin's high-resolution crystal structure (Palczewski *et al.*, 2000). In particular, the IL4, also known as α-helix 8, is thought to expand the apparent surface of rhodopsin and facilitate the binding of G protein subunits. Based on these considerations and in agreement with our previous results, we predict that the insertion of EL3 of the D1B receptor would likewise rescue the total functional receptor number of the D1A-IL4B chimera. Indeed, our findings indicate that interfering intramolecular interactions between the EL3 and CT regions, possibly mediated by the

IL4 segment, participate in the regulation of total functional receptor number of D1-like receptors in HEK 293 cells. Moreover, the opposing influences of the EL3 and CT regions on receptor number are also evident in the D1B-EL3CTA chimera. In this particular case, the EL3 region of the D1A subtype exerts on the D1B receptor a decreased structural stability, while the CT increases it. Our findings pertaining to the regulation of structural stability of the D1-like receptors may be of clinical relevance, as alteration of the D1-like receptor number in the prefrontal cortex of schizophrenic patients is believed to be related to negative symptoms and cognitive deficits seen in schizophrenia (Okubo *et al.*, 1997; Abi-Dargham *et al.*, 2002). On the whole, inferences about the role of distinct receptor domains in GPCR stability provide valuable insight into mechanisms regulating the cell surface expression of GPCRs. For instance, the third intracellular loop has been shown to play a central role in stabilization of the α_{2A}-AR on the basolateral surface of the polarized MDCKII cells (Edwards and Limbird, 1999). Improved cell surface expression through surface stabilization of structurally unstable receptors not only enhances neuronal responsiveness, but also provides a potential site for regulation of GPCR function. Indeed, ligand-dependent receptor stabilization / upregulation has been demonstrated for a number of GPCRs in a variety of cell types (Betuing *et al.*, 1997; Gether *et al.*, 1997a; Lee *et al.*, 1997; Samama *et al.*, 1997; Rasmussen *et al.*, 1999; Morello *et al.*, 2000; Wilson and Limbird, 2000).

In conclusion, the results presented in this thesis aim to elucidate the complex structure – function relationships that govern the functional properties of the D1-like receptors. In particular, the study provides an insight into the D1-like dopaminergic receptor activation by means of a novel molecular interplay between an extracellular

region, EL3, and an intracellular region, CT. In addition, we have narrowed down the region of the CT involved in the many aspects of the D1-like receptor signaling to the N-terminal segment, the IL4. Bearing in mind that the chimeric receptor approach has proven tremendously useful in providing meaningful insight into studies of subtype selectivity, it is seldom specific enough to identify key amino acid residues involved in ligand binding and G protein coupling properties of GPCRs, for which single point mutation studies are indicated. Consequently, studies are underway in our laboratory to clarify further the role of IL4 residues in the activation properties of the D1-like dopaminergic receptors. Taken together, the results presented in this thesis elucidate the molecular basis of D1-like receptor activation and may be of potential physiological relevance for the treatment of pathological conditions caused by a compromised D1-like receptor function (Emilien *et al.*, 1999).

CHAPTER 5

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APPENDIX 1

Graphical representation of the cloning strategy used to create chimeric D1-like receptors described in the manuscript titled "Insight into the mechanism of G protein-coupled receptor activation. Evidence for a molecular interplay between the third extracellular loop and cytoplasmic tail of D1-like dopaminergic subtypes."

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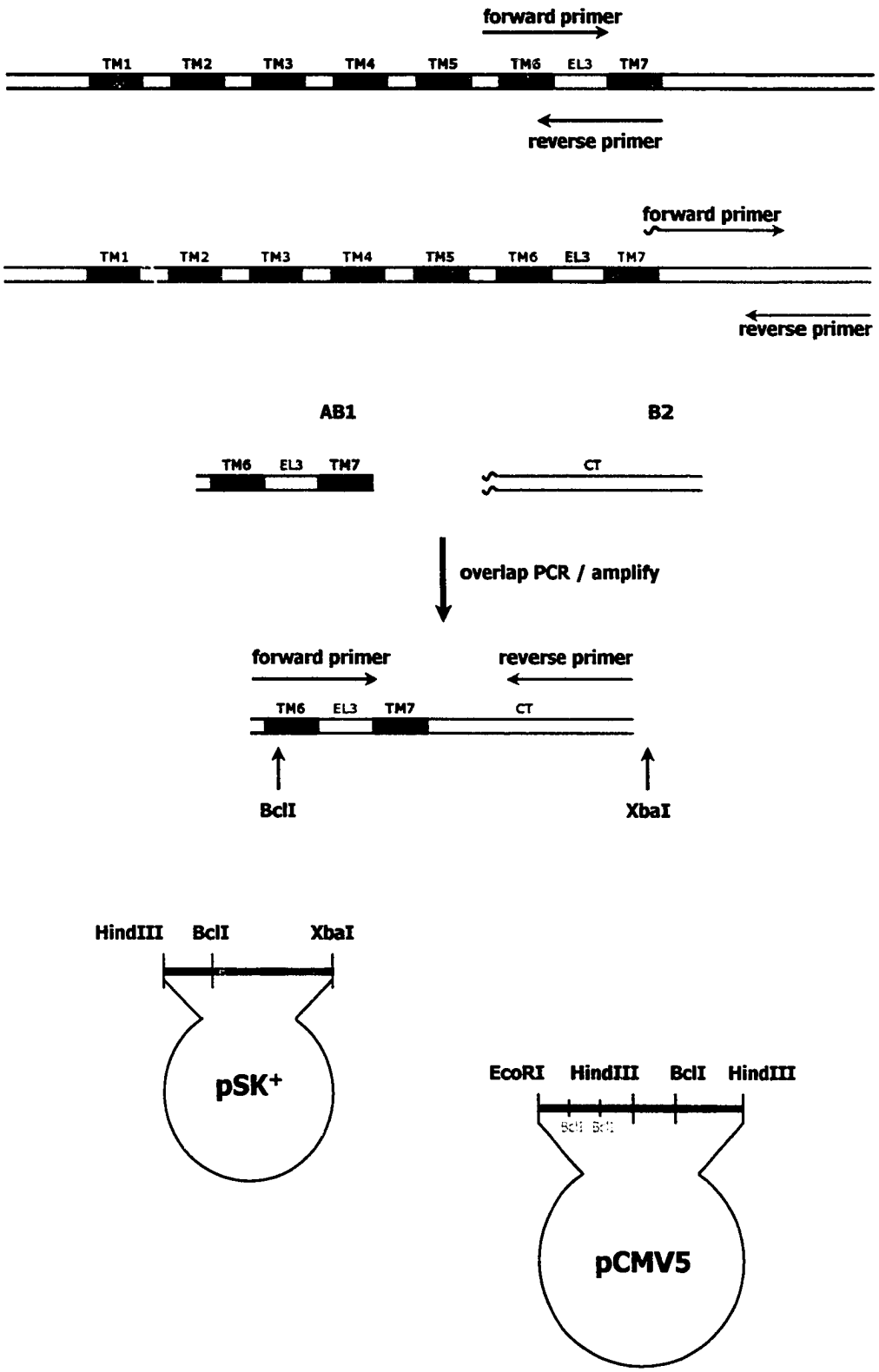
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EL3
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TM5
TM6
EL3
TM7
reverse primer
AB1
B2
TM6
EL3
TM7
CT
overlap PCR / amplify
forward primer
reverse primer
TM6
EL3
TM7
CT
BclI
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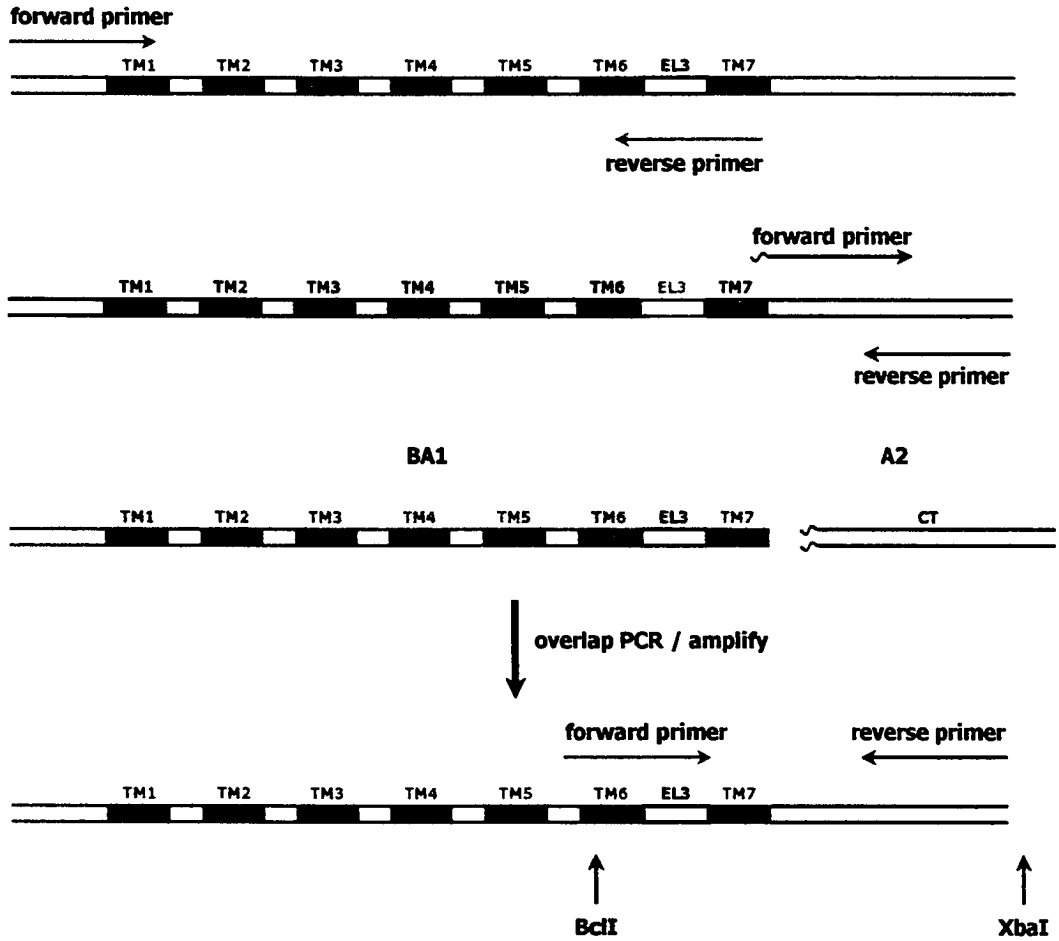
D1B-EL3CTA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reverse primer
forward primer
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TM6
EL3
TM7
reverse primer
BA1
A2
TM1
TM2
TM3
TM4
TM5
TM6
EL3
TM7
CT
overlap PCR / amplify
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reverse primer
TM1
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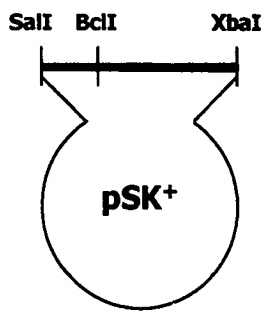
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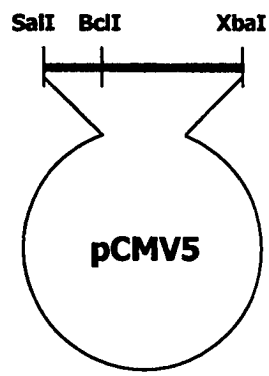
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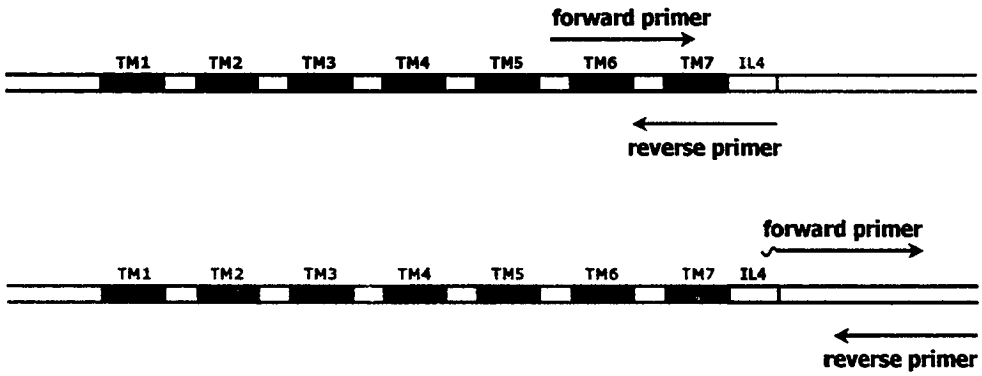
pCMV5

APPENDIX 2

Graphical representation of the cloning strategy used to create chimeric D1-like receptors described in the manuscript titled "Role of the cytoplasmic helix-8 region of D1-like dopaminergic receptors in conferring subtype-specific ligand binding and G protein coupling properties."

D1A-IL4B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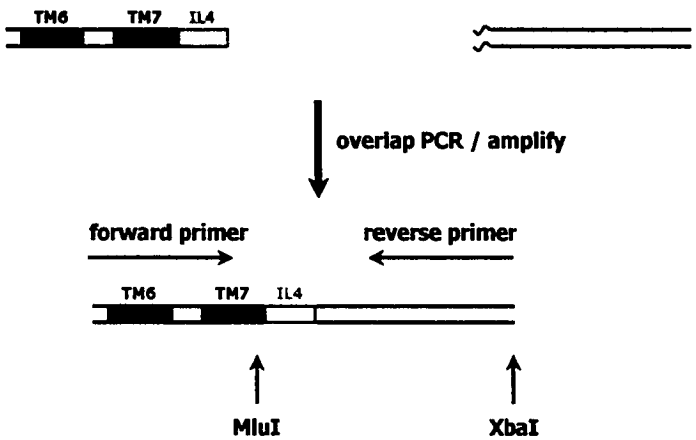
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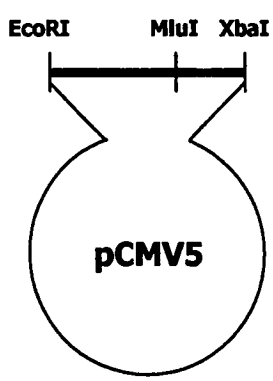
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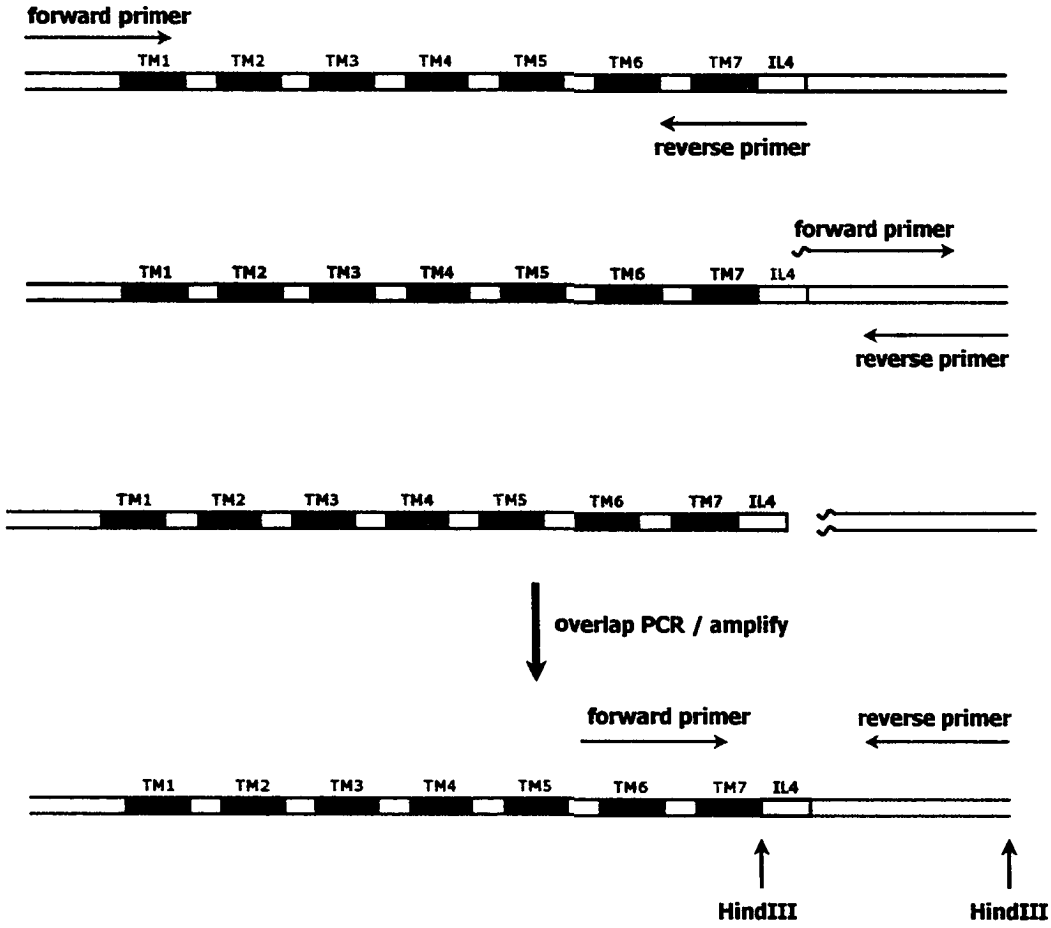
pCMV5

D1B-IL4A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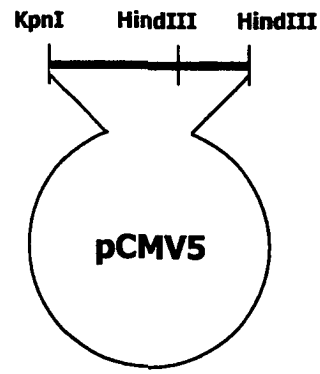
TM7
IL4
reverse primer
TM1
TM2
TM3
TM4
TM5
TM6
TM7
IL4
overlap PCR / amplify
forward primer
reverse primer
TM1
TM2
TM3
TM4
TM5
TM6
TM7
IL4
HindIII
HindIII


KpnI
HindIII
HindIII
pCMV5