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Delineation of the Structure-Function Relationships within the Fourth Intracellular Loop of Dopamine D1-Like Receptors

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ABSTRACT

The D1-like receptors are categorized into D1A/D1 and D1B/D5 subtypes, which belong to the superfamily of G protein-coupled receptors. Notwithstanding a high degree of homology in their primary sequences, D1A/D1 and D1B/D5 receptors display distinct ligand binding, agonist-independent and dependent G protein coupling properties. Previous mutational studies have suggested that the cytoplasmic tail (CT) is an important structural domain, which bestows D1-like subtype-specific functional properties. The present study focuses the attention on a region of CT known to participate in the formation of a fourth intracellular loop (IL4). The central objective of this study is to investigate the potential role of variant residues within IL4 region in regulating distinct D1-like functional properties. Results obtained from ligand binding and agonist-dependent G protein coupling studies using chimeric receptors reveal a crucial role of the N-terminal segment of IL4 (IL4N) in regulating receptor expression, ligand binding and dopamine (DA) potency of D1-like subtypes. Moreover, results show that the C-terminal segment of IL4 (IL4C) regulate the extent of IL4N-mediated effects on DA binding and potency mostly through interfering intramolecular interactions. Meanwhile, agonist-independent G protein coupling data suggest that the D1-like subtype-specific agonist-independent activity relies exclusively on intramolecular interactions regulated by IL4N segment. Taken together, the present study underscores the important roles of IL4N and IL4C segments of D1-like receptors in regulating their distinct ligand binding and G protein coupling properties, a finding that may prove beneficial for the development of D1A/D1 and D1B/D5 subtype-specific ligands.

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LIST OF ABBREVIATIONS

AA	- Arachidonic acid
AC	- Adenylyl cyclase
α_{GDP}	- GDP bound G protein α subunit
α_{GTP}	- GTP bound G protein α subunit
Bmax	- Maximal binding capacity
BUTA	- (+)-Butaclamol
CA	- [^{3}H]cAMP formed
cAMP	- cyclic 3', 5'-adenosine monophosphate
CNS	- Central nervous system
CT	- Cytoplasmic tail
DA	- Dopamine
DAG	- Diacylglycerol
DNA	- Deoxyribonucleic acid
EC_{50}	- Half-maximal effective concentration
EDTA	- Ethylenediaminetetraacetic acid
EL	- Extracellular loop
FBS	- Fetal bovine serum
FLU	- Cis-flupentixol
GAP	- GTPase-activating protein
$G\alpha_s$	- α subunit of stimulatory G protein
$G\alpha_i$	-α subunit of inhibitory G protein
GDP	- Guanosine diphosphate

List of abbreviations

GEF	- Guanine exchange factors
G protein	- GTP-binding protein
GPCR	- G protein-coupled receptor
GRK	- G protein-coupled receptor kinase
GTP	- Guanosine triphosphate
H8	- Helix 8
HBS	- HEPES-buffered saline
HEK	- Human embryonic kidney
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
[^{3}H]-SCH	- N-[methyl-^{3}H]-SCH23390
IBMX	- 1-methyl-3-isobutylxanthine
IL	- Intracellular loop
IL2	- The second intracellular loop
IL3	- The third intracellular loop
IL4	- The fourth intracellular loop
IL4A	- The IL4 region of D1A receptor
IL4B	The IL4 region of D1B receptor
IL4N	- The N-terminal segment of IL4 region
IL4C	- The C-terminal segment of IL4 region
IL4NA	- The N-terminal segment of IL4 region of D1A receptor
IL4CA	- The C-terminal segment of IL4 region of D1A receptor
IL4NB	- The N-terminal segment of IL4 region of D1B receptor
IL4CB	- The C-terminal segment of IL4 region of D1B receptor

List of abbreviations

IP_3	- Inositol triphosphate
Kd	- Equilibrium dissociation constant for N-[methyl-^{3}H]-SCH23390
Ki	-Inhibitory constant
LH	- Luteinizing hormone
MEM	- Minimal essential medium
mRNA	- Messenger ribonucleic acid
PBS	- Phosphate-buffered saline
PCR	- Polymerase chain reaction
PET	- Positron emission tomography
PI	-.Phosphoinositide
PLA_2	- Phospholipase A_2
PLC-β	- Phospholipase C-β
PKA	- Protein kinase A
PKC	- Protein kinase C
PTH	- Parathyroid hormone
PTHrP	- Parathyroid hormone related peptide
R	- GPCR inactive conformation
R*	- GPCR active conformation
RGS	- Regulator of G protein signaling
SCH23390	- R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5,-tetrahydro-1H-3-benzazepine
SCH	- SCH23390
TM	- Transmembane

TRL - Terminal receptor locus

TU - Total uptake

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CHAPTER ONE – INTRODUCTION

PART I – BACKGROUND

1. G Protein-Coupled Receptors

1.1. Diversity of G protein-coupled receptors

Cells use receptors to respond to external stimuli. Receptors are responsible for transmitting extracellular signals and initiating specific intracellular effects. Among many classes of receptors, G protein-coupled receptors (GPCRs) are cell surface receptors that initiate intracellular responses via coupling to heterotrimeric GTP-binding proteins (G proteins). GPCRs constitute a superfamily, which is known to form one of the largest protein families in nature (Gether 2000). There are more than 1000 receptors belonging to the superfamily and 1-2% of total genes in the mammalian genome code for GPCRs (Gether 2000). A wide variety of ligands, ranging from neurotransmitters, hormones, polypeptides to sensory stimuli (e.g. odorants, light sensitive retinal), are known to bind to GPCRs. Binding of ligands to GPCRs gives rise to numerous biological effects, including neurotransmission, perception of light, endocrine and exocrine gland release (Gutkind 1998). Moreover GPCRs serve as an important target for pharmaceutical agents. More than 50% of drugs exert their therapeutic effects through modulation of GPCRs (Palczewski 2002).

1.2. A common structural feature of GPCRs

GPCRs share a common structural feature of seven α-helical transmembrane (TM) domains. Hence, GPCRs are also referred to as heptahelical receptors or seven transmembrane receptors. Seven TM helices (TM1-TM7) are linked by three extracellular loops (EL1-EL3) and three intracellular loops (IL1-IL3) (Fig. 1). The amino terminus is on the extracellular side, while the carboxyl terminus or the cytoplasmic tail (CT) is on the intracellular side (Fig. 1). GPCRs can be further categorized into three major families (family A, B and C) and three minor families (family D, E, and F) based on primary sequence similarity (Horn *et al.* 1998). Family A, known as rhodopsin/β_2-adrenergic receptor-like family, is the largest family and accounts for ~90% of all GPCRs.

1.3. A common functional feature of GPCRs

As the name suggests, a common functional feature shared by all GPCRs is that they can interact with G proteins following agonist binding. In general, GPCR domains involved in agonist binding vary among GPCRs. For example, binding domains of the receptors for large peptides and hormones are mostly found in the extracellular loops, whereas those for catecholamines are found in TM regions (Gether and Kobilka 1998; Gether 2000). Binding of agonists to GPCRs causes conformational changes in TM domains. For instance, the movement of TM3 and TM6 has been demonstrated in β_2-adrenergic receptor upon agonist binding (Gether *et al.* 1997b, Rasmussen *et al.* 1999). Changes in the conformation of TM domains subsequently lead to changes in the conformation of intracellular domains, uncovering previously buried G protein coupling

sites. G protein coupling domains of GPCRs are located on the intracellular side, including the second and third intracellular loops (IL2 and IL3) as well as the membrane proximal part of CT (Strader *et al.* 1994; Huang *et al.* 1995;Liu and Wess *et al.* 1996; Nasman *et al.* 1997; Verrall 1997; O'Dowd *et al.* 1988; Marin *et al.* 2000).

1.4. GPCR signaling via G proteins

1.4.1. G protein cycles of activation and inactivation

G proteins constitute a major intracellular signaling partner of GPCRs and relay signals through cycles of activation and inactivation. G proteins are heterotrimeric proteins composed of α, β and γ subunits. β and γ subunits form a dimer and can be considered as one functional unit. In the inactive state, βγ dimer associates with GDP bound form of α subunit (α_{GDP}). The interaction of an activated receptor with the α_{GDP}·βγ complex promotes the exchange of GDP with GTP on α subunit. This process activates the G protein, resulting in a dissociation of GTP bound α subunit (α_{GTP}) and βγ dimer. Both α_{GTP} and βγ dimer can transduce signals through interaction with downstream effectors (e.g. effector enzymes and ion channels). α_{GTP} has an intrinsic GTPase activity, which is responsible for hydrolyzing GTP to GDP on α subunit. Hydrolysis of GTP to GDP inactivates α subunit and restores α subunit high binding affinity for βγ dimer. Then α_{GDP} re-associates with βγ dimer to be ready for another cycle of activation.

1.4.2. The classification of G protein subunits and their downstream effectors

The classification of G proteins is based on their α subunits. Twenty three different α subunits can be grouped into four classes (α_s, α_i, α_q, α_{12}) based on their primary

sequence similarity (Simon *et al.* 1991; Nurnberg et al. 1995). Moreover, specific bacteria toxins provide useful tools to study α_s and α_i activities. For example, cholera toxin ADP-ribosylates α_s and blocks its intrinsic GTPase activity, activating it, whereas pertussis toxin ADP-ribosylates α_i and uncouples $G\alpha_i$ from the receptor and effector, inactivating it. At present six β subunits and twelve γ subunits have been identified (Gautam *et al.* 1998; Schwindinger and Robishaw 2001). Although potential combinations of $\beta\gamma$ dimers are diverse, some of them do not exist in nature. For example, β_2 can not form a dimer with γ_1 (Schmidt 1992).

It is well documented that the basic functional elements of G protein signaling include GPCRs, G proteins, and downstream effectors (Fig. 2). Both GTP bound α subunits and free $\beta\gamma$ dimers are known to transduce intracellular signaling cascades through interaction with their downstream effectors (Clapham *et al.* 1993). Particularly, α subunits are found to interact with effector enzymes, which in turn lead to the activation of second messengers. For instance, effector enzymes (as shown in Fig. 2) such as adenylyl cyclase (AC), phospholipase A_2 (PLA_2) and phospholipase C-β (PLC-β) produce downstream second messengers such as cAMP, arachidonic acid (AA), diacylglycerol (DAG) and inositol triphosphate (IP_3). Furthermore, individual α subunit families display selectivity to activate or inhibit specific effector enzymes. α_s stimulates AC activity, whereas α_i inhibits AC activity. Previously, $\beta\gamma$ dimers were believed merely to stabilize α subunits in the inactive form. However, studies have now established that $\beta\gamma$ dimers are also actively involved in regulating downstream effectors, such as potassium and calcium channels, AC, and PLC-β (Fig. 2) (Tang *et al.* 1991; Clapham *et al.* 1993; Wickman *et al.* 1994).

1.4.3 The regulation of G protein signaling

The duration of G protein signaling is determined by the life span of α_{GTP} (Siderovski 2000). The balance between the active (α_{GTP}) and inactive forms (α_{GDP}) of α subunit is highly regulated. Guanine exchange factors (GEFs) facilitate exchange of GDP with GTP on α subunits and keep G proteins in the active state. In fact, GPCRs serve as GEFs. Conversely, GTPase activating proteins (GAPs) inactivate G proteins by catalyzing the intrinsic GTPase activity of α subunits. Some effectors such as PLC-β play the role of GAPs. A newly identified family of regulators of G protein signaling (RGS) is found to have multiple functions, including GAP-like activity. Indeed, RGS are shown to display GTPase catalyzing properties when interacting with α_q, α_i, α_s and α_{12} (Berman *et al.* 1996; Ross and Wilkie 2000; Well *et al.* 2002; Day *et al.* 2003; Roy *et al.* 2003).

1.5. Mechanisms underlying GPCR activation

As we all know, GPCR activation follows agonist binding. Additionally, a body of evidence has shown that GPCR activation can occur in the absence of agonists. For instance, α_{1B}-adrenergic receptor carrying a mutation in the C-terminal region of the IL3 has been shown to activate polyphosphoinositide hydrolysis via G protein coupling in the absence of agonists (Cotecchia *et al.* 1990; Kjelsberg *et al.*1992). Furthermore, certain antagonists (inverse agonists) are able to reduce this GPCR agonist-independent activity. With the growing body of experimental evidence regarding the molecular basis for GPCR activation, studies have developed and updated different models of GPCR activation.

1.5.1 Two-state model

The two-state model has been expanded to take into account the isomerization of GPCRs in the absence of agonist and is known as the extended ternary complex model (Samama *et al.* 1993). Here, I will refer to the two-state model for sake of simplicity. The two-state model helps explaining the mechanisms underlying the actions of various types of ligands, including full and partial agonists, full and partial inverse agonists, and antagonists (neutral), as well as GPCR agonist-independent activity (as reviewed in Gether 2000). The two-state model suggests that GPCRs can exist in an equilibrium between two interchangeable conformations, the inactive conformation (R) and the active conformation (R*) (Fig. 3). In the R state GPCRs show low affinity for G proteins, whereas in the R* state GPCRs bind to G proteins with high affinity. The transition between R and R* is termed isomerization. In the absence of agonists, a GPCR predominantly assumes R by intramolecular constraints, which prevent the efficient G protein coupling of the receptor. Full agonist binding releases the intramolecular constraints and can stabilize optimally receptor in the active conformation, driving completely the equilibrium towards R* (Fig. 3). Conversely, full inverse agonists stabilize optimally the receptor in the inactive receptor conformation, shifting fully the equilibrium towards R (Fig. 3). Partial agonists stabilize the receptor in R* conformation less efficiently as compared with full agonists (Fig. 3). Similarly, partial inverse agonists are less efficient than full inverse agonists in the ability to stabilize R conformation (Fig. 3). Antagonists, which are neutral ligands, do not change the receptor conformation, leaving the equilibrium unchanged. However, neutral antagonists interfere with the

effects of agonists and inverse agonists by simply blocking the GPCR ligand binding sites.

In the absence of agonists, GPCRs can spontaneously adopt R* because of the intrinsic thermodynamic activity of receptors. R to R* isomerization in the absence of ligands is termed constitutive activity or agonist-independent activity, which underlies the basal G protein coupling properties of GPCRs (Seifert and Wenzel-Seifert 2002). Constitutive activities vary from low to very high according to specific receptors (Seifert and Wenzel-Seifert 2002). Based on the two-state model, agonists increase basal G protein coupling activity of GPCRs; antagonists do not change basal activity; inverse agonists decrease basal activity (Seifert and Wenzel-Seifert 2002). Moreover, certain GPCR mutations can disrupt intramolecular constraints and enhance the isomerization rate of R to R* in the absence of agonists. Hence, these mutants display high constitutive activities and are referred to as constitutively active mutants. The two-state model indicates that GPCRs transit only between one active receptor conformation and one inactive receptor conformation. However, recent evidence suggests that GPCR activation can not be fully explained by the two-state model.

1.5.2. Multistate model

Multistate model is also termed the allosteric ternary complex model. The multistate model suggests that GPCRs bear multiple active and inactive states (as reviewed in Gether 2000, Kenakin 2003). Evidence shows that not all agonists stabilize GPCRs into one uniform active conformation and trigger the same signaling pathway. Instead, a given agonist can selectively stabilize receptor into the agonist-specific conformation, which

favors a particular signaling pathway. For example, some agonists, binding to serotonin 5-HT_{2C} receptor, preferentially activated PLC that mediated inositol phosphate accumulation signaling pathway, whereas others preferentially stimulated PLA_2 that mediated AA release signaling pathway (Berg *et al* 1998). Besides multiple agonist-specific receptor conformations, multistate model is also supported by studies on constitutively active mutants. One study has demonstrated that the distinct patterns of phosphorylation and agonist-induced internalization displayed by two constitutively active α_{1B}-adrenergic mutants, in which either Asp^{142} in TM3 was replaced by Glu or Ala^{293} in IL3 was substituted by Glu (Mhaouty-Kodja *et al* 1999). These results reflect distinct active receptor conformations adopted by constitutively active mutants. As a result, the distinct active receptor conformations may be differently recognized by regulatory molecules (e.g. G protein-coupled receptor kinase and arrestin), resulting in divergent regulatory effects (e.g. phosphorylation and internalization) (Mhaouty-Kodja *et al* 1999). Altogether, growing experimental evidence supports the multistate model of GPCR activation.

1.6. GPCR constitutive activity

1.6.1. General properties of GPCR constitutive activity

To date, increasing number of wild-type and mutant receptors with high constitutive activities has been identified (Cotecchia *et al.* 1990; Kjelsberg *et al.* 1992; Samama *et al.* 1993; Tiberi and Caron 1994). Computer simulations and experimental data obtained from constitutively active GPCRs help to define several general properties of constitutive activity: (i) generation of a signal in the absence of agonist occupancy; (ii) increased

agonist affinity for binding to the receptor; (iii) increased potency of agonists for stimulation or inhibition of the effector response; and (iv) increased intrinsic activity of partial agonists (Lefkowitz et al. 1993; Tiberi and Caron 1994; Scheer and Cotecchia 1997).

1.6.2 Constitutive activity in pathologies and normal physiological functions

GPCR constitutive activity is a potential cause of various diseases. Naturally occurring constitutively active GPCR mutants found in rhodopsin, LH receptor, Ca^{2+} receptor, PTH/PTHrP receptor are associated with retinitis pigmentosa, familial male precocious puberty, autosomal dominant hypoparathyroidism and Jansen-type metaphyseal chondrodysplasia (Spiegel 1996). Moreover, transgenic mice with cardiac specific expression of constitutively active α_{1B}-adrenergic receptor mutant have been shown to develop cardiac hypertrophy (Milano *et al.* 1994). In addition, constitutively active GPCRs encoded by KSHV (Kaposi's sarcoma-associated herpesvirus) have shown to stimulate cellular proliferation in transfected cells and induce tumor formation in transgenic mice (Arvanitakis *et al.* 1997; Yang *et al.* 2000). In general, a common property shared by all disease-causing constitutive GPCR mutants is their enhanced basal G protein coupling activities (Seifert and Wenzel-Seifert 2002). Thus clarification of the potential involvement of GPCR constitutive activity in diseases is of clinical importance because inverse agonists could be used to reduce constitutive activity of mutant GPCRs implicated in the etiology of pathologies. Therefore, inverse agonists may be useful as therapeutic agents for treating high receptor constitutive activity associated diseases.

In addition to its role in causing potential various diseases, GPCR constitutive activity is also involved in regulating normal physiological functions. Constitutively active wild-type GPCRs have been identified in recombinant systems as well as native systems. Two isoforms of histamine H_3 receptors (H_{3S} and H_{3L}) can couple to $G_{i/o}$ proteins, which lead to AC inhibition and PLA_2 activation. When expressed in Chinese hamster ovary cells (recombinant system), both H_{3L} and H_{3S} receptors were shown to reduce cAMP accumulation and enhance AA release in the absence of agonists (Morisset *et al.* 2000). Compared with recombinant systems, native systems, particularly the intact organs, are more important because these systems allow analysis of endogenous receptors at physiological expression levels (Seifert and Wenzel-Seifert 2002). Further study on the H_{3L} and H_{3S} receptors has demonstrated that the two receptors were both expressed in rodent brain and may be involved in regulating autoreceptor function in histaminergic neurons in vivo (Morisset *et al.* 2000). More specifically, the inverse agonist of H_{3L} and H_{3S}, which decreases basal activity of the two receptors, was shown to reduce autoreceptor function. In contrast, the agonist of H_{3L} and H_{3S} receptors, which increases basal activity of the two receptors, was shown to enhance autoreceptor function (Morisset *et al.* 2000). The antagonist (neutral) was shown to block the opposite effects of agonists and inverse agonists (Morisset *et al.* 2000). These results suggest that constitutive activities of H_{3L} and H_{3S} receptors control histamine neuron activity in vivo (Morisset *et al.* 2000). Further support for physiological importance of constitutive activity comes from the natural existence of different degrees of constitutive activities displayed by specific members within a given group of GPCRs (Seifert and Wenzel-Seifert 2002). In particular, a pair of prototypes is human D1A/D1 and D1B/D5 receptors, both of which

belong to dopamine receptors and lead to AC activation (discussed later in details). The D1B receptor displays significant higher constitutive activity in recombinant systems in comparison with the D1A receptor (Tiberi and Caron 1994). The constitutive activity of the D1B/D5 receptor may be required to sustain cell basal activity. Indeed, a study has shown that constitutively active D1B receptors led to the production of cAMP in an agonist-independent manner in atrial natriuretic factor (ANF)-producing neurons of rat hypothalami. This mechanism is thought to be important for controlling the ANF-producing neuron activity (Lee *et al.* 1999).

1.7. Post-translational modifications and receptor desensitization

GPCRs are subjected to post-translational modifications, including N-glycosylation, phosphorylation and palmitoylation. Asparagine residues of GPCRs constitute a major target for N-glycosylation. This modification is believed to be involved in the regulation of receptor surface expression (Russo *et al.* 1991; Couvineau *et al.* 1996; Buhlmann *et al.* 2000; Zhou *et al.* 2000), ligand binding and intracellular signal transduction (Fukushima *et al.* 1995; Zhou *et al.* 2000).

Phosphorylation of GPCRs initiates receptor desensitization. GPCR desensitization is defined as a diminishing responsiveness of GPCRs following repeated or continuous exposure to agonists. The desensitization occurs as a consequence of GPCR uncoupling from G proteins when serine/threonine residues are phosphorylated in the CT and intracellular loops of GPCRs (Ferguson 2001). The phosphorylation process is catalyzed either by second messenger-dependent kinases, such as protein kinase A (PKA) and protein kinase C (PKC), or by G protein-coupled receptor kinases (GRKs). GRK-

mediated receptor phosphorylation enhances the binding of phosphorylated receptors to arrestins, another important regulatory molecule involved in mediating GPCR desensitization. Binding of arrestins to GPCRs also triggers GPCR internalization via clathrin-coated pits and terminates G protein-mediated signaling (Ferguson 2001). Moreover, arrestins are not only involved in terminating G protein-mediated signaling of GPCRs but also act as adaptor proteins that link GPCRs to new signaling pathways (Hall *et al.* 1999).

Besides N-glycosylation and phosphorylation, palmitoylation is also an important post-translational modification, in which the GPCRs are modified by covalent attachment of the fatty acid palmitate through thioester bonds. The cysteine residues, which are highly conserved in the N-termini of CTs of GPCR family A receptors, constitute a major target for palmitoylation. For example, palmitoylation of such residues has been identified in rhodopsin (Ovchinnikov *et al.* 1988), β_2-adrenergic receptor (O'Dowd *et al.* 1989) and D1A receptor (Ng *et al.* 1994a). Palmitoylation facilitates CT association with membrane. With respect to its functions, palmitoylation is believed to play an important role in protein processing and targeting (reviewed in Qanbar and Bouvier 2003). Moreover, due to its reversible property, palmitoylation may be involved in regulating receptor properties (Qanbar and Bouvier 2003). One study has shown that removal of palmitoylation of β_2-adrenergic receptor significantly increased ligand-independent receptor phosphorylation (Moffett *et al.* 1996). Interestingly, primary sequences of β_2-adrenergic receptor show that a PKA phosphorylation site is located immediately downstream of the palmitoylation site. It is proposed that palmitoylation may shape β_2-adrenergic receptor in a conformation that covers the adjacent PKA site (Moffett *et al.*

1996). Depalmitoylation may induce a receptor conformational change that uncovers the previously buried PKA phosphorylation site and causes increased phosphorylation of β_2-adrenergic receptor (Moffett *et al.* 1996). The importance of palmitoylation has been also evaluated for other family A receptors. Removal of palmitoylation sites of rhodopsin did not affect the interaction of the receptor with G proteins (Karnik *et al.* 1993). Similar result was also obtained with D1A receptor. The mutant D1A receptors with eliminated palmitoylation sites were essentially identical in comparison with wild-type receptor in terms of their ligand binding and G protein coupling properties (Jin *et al.* 1997). Further analysis of the primary sequences of rhodopsin and D1A receptors reveals that no phosphorylation site is adjacent to the equivalent cysteines (Jin *et al.* 1997). Therefore, receptor conformational changes induced by unpalmitoylation may have little effects on phosphorylation properties and the responsiveness of rhodopsin and D1A receptor (Jin *et al.* 1997). Taken together, each GPCR possesses its own detailed structural feature. The effects of palmitoylation on GPCR functions may vary among individual GPCRs (Jin *et al.* 1997).

In summary, GPCRs share a common structural feature of seven TM domains and a common functional feature of G protein coupling. On the other hand, GPCRs show a great diversity, such as a variety of ligands in nature found out to bind to GPCRs. Their diversity is further augmented by the fact that receptors for the same ligand can include many subtypes. Moreover, these subtypes initiate distinct intracellular signaling cascades. One of the prototypes is the dopaminergic receptors whose endogenous agonist is dopamine (DA). Studies on DA receptors provide insight into how an endogenous ligand

mediates physiological functions via binding to different subtypes of the same receptor family.

2. Dopamine Receptors

2.1. Dopamine system

DA is a major catecholamine neurotransmitter both in the central nervous system (CNS) and in the periphery. In mammalian brain, DA system regulates a variety of physiological functions. Dysfunction of the system is implicated in etiology of many well known neurological and neuropsychiatric disorders, such as Parkinson's disease, schizophrenia, and drug addiction. Thus, the study of DA system is critical to understand the etiology, phenotypic expression and treatment of these disorders.

The DA system consists of three principle neuronal pathways. The nigrostriatal pathway starts off from the substantia nigra and projects into the striatum. It controls locomotion. Dysregulation of the pathway is believed to be the underlying cause of Parkinson's disease. The mesolimbicortical pathway stems from ventral tegmental area and projects into limbic system and prefrontal cortex. This pathway is responsible for regulating emotion, cognition and reward. Dysfunction of this pathway is associated with schizophrenia and drug addiction. The tuberoinfundibular pathway originates from hypothalamus and projects to pituitary. This pathway is important in controlling neuroendocrine secretion, such as prolactin secretion (Kandel *et al.* 1991).

2.2. Classification of DA receptors

DA produces a broad variety of physiological effects through binding to members of DA receptor family. D1 and D2 receptors were the first DA receptors identified on the basis of pharmacological and biochemical evidence (Spano *et al.* 1978). Gene cloning brought new insight into DA receptor research (Missale *et al.* 1998). In addition to D1 and D2 receptors, three novel mammalian DA receptors were also cloned, including D3, D4, D5 receptors (Sokoloff *et al.* 1990; Tiberi *et al.* 1991; Sunahara *et al.* 1991; Weinshank *et al.* 1991; Grandy *et al.* 1991; Van Tol *et al.* 1992;). The D1 and D5 receptors are also referred to as D1A and D1B receptors, respectively. All mammalian DA receptors are categorized into two subfamilies, D1-like receptors and D2-like receptors, based on their different primary structure, gene structure, pharmacological profiles and signal transduction. D1-like receptors are composed of D1A/D1 and D1B/D5 subtypes and D2-like receptors are divided into D2, D3 and D4 subtypes (Table 1).

2.2.1. The primary structure and gene structure of DA receptors

DA receptors belong to GPCR family A (rhodopsin/β_2-adrenergic receptor-like receptors). A majority of receptors in family A, including D1A/D1 receptor and rhodopsin, have shown to contain one or more highly conserved palmitoylated cysteines, which facilitate CT association with membrane. Since these cysteines are located in the N-termini of CTs, a loop is believed to exist, encompassing from the carboxyl terminus of TM7 to palmitoylated cysteines, namely the fourth intracellular loop (IL4) (Konig *et al.* 1989). However, surprisingly, IL4 formation does not require the contribution of palmitoylation (Yeagle *et al.* 1996). The recently obtained crystal structure of rhodopsin

showed that the IL4 actually adopts α-helical conformation and is then termed helix 8 (H8) (Palczewski 2000). The unique structure of IL4 may reflect its important role in regulating GPCR functions (Palczewski 2000).

Studies on DA receptor primary structures reveal differences in the overall topology of the two subfamilies. Compared with D2-like receptors, D1-like receptors have short IL3s and long CTs (Missale *et al.* 1998) (Table 1). Members of each subfamily share high degree of homology in TMs: 82% identity shared by D1A and D1B receptors, 75% identity found between D2 and D3 receptors and 53% identity observed between D2 and D4 receptors (Jarvie and Caron 1993) (Table 1).

The five DA receptors are encoded by five different genes. Analysis of gene structures shows that D1-like receptor genes do not contain introns in their coding sequences, whereas D2-like receptor genes have several introns (Table 1). The existence of introns in the coding sequences of D2-like receptors gives rise to a variety of splice variants. The D2 receptor cloning reveals the existence of two splice variances, $D2_{short}$ and $D2_{long}$ (Dal Toso *et al.* 1989; Giros *et al.* 1989; Monsma et al. 1989) (Table 1). Splice variants for D3 receptors have been identified and shown to encode functional and nonfunctional proteins (Giros *et al.* 1991; Snyder *et al.* 1991; Fishburn *et al.* 1993). Besides splice variants, the existence of polymorphic variants in the coding sequence of human D4 receptors has also been recognized (Van Tol *et al.* 1992). In addition, two related pseudogenes of D1B receptors have also been isolated. They encode truncated, nonfunctional forms of the D1B receptors (Grandy *et al.* 1991).

2.2.2 Pharmacological profiles and signal transduction of the DA receptor subfamilies

Pharmacological differentiation of the two DA receptor subfamilies is achieved by their selective ligands. Members of D1-like receptors bind to their prototype antagonist SCH23390 with high affinity; whereas members of D2-like receptors bind to their prototype antagonist spiperone with high affinity (Table 1). However, no compounds are available to identify specific subtypes of D1-like receptors. Moreover, no ligands can distinguish two D2 receptor splice variants. Some agonist and antagonists selectively binding to either D3 (Rivet *et al.*1994; Scheideler et al. 1997) or D4 (Van Tol *et al.* 1991; Nayak and Cassaday 2003) receptors have been developed. These compounds help distinguishing D3 and D4 subtypes from D2 receptors.

In terms of signal transduction, D1-like receptors and D2-like receptors display distinct regulatory effects on AC activities. In particular, D1-like receptors can activate AC via coupling to $G\alpha_s$ proteins; whereas D2-like receptors can inhibit AC via coupling to $G\alpha_i$ proteins (Table 1). In addition to inhibition of AC, it is well documented that D2-like receptors can activate potassium channels, leading to cell hyperpolarization (Einhorn *et al.* 1991; Liu *et al.* 1994). It has been reported that D1-like receptor agonists can activate PLC-mediated phosphoinositide (PI) hydrolysis in brain (Felder *et al.* 1989a; Undie and Friedman 1990, 1992; Undie *et al.* 1994) and in kidney (Felder *et al.* 1989b). However, stimulation of PI hydrolysis failed in the recombinant systems expressing cloned D1-like receptors. The human genome sequences have excluded the potential existence of novel D1-like receptors. So the most likely explanation of this discrepancy between native systems and recombinant systems is that the functional effects observed

after D1-like agonist treatment may be caused by receptor(s) other than D1-like receptors (Montague *et al.* 2001). Alternatively, recombinant systems may lack of one or more downstream signaling partner(s) of D1-like receptors essential for the stimulation of PLC-mediated PI hydrolysis pathway.

2.3. Molecular basis for the regulation of DA receptor functions

Some functional properties of DA receptors include ligand binding, G protein coupling, post-translational modifications, desensitization, and internalization. Moreover, studies on structure-function relationships revealed that DA receptor functional properties are regulated by specific structural determinants.

2.3.1. DA receptor domains involved in ligand binding

A model for ligand binding to β_2-adrenergic receptor is well documented (Strader *et al.* 1988; Strader *et al.* 1989). Structural similarities among catecholamine receptors allow the information gained from β_2-adrenergic receptor to define the ligand binding profile of DA receptors. The binding model states that TMs of catecholamine receptor form a ligand binding pocket (Gether and Kobilka 1998; Gether 2000). In the binding pocket, several residues are highly conserved in catecholamine receptors and are proposed to be crucial in ligand binding. They include the highly conserved Asp residue in TM3 and two Ser residues in TM5 of catecholamine receptors. Mutations of the Asp residue led to reduced binding affinity of catecholamine ligands to β_2-adrenergic receptor (Strader *et al.* 1988) as well as binding of agonist and antagonist to D2 receptor (Mansour *et al.* 1992). The underlying mechanism may be that negatively charged carboxyl group

of Asp^{113} binds to positively charged amine group of catecholamine (Strader *et al.* 1988). Moreover, mutations of conserved Ser residues in TM5 of β_2-adrenergic receptor significantly attenuated the receptor's ability to bind to catecholamine agonists (Strader *et al.* 1989). The two Ser residues are proposed to form hydrogen bounds with carboxyl group of catecholamine (Strader *et al.* 1989). Mutations of these highly conserved Ser residues in D1A receptors abolished DA affinity but led to varied effects on other dopaminergic ligand binding (Pollock *et al.* 1992). Taken together, these results not only indicate the crucial roles of highly conserved residues (Asp residue in TM3 and Ser residues in TM5) in mediating ligand binding properties of catecholamine receptors but also reflect on the importance of individualized ligand binding pockets for DA and β_2-adrenergic receptors.

2.3.2. DA receptor domains involved in G protein coupling

With respect to G protein coupling domains, and consistent with most other GPCRs, DA receptors contain G protein coupling sites located on the intracellular site. In particular, IL3 of D1A/D1 receptor plays a crucial role in $G\alpha_s$ coupling and AC activation (Kozell *et al.* 1994), whereas both IL2 and IL3 of D2 receptor are critical for $G\alpha_i$ coupling and AC inhibition (Kozell *et al.* 1994; Lachowicz *et al.* 1997).

2.3.3. DA receptor regions involved in desensitization and internalization

Desensitization and internalization are two processes critical for controlling GPCR responsiveness. As mentioned previously, GPCR desensitization occurs following

phosphorylation by either second messenger-dependent kinases (e.g. PKA) or G protein-coupled receptor kinases (GRKs). D1A/D1 receptor is the best studied DA receptor regarding receptor phosphorylation and desensitization (Tiberi *et al.* 1996; Jiang and Sibley 1999; Lamey *et al.* 2002; Jackson *et al.* 2002). A mutational study has revealed that Thr^{268} in the IL3 serves as the substrate for PKA-mediated phosphorylation (Jiang and Sibley 1999). Phosphorylation of Thr^{268} led to agonist-induced desensitization (Jiang and Sibley 1999). Meanwhile, variant residues in the CT are shown to be substrates for GRK-mediated phosphorylation (Tiberi *et al.* 1996; Lamey *et al.* 2002; Jackson *et al.* 2002). Furthermore, phosphorylation of Thr^{360} in the CT of D1A receptor is shown to result in receptor desensitization, whereas phosphorylation of Thr^{446}, Thr^{439}, and Ser^{431} of the CT are involved exclusively in receptor internalization (Lamey *et al.* 2002). These results suggest that desensitization and internalization may be regulated by different amino acid residues in the CT of D1A receptor (Jackson *et al.* 2002; Lamey *et al.* 2002).

2.3.4. Palmitoylation of DA receptors

Besides phosphorylation, palmitoylation is another important post-translational modification of DA receptors. D1A and D2 receptors have been demonstrated to undergo palmitoylation (Ng *et al.* 1994a, b; Jin *et al.* 1999b). The palmitoylation sites of D1A receptors are found at Cys^{347} and Cys^{351} residues located at the N-terminal part of the CT (Jin *et al.* 1999). The palmitoylaiton of D1B/D5, D3 and D4 receptors remains to be explored.

3. Focus on D1-Like Receptors

D1-like receptors are composed of D1A/D1 and D1B/D5 subtypes, which share similar gene structure, pharmacological profiles and signal transduction properties. However, notwithstanding these similarities, the two subtypes display striking differences, such as receptor distribution and functional properties. Differences displayed by D1A/D1 and D1B/D5 subtypes may reflect the unique roles of the two D1-like subtypes in DA-mediated physiological functions.

3.1. D1-like receptor distribution

3.1.1. mRNA distribution of D1-like receptor

D1A/D1 receptor is the more broadly distributed receptor in CNS as compared with D1B/D5 receptors (Dearry *et al.* 1990; Fremeau *et al.* 1991; Weiner *et al.* 1991). In situ hybridization reveals that D1A mRNA is most abundant in striatum, nucleus accumbens and olfactory tubercles (Dearry *et al.* 1990; Fremeau *et al.* 1991; Weiner *et al.* 1991) (Table 1). Cells expressing D1A mRNA are also detected throughout cerebral cortex, hypothalamus, and thalamus (Fremeau *et al.* 1991). In striking contrast to D1A receptor, only small amount of D1B mRNA is present in striatum (Tiberi *et al.* 1991; Meador-Woodruff *et al.* 1992) but expresses at high levels in hippocampus, lateral mammalian nuclei, and parafascicular nucleus of the thalamus (Tiberi *et al.* 1991; Meador-Woodruff *et al.* 1992) (Table 1).

Studies on peripheral distribution of DA receptors reveal that both D1-like subtypes are present in kidney (Nash *et al.* 1993). Additionally, D1A receptors are located in adrenal glands and gastrointestinal tract (Aherne *et al.* 1997; Vaughan *et al.* 2000).

3.1.2. Protein distribution of D1-like receptors

The mRNA distribution can not reflect the actual amount and distribution of translated protein, because mRNA is restricted to the cell body and the amount and location of proteins are also affected by many other factors (e.g. synthesis, turnover, intracellular transport and protein trafficking) (Ciliax *et al.* 2000). The immunocytochemistry technique provided insight into the protein distribution of D1-like receptors. Protein distribution of D1-like receptors reveals that the two subtypes display different cellular and subcellular localization in some brain tissues. More specifically, cellular analysis on D1-like receptors reveals that D1A receptor is more abundant than D1B receptor in medium spiny neuron of caudate nucleus and in neuropil of the substantia nigra pars reticulata, whereas D1A receptors express to a lesser extent than D1B receptors in cholinergic neurons of basal forebrain (Bergson *et al.* 1995a, b). Moreover, only D1B receptor but not D1A receptor was detected in cholinergic interneurons of caudate nucleus (Bergson *et al.* 1995a, b). Subcellular analysis on D1-like receptors shows that the two receptors display different subcellular localization in cerebral cortex and hippocampus (Bergson *et al.* 1995a, b; Ciliax *et al.* 2000). In particular, D1A receptor is located on dendritic spines of pyramidal neurons in cerebral cortex and hippocampus, while D1B receptor is located on dendritic shafts of these neurons (Bergson *et al.* 1995a).

With respect to peripheral protein distribution, D1-like receptors are located in retina, kidneys and lungs (Kobayashi *et al.* 1995; Nguyen-Legros *et al.* 1997, 1999; Amenta *et al.* 2002). A recent study on systemic arteries revealed that faint D1A receptor and moderate D1B receptor were detected in smooth muscle of tunica media of pial and

mesenteric artery branches (Amenta *et al.* 2000). Additionally, D1B receptors are found in platelets (Ricci *et al.* 2001).

3.2. The roles of D1-like receptors in normal physiologies and pathologies

3.2.1. D1-like receptor knockout studies

The distinct distribution of D1-like receptors in the CNS as mentioned above may suggest their unique roles in physiological functions (Bergson *et al.* 1995a, b). However, due to the lack of highly selective agonists and antagonists, the knowledge regarding the functional roles of each D1-like subtype is quite limited. Genetic approaches aiming to manipulate the expression of specific D1-like receptor subtypes have been used as alternatives. Gene targeting using homologous recombination techniques (knockout) is one of the widely used genetic approaches. Thus observation on phenotypes of knockout mice may provide insight into the roles of a particular D1-like receptor subtype in mediating physiological functions (Sibley 1999).

D1A knockout mouse models have been constructed by two independent research groups (Xu *et al.* 1994b; Drago *et al.* 1994). The D1A knockout mice showed significantly retarded growth as well as reduced body weight and brain size (Xu *et al.* 1994b; Drago *et al.* 1994). In terms of locomotor activity, one research group showed that the locomotor activity of D1A knockout mice was not significantly different from that of the wild-type mice (Drago *et al.* 1994), whereas the other research group observed that D1A knockout mice were hyperactive (Xu *et al.* 1994b). This discrepancy may be due to different genetic backgrounds of these mice (Drago *et al.* 1994; Xu *et al.* 1994b). Surprisingly, the phenotypes of D1A knockout mice in locomotor activity seem to be

contradictory from the well believed stimulatory role of D1A receptors in locomotion (Sibley 1999; Drago *et al.* 1994; Xu *et al.* 1994b). The lack of locomotion deficiency in D1A knockout mice may be due to compensatory mechanisms activated by the D1A receptor deletion (Xu *et al.* 1994b). In the other words, adaptive changes may occur to compensate for the absence of D1A receptor functions. Besides locomotor activity, the potential role of D1A receptors in mediating effects of psychostimulants (e.g. cocaine) was investigated in knockout mice (Xu *et al.* 1994a). Some effects of cocaine, such as hyperactivity and stereotyped behaviors were abolished in D1A knockout mice (Xu *et al.* 1994a). Thus, D1A receptor may also be crucial in mediating certain cocaine-induced effects (hyperactivity and stereotyped behaviors). Moreover, Morris water task indicates that D1A knockout mice exhibited impaired learning and memory abilities, suggesting the involvement of D1A receptor in learning and memory processes (Smith *et al.* 1998). In addition, since D1A receptors are expressed in renal proximal tubule, the importance of this receptor in the control of blood pressure was also investigated in mice lacking D1A receptor (Albrecht *et al.* 1996). D1A knockout mice were shown to develop high blood pressure, raising the possible involvement of D1A receptor malfunction in the development of hypertension (Albrecht *et al.* 1996).

The potential role of D1B receptor in physiology functions was also assessed by D1B knockout mice. The D1B knockout mice were viable, grow normally and fertile (Holmes *et al.* 2001; Hollon *et al.* 2002). However, D1B knockout mice were shown to be hypertensive, most likely due to increased sympathetic tone (Hollon *et al.* 2002). Further study reveals that the increased sympathetic tone may be caused by the abnormal activation of vasopressin V_1 and non-NMDA glutamatergic receptor mediated neuronal

pathways in D1B knockout mice (Hollon *et al.* 2002). These results suggest that the D1B receptor regulates neuronal pathways essential for the mediation of blood pressure, a finding that may provide new insight into the underlying causes of hypertension (Hollen *et al.* 2002). In addition, the potential role of D1B receptor in mediating effects of cocaine was investigated. Results show that locomotor stimulus effects of cocaine in D1B knockout mice was significantly lower than that in wild-type mice, suggesting that D1B receptor is involved in mediating locomotor stimulus effects of cocaine (Elliot *et al.* 2003). In terms of locomotor activity, the phenotypes of D1B knockout mice did not support strongly the important functional role in locomotor activity, since D1B knockout mice were essentially identical to wild-type mice (Holmes *et al.* 2001). However, we should bear in mind that compensatory mechanisms may occur in response to the elimination of D1B receptors and obscure the actual changes caused by a D1B receptor deficiency. Furthermore the distinct CNS distribution as well as functional properties (discussed later) of D1B receptor implicate the unique role of D1B receptor in physiological functions. Indeed, by using antisense oligonucleotides to D1B/D5 receptor, which specifically suppress the D1B receptor expression in the rats brain, evidence has shown the potential inhibitory effect of D1B receptor on locomotion (Dziewczapolski *et al.* 1998) and the potential physiological role of the D1B receptor in mediating female rat sexual behavior (the reproductive behavior lordosis) (Apostolakis *et al.* 1996).

3.2.2. The potential role of D1-like receptors in pathologies

Not only D1-like receptors are engaged in numerous physiological functions, but also impaired D1-like receptor signaling is associated with many diseases in both CNS and

periphery. Although one hypothesis proposes the association of overactive D2 receptors with positive symptoms of schizophrenia, recent evidence shows the involvement of D1-like receptors in schizophrenia (Okubo *et al.* 1997). More specifically, D1-like receptors, located at the prefrontal cortex (PFC), take part in mediating working memory function (Arnsten *et al.* 1994; Friedman *et al.* 1999). One of the characteristics observed in schizophrenia is impaired working memory function (Goldman-Rakic 1994). Recently, positron emission topography (PET) revealed the decreased expression of D1-like receptors in PFC of schizophrenia patients (Okubo *et al.* 1997). This reduced amount of D1-like receptor in PFC is believed to relate to cognitive deficits and negative symptoms of schizophrenia (Okubo *et al.* 1997). Therefore, agents aiming to enhance the activities of D1-like receptors may be beneficial for treating this disease. A promising candidate is estrogen, which augments D1B receptor expression in the brain (Lee *et al.* 2001). Indeed, one group reported that female patients with schizophrenia, treated with estrogen combined with neuroleptic drugs showed significantly improvement in psychotic symptoms in comparison with female patients treated with neuroleptic drugs alone (Kulkarni *et al.* 1996).

Defects in D1-like receptor function may also underlie the cause of hypertension. As mentioned in knockout studies, mice lacking either D1-like subtype developed hypertension. However, abnormal D1A and D1B receptors may lead to hypertension through different mechanisms. On the one hand, dysfunction of D1B receptor may cause hypertension most likely through increased sympathetic tone (see D1B knockout studies), and one the other hand, impaired D1A receptor signaling in renal proximal tubule may be related to some forms of essential hypertension (Albrecht *et al.* 1996). In kidney, D1A

receptors on renal proximal tubule play a crucial role in regulating sodium reabsorption by inhibiting sodium transporters, such as Na^+/H^+ exchanger (reviewed in Carey 2001). This inhibitory effect is mainly due to AC stimulation and the subsequent activation of cAMP/PKA pathway (Felder *et al.*, 1990a, b; Glahn *et al.*, 1993; Debska-Slizien *et al.*, 1994). Studies have revealed uncoupling of D1A receptor with downstream G protein and effectors in both rat and human models of hypertension (Felder *et al.* 1990; Sanada *et al* 1999). Moreover, the uncoupling of D1A receptor may be due to high levels of constitutive or basal receptor phosphorylation and subsequently receptor desensitization (Jose *et al.* 1996; Sanada *et al.* 1999;Asghar *et al.* 2002). Indeed, a polymorphic variant of GRK4 (a subtype of GRK family) has been implicated in the desensitization of D1A receptor in human renal proximal tubule (Watanabe *et al.* 2002).

In summary, a body of evidence has shown the important physiological and pathological roles of D1-like receptors. Further investigation on D1-like receptor signaling is crucial for better understanding D1-like receptors in normal physiological functions and disease associated states.

3.3. The constitutive activity of D1B receptor and its potential role in physiological function

Studies on D1-like receptor signal transduction reveal that D1B receptor displays significantly higher constitutive activity (or agonist-independent activity) in comparison with D1A receptor (Tiberi and Caron 1994). Furthermore, the constitutive activity of D1B receptor is believed to play a role in regulating physiological functions. Specifically, a study has shown that estrogen induces increased expression of constitutively active

D1B receptors, which generate cAMP in a ligand-independent manner. The constitutive activity of the D1B receptor is proposed to mediate estrogen-induced stimulation on atrial natriuretic factor release and allows estrogen to exert its effect on female sexual behavior (Lee *et al.* 1999). Further study reveals that progesterone, another ovarian steroid, can facilitate the effect of estrogen on enhancing D1B receptor expression (Lee *et al.* 2000).

Ideally, D1B selective ligands that have the ability to modify the constitutive activity of D1B receptor will prove valuable to study the constitutive activity of D1B receptors both in vitro and in vivo. However, until now, no such compounds have been developed. Thus studies investigating the underlying molecular basis of the constitutive activity of D1B receptors may aid in the development of selective ligands that reduce D1B receptor constitutive activity.

3.4. Studies on structure-function relationships of D1-like receptors

Besides their different levels of constitutive activity, the D1A and D1B receptors also display other distinct functional properties (Tiberi and Caron 1994). In particular, studies on ligand binding properties of D1-like receptors show that D1B receptors display a higher affinity for agonists and a lower affinity for antagonists as compared with D1A receptors. Investigation on G protein coupling properties of D1-like receptors indicates that D1B receptors exhibit an increased constitutive activity (or agonist-independent activity), an enhanced DA potency and a decreased DA-mediated maximal activation in comparison with D1A receptors. However, the structural determinants of D1-like receptors underlying these different functional properties are not yet fully understood. So

far, investigation of structure-function relationships of D1-like receptors has mostly relied on mutagenesis studies.

Pro residues are well conserved in TM helices of GPCRs. One study suggested that X-Pro peptide bonds of D1A receptor (X is the residue that locates immediately adjacent to Pro residue on the amino-terminal side) are critical for ligand binding and G protein coupling (Cho *et al.* 1996). In particular, researchers substituted the X residues in X-Pro peptide bonds and compared the functional properties of mutant receptors with the wild-type D1A receptors. Mutant receptors, in which X residue (either Ile^{205} in TM5 or Leu^{286} in TM6) was replaced with Tyr residue, displayed significantly decreases in dopaminergic agonist binding affinity and DA efficacy as compared with wild-type D1A receptors (Cho *et al.* 1996). Interestingly, substitution of Leu^{286} with Ala led to a mutant receptor with enhanced agonist-independent activity and increased agonist potency (Cho *et al.* 1996). These results suggest that X-Pro bonds may be involved in mediating receptor ligand binding pocket formation and thermodynamic transition between the active and inactive receptor conformation (Cho *et al.* 1996).

Furthermore, in light of the role of the C-terminal region of IL3 of α_{1B}-adrenergic receptor in constitutive activity (Kjelsberg *et al.* 1992; Lefkowitz *et al.* 1993), the potential regulatory roles of residues located in this region of D1-like receptors have been investigated. In the C-terminal region of the IL3 of D1-like receptors, primary sequences of D1A and D1B receptors are identical except for two amino acids, Phe^{264}/Arg^{266} in the D1A receptor and ILe^{288}/Lys^{290} in the D1B receptor (Charpentier *et al.* 1996). The substitution of D1A receptor residue Phe^{264} by D1B receptor counterpart ILe^{288} led to a chimeric D1A receptor displaying increased DA affinity, DA potency and agonist-

independent activity as compared with wild-type D1A receptor (Charpentier *et al.* 1996). In the opposite fashion, the replacement of D1B receptor residue ILe^{288} by the D1A receptor residue Phe^{264} resulted in a chimeric D1B receptor exhibiting a decreased DA affinity, DA potency and agonist-independent activity as compared with wild-type D1B receptor (Charpentier *et al.* 1996). The exchange of Arg^{266}/Lys^{290} between D1A and D1B receptors did not induce any noticeable changes in functional properties of chimeric receptors as compared with their cognate wild-type receptors (Charpentier *et al.* 1996). These results demonstrated that D1A receptor residue Phe^{264} and D1B receptor residue ILe^{288} are involved in regulating D1-like subtype-specific functional properties. However, the reciprocal swap of these two residues did not lead to a complete reversal of D1A and D1B receptor phenotypes (Charpentier *et al.* 1996). Therefore, other structural determinants may also take part in regulating D1-like subtype-specific functional properties.

Previously, it has been shown that mutations, found in extracellular, transmembrane, other intracellular domains of GPCRs, could also lead to constitutively active mutant receptors (Robinson *et al.* 1992; Robbins *et al.* 1993; Rao *et al.* 1994; Matus-Leibovitch *et al.* 1995; Spalding *et al.* 1995). Hence, these regions may also contain structural determinants regulating D1-like subtype-specific constitutive activity. Based on this assumption, our laboratory has studied the role of structural determinants in a region of D1-like receptors termed the terminal receptor locus (TRL). The TRL is the region of the receptor composed of TM6 and TM7 as well as EL3 and CT (Iwasiow *et al.* 1999). Several sets of chimeric receptors were constructed by switching either entire TRL or EL3 or CT sequences between D1A and D1B receptors (Iwasiow *et al.* 1999; Jackson *et*

al. 2000; Tumova *et al.* 2003). The functional properties of chimeric D1-like receptors were then compared with those of wild-type receptors. In terms of ligand binding properties, the insertion of the D1B-TRL into D1A receptor led to the chimeric D1A receptor displaying an enhanced DA affinity, which is reminiscent of the D1B receptor. However, the addition of the D1A-TRL into D1B receptor resulted in the chimeric D1B receptor exhibiting a reduced DA affinity, which is reminiscent of the D1A receptor (Iwasiow *et al.* 1999). In a similar fashion, the exchange of CT resulted in a complete switch of DA affinity (Jackson *et al.* 2000). However, the exchange of EL3 region only led to a partial swap of DA affinity (Iwasiow *et al.* 1999). Taken together, the binding results suggest that CT is the TRL region regulating DA affinity (Jackson *et al.* 2000).

Our laboratory has also assessed the G protein coupling properties such as agonist-independent activity and dependent activity (e.g. DA-mediated maximal activation and DA potency) of wild-type and chimeric D1-like receptors. With respect to agonist-independent activity, chimeric D1A receptor harboring the D1B-TRL displayed an enhanced agonist-independent activity, which is indistinguishable from the D1B receptor. Chimeric D1B receptor bearing the D1A-TRL exhibited a decreased agonist-independent activity, which is indistinguishable from the chimeric D1A receptor (Iwasiow *et al.* 1999). Similarly, the exchange of CT also resulted in a complete switch of agonist-independent activity, suggesting that the CT is the structural determinant of TRL in controlling agonist-independent activity (Jackson *et al.* 2000).

In terms of agonist-dependent activity, chimeric D1A receptor with D1B-TRL displayed an increased DA potency, which is reminiscent of D1B receptor. Chimeric D1B receptor harboring D1A-TRL exhibited a decreased DA potency, which is

essentially similar to that of D1A receptors (Iwasiow *et al.* 1999). However, swapping of EL3 only led to a partial switch of DA potency (Iwasiow *et al.* 1999). Intriguingly, DA potency was decreased in cells expressing the CT chimeras (D1A-CTB and D1B-CTA) (Jackson *et al.* 2000). Since the swap of TRL led to a complete reversal of DA potency (Iwasiow *et al.* 1999), other structural determinants within TRL may play a role in "rescuing" reduced DA potency displayed by the CT chimeras (Jackson *et al.* 2000; Tumova *et al.* 2003). Indeed, a recent study from our laboratory revealed that the insertion of EL3-D1B into the chimeric D1A-CTB receptor "rescued" DA potency of this chimera to a level similar to that of D1B receptors (Tumova *et al.* 2003). Similar results were obtained using D1B-EL3CTA chimera (Tumova *et al.* 2003). Altogether, these results indicate that a molecular interplay between the EL3 and CT regions controls DA potency (Tumova *et al.* 2003).

With respect to DA-mediated maximal activation of AC, no changes were observed following the swap of TRL regions. Specifically, the higher DA-mediated maximal activation of AC elicited by wild-type D1A receptors was maintained in cells expressing chimeric D1A-TRLB receptors (Iwasiow *et al.* 1999). Similarly, the lower DA-mediated maximal activation of AC observed in cells expressing wild-type D1B receptors remains unchanged in cells expressing the D1B-TRLA chimera (Iwasiow *et al.* 1999). These findings suggest that other structural determinants located outside TRL may be required for mediating DA-mediated maximal activation. Alternatively, it is possible that specific regions within TRL may contribute to the activity (Iwasiow *et al.* 1999). However, the exchange of the relatively large TRL region may have obstructed the identification of these structural determinants (Iwasiow *et al.* 1999). Indeed, the exchange of EL3 region

led to a full swap of DA-mediated maximal activation of AC (Iwasiow *et al.* 1999). In contrast, DA-mediated maximal activation was increased in cells expressing both CT chimeras (Jackson *et al.* 2000). Additionally, the complete switch of DA-mediated maximal activation of AC induced by the swap of EL3 region was not observed when exchanging both the EL3 and CT regions. This result indicated that specific residues or motifs within the CT may antagonize the effect of the EL3 on the regulation of agonist-mediated maximal activation.

Another interesting finding is that insertion of D1B-CT into the D1A receptor significantly reduced receptor number of this D1A chimera (D1A-CTB chimera) (Jackson *et al.* 2000). This finding indicated that CT of D1B receptor may take part in regulating receptor stability and determining functional D1-like receptor expression (Jackson *et al.* 2000). Moreover, the addition of the EL3 of D1B into the D1A-CTB chimeric receptor led to a total rescue of the loss of receptor density for this chimera (Tumova *et al.* 2003). This further demonstrated the importance of the molecular interplay between the EL3 and CT regions in regulating receptor number (Tumova *et al.* 2003).

In light of the crucial role of CT in controlling functional properties of D1-like receptors, further efforts are needed to narrow down the specific sequence motifs or particular amino acid residues within CT involved in the regulation of ligand binding and G protein coupling properties. A site-directed mutagenesis has shown that replacement of highly conserved residue in the CT of D1B receptor, Gln^{439}, with Ala/Ile resulted in a drastic increase of DA affinity (Demchyshyn *et al.* 2000). Besides the potential role of Gln^{439} in mediating the DA binding affinity of D1B receptor, several lines of evidence

also suggest the potential involvement of IL4 in conferring subtype-specific ligand binding and G protein coupling properties of D1-like receptors. As mentioned earlier, the fourth intracellular loop (IL4) is a CT region, which is also known as H8 based on its unique α-helical conformation revealed by crystal structure of rhodopsin (Palczewski 2000). The important role of IL4 in mediating receptor-G protein activation has been demonstrated in many receptors in GPCR family A. In particular, substitution of IL4 of rhodopsin, more specifically the N-terminal region of IL4, with corresponding sequence of β_2-adrenergic receptor reduced mutated receptor's ability to activate G protein transducin (Gt) (Marin *et al.* 2000). An additional study provides direct evidence that the N-terminal region of IL4 serves as the binding site for G protein α subunit (Ernst *et al.* 2000). In addition to rhodopsin, IL4 of β_2-adrenergic receptor is proven to play an important role in G protein coupling of the receptor (O'Dowd *et al.* 1988). Substitution or deletion of specific amino acid residues within this region resulted in a diminished agonist potency and agonist-mediated maximal activation of β_2-adrenergic receptor (O'Dowd *et al.* 1988). Moreover, a recent truncation study in our laboratory revealed that truncating CT at Cys^{351} residue, and thus maintaining IL4 of D1A receptors led to a truncated receptor displaying enhanced DA affinity and DA-mediated maximal activation (Chaar *et al.* 2002). This study suggests that IL4 may be the CT domain regulating D1-like subtype-specific functional properties.

PART II – OBJECTIVE AND HYPOTHESIS

In addition to the truncation study mentioned above, our laboratory has also used a mutational approach to investigate the potential role of IL4 in the regulation of D1-like

subtype-specific ligand binding and G protein coupling properties. More specifically, two chimeric D1-like receptors were constructed by swapping of IL4 between D1A and D1B receptors. Preliminary data obtained from IL4 mutational studies revealed a potential role of IL4 in controlling distinct functional properties of D1-like receptors (Tumova and Tiberi, unpublished data). During my master studies, I focus my efforts on investigating further the structural determinants within IL4 of D1-like receptor implicated in regulating the distinct functional properties of the two D1-like subtypes. Primary sequences of D1-like receptor show that two palmitoylated cysteines are located in IL4 of D1A receptor. Likewise, two cysteine residues are also found in similar locations of the IL4 of D1B receptors. The two cysteines were used as borders to define N- and C- terminal parts or segments of IL4, namely IL4N and IL4C (Fig. 4). Most importantly, IL4N and IL4C of D1A and D1B receptors share low primary sequence identities (Fig. 4). These differences may thus account for the distinct functional properties of D1-like receptors. The ***objective*** of my project is to study the role of the N- and C-terminal segments of IL4 in the regulation of the functional properties of the D1-like receptors. The underlying ***hypothesis*** is that differences found in the primary amino acid sequences of the N- and C-terminal ends of IL4 of the D1A and D1B receptors confer D1-like subtype-specific ligand binding and G-protein coupling properties.

Figure 1: The schematic representation of GPCRs

The common feature of GPCRs is composed of seven α-helical transmembrane domains. Three extracellular loops (EL1, EL2, EL3) and amino terminus are on the extracellular side, whereas three intracellular loops (IL1, IL2, IL3) and carboxyl terminus are situated on the intracellular side.

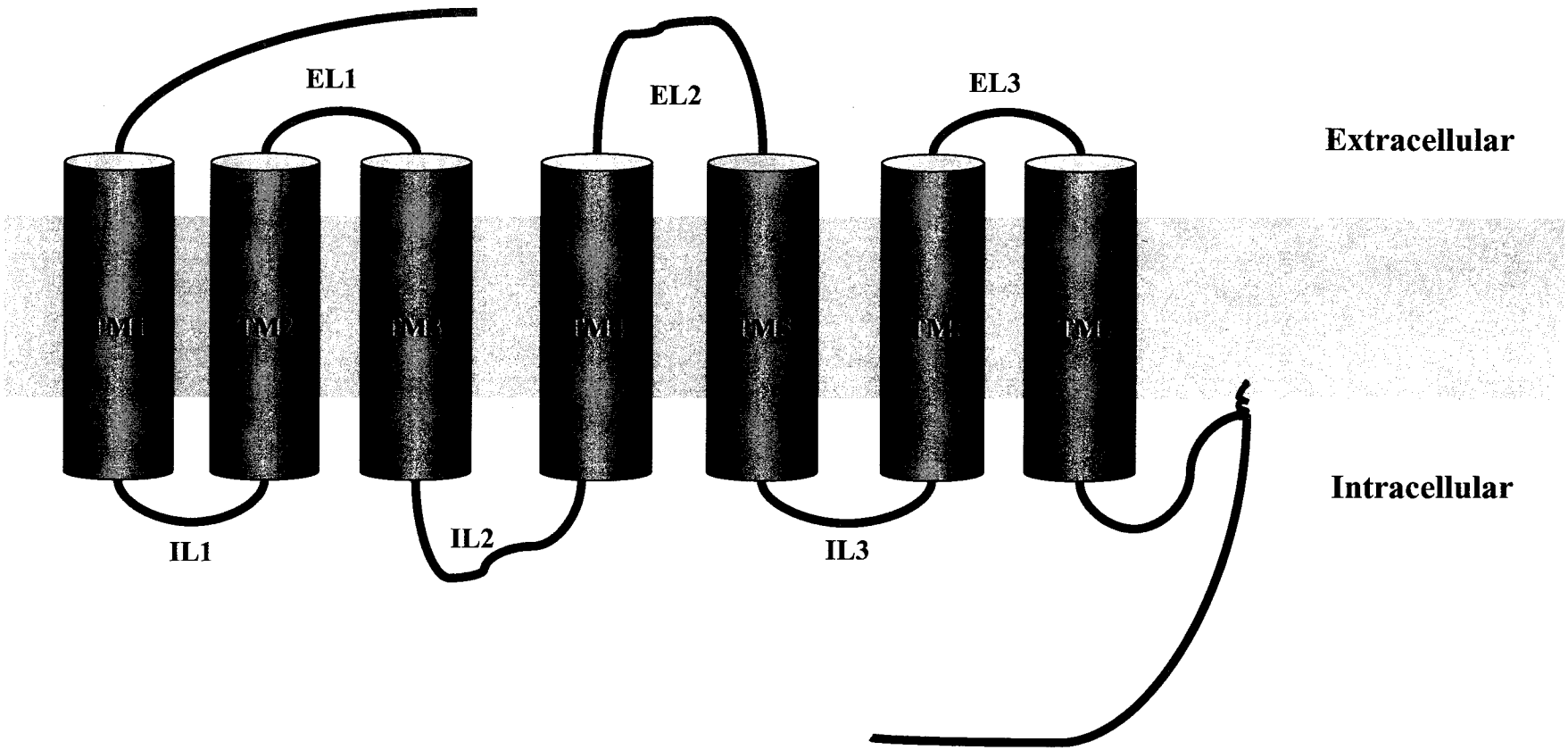
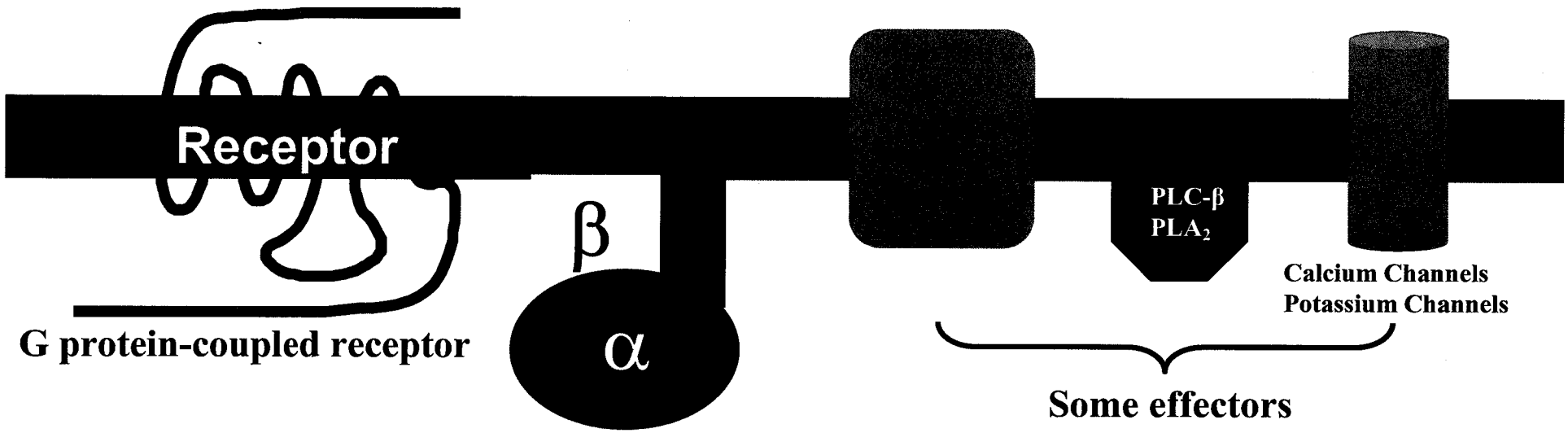

EL1
EL2
EL3
Extracellular
TM1
TM2
TM3
TM4
TM5
TM6
TM7
Intracellular
IL1
IL2
IL3

Figure 2: Basic functional elements of G protein signaling

The elements include GPCRs, G proteins and effectors.



G protein:

23 α subunits
6 β subunits
12 γ subunits

Figure 3: Regulation of the position of the equilibrium between an inactive state (R) and active state (R*) by classes of pharmacological ligands

Full agonist binding can release the intramolecular constraints and can stabilize optimally receptor in the active conformation, driving completely the equilibrium towards R*. Conversely, full inverse agonists stabilize optimally the receptor in the inactive receptor conformation, shifting fully the equilibrium towards R. Antagonists do not change GPCR conformation, leaving this equilibrium unchanged. Partial agonists and partial inverse agonists are less efficient in comparison with full agonists and full inverse agonists in terms of stabilizing GPCRs in R* and R, respectively.

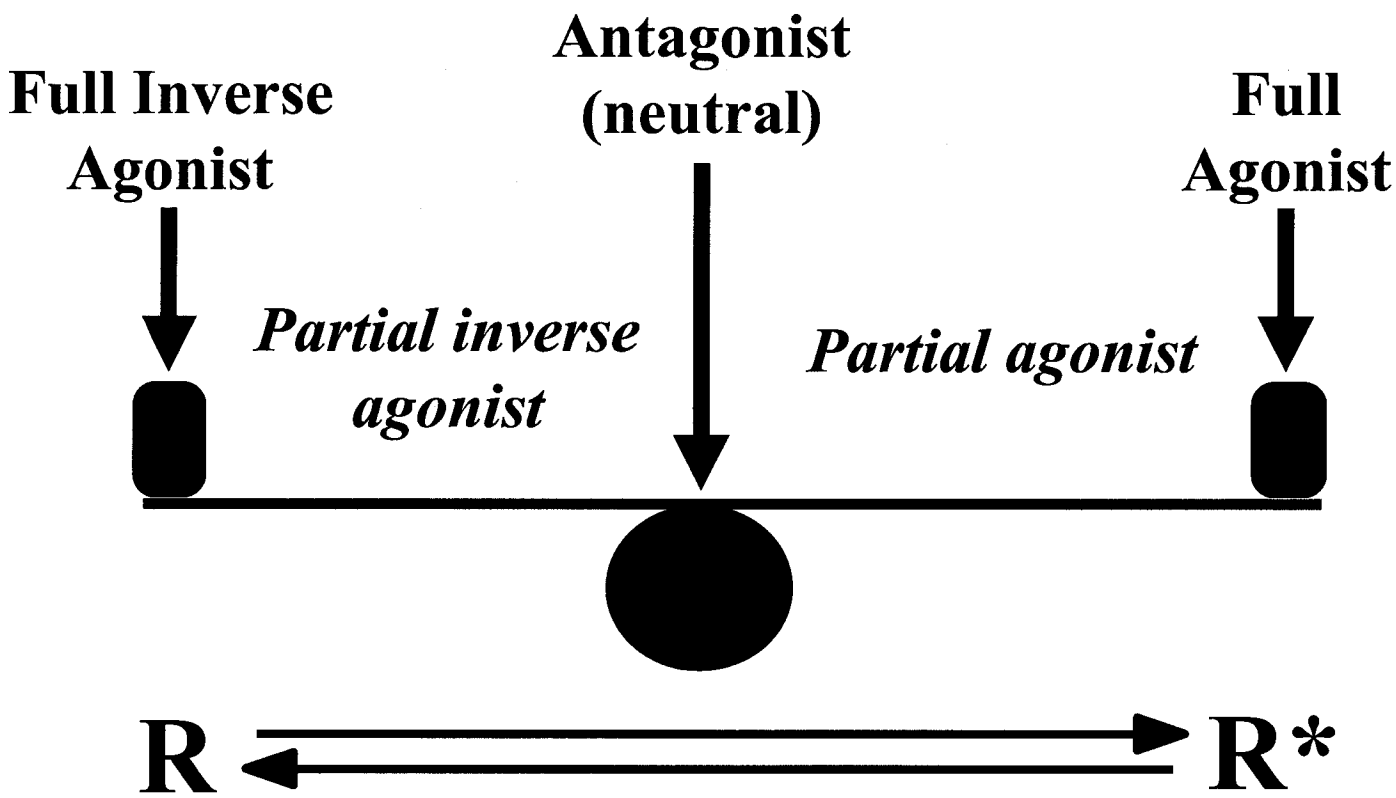

Full Inverse Agonist
Antagonist (neutral)
Full Agonist
Partial inverse agonist
Partial agonist
R
R*

Table 1: Classification of DA receptor subtypes based on primary structure, gene structure, pharmacological profiles and signal transduction studies

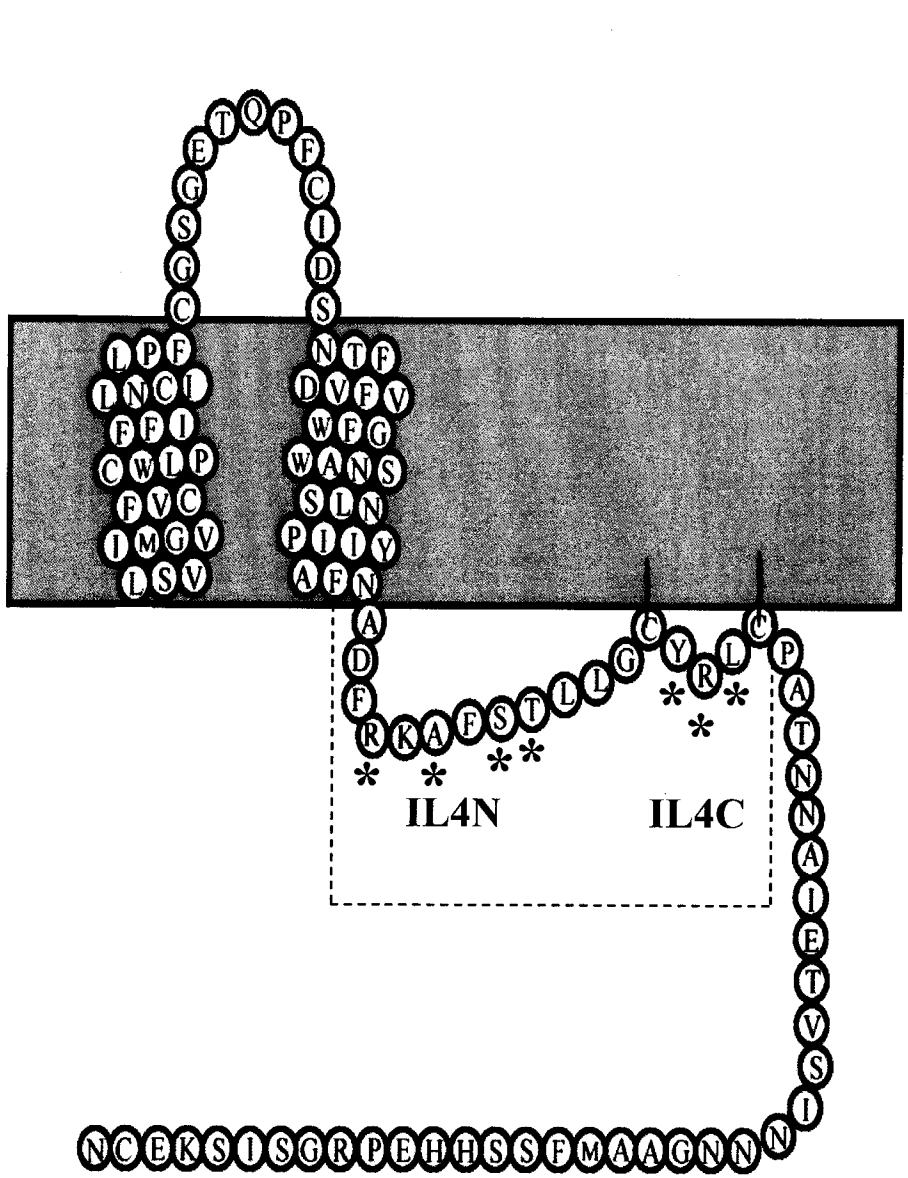
All mammalian DA receptors are categorized into two groups: D1-like receptors and D2-like receptors. This classification is based on differences in gene and primary structure, pharmacological profiles and signal transduction of DA receptors.

Table 1

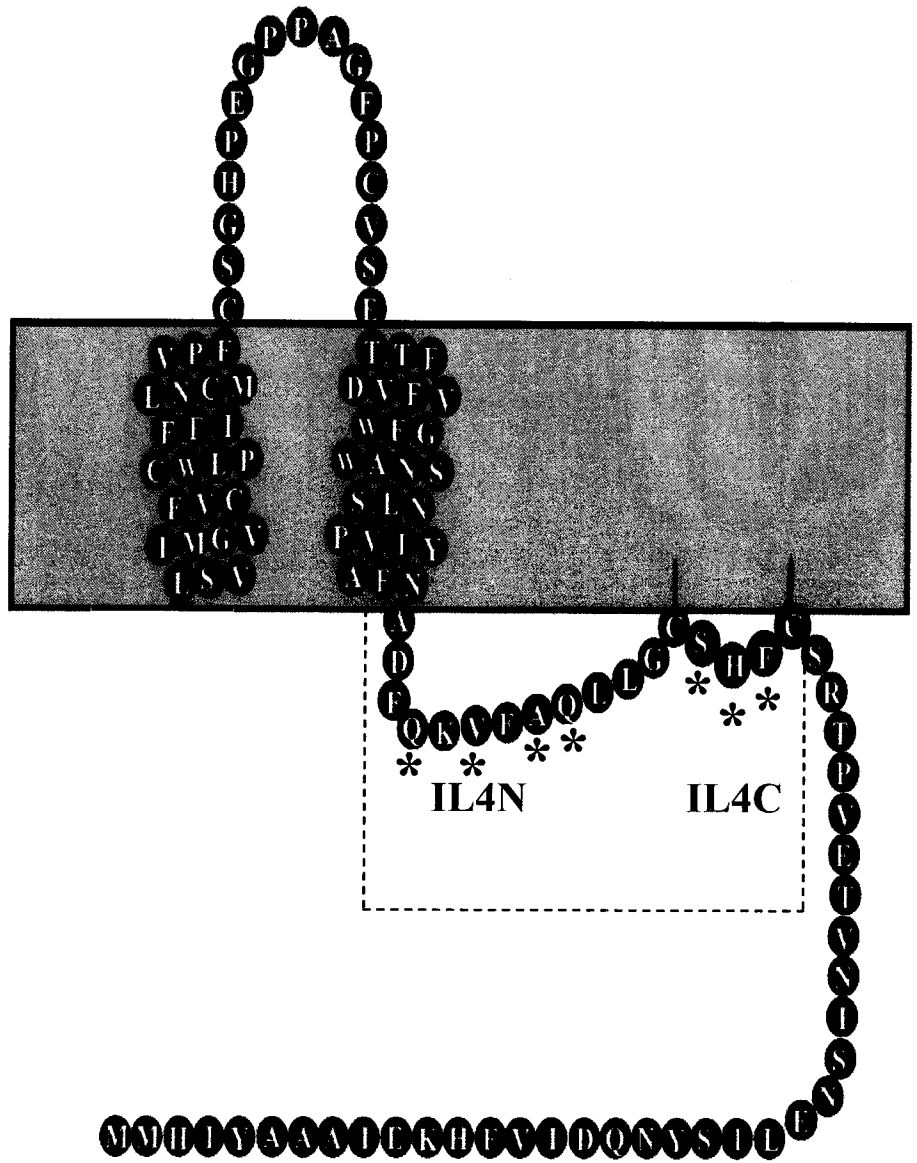
	D1-like receptors		D2-like receptors		
	D1A/D1	D1B/D5	$D2_{short/long}$	D3	D4
Antagonists with high affinity	SCH23390	SCH23390	Spiperone	Spiperone	Spiperone
Homology in TMs					
with D1 receptor	100	82	44	44	42
with D2(short)	44	49	100	75	53
Signal transduction	Activate AC	Activate AC	Inhibit AC	Inhibit AC	Inhibit AC
Gene intron in human coding sequences	None	None	Six	Five	Three
Organization of primary sequence					
IL3	Short	Short	Long	Long	Long
CT	Long	Long	Short	Short	Short
Receptor localization highest mRNA level in brain region	Striatum Nucleus accumbens Olfactory tubercles	Hippocampus Lateral mammalian nuclei Parafascicular nucleus of the thalamus	Striatum Nucleus accumbens Olfactory tubercle	Nucleus accumbens Olfactory tubercle Islands of calleja	Thalamus, Hypothalamus Frontal cortex, Amygdala Hippocampus

Figure 4: Schematic representation of the amino acid sequence of the IL4 region of D1A and D1B receptors

Empty circle represent the amino acids of the D1A receptor; Black-filled circles represent the amino acids of the D1B receptor. The IL4 regions of D1-like receptors are surrounded by black broken lines. Circles marked with asterisk represent amino acids that are different in IL4 regions of the D1A and D1B receptors. The IL4N primary sequence spans form Ala^{335} to Cys^{347} in the D1A subtype and from Ala^{363} to Cys^{375} in the D1B subtype, whereas IL4C spans from Cys^{347} to Cys^{351} in the D1A receptor and from Cys^{375} to Cys^{379} in the D1B receptor.



D1A



D1B

CHAPTER TWO – MATERIALS AND METHODS

1. Drugs

N-[methyl-^{3}H]-SCH23390 ([^{3}H]-SCH; 66-81 Ci/mmol), [^{3}H]-adenine (27-29 Ci/mmol) and [^{14}C]-cAMP (252-275 mCi/mmol) were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). DA, cis-flupentixol (FLU), (+)-butaclamol (BUTA), R-(+)-SCH-23390 (SCH) and 1-methyl-3-isobutylxanthine (IBMX) were purchased from Sigma/Research Biochemicals International (Oakville, Ontario, Canada).

2. Construction of Chimeric Human D1A and D1B Receptors

The IL4N primary sequence spans form Ala^{335} to Cys^{347} in the D1A subtype and from Ala^{363} to Cys^{375} in the D1B subtype, whereas IL4C spans from Cys^{347} to Cys^{351} in the D1A receptor and from Cys^{375} to Cys^{379} in the D1B receptor (Fig. 5). The IL4N sequences of the D1A and D1B receptors were exchanged to create two chimeric IL4N receptors referred to as D1A-IL4NB and D1B-IL4NA, respectively (Fig. 5 and 6). Similarly, the IL4C sequences of the D1A and D1B receptors were swapped to generate two chimeric IL4C receptors referred to as D1A-IL4CB and D1B-IL4CA (Fig. 5 and 6). Construction of chimeric receptors was performed by gene splicing using a PCR-based overlap extension approach (Horton *et al.* 1990). More specifically, wild-type receptors and existing chimeras (D1A-CTB and D1B-CTA) in which the entire CT was swapped

between the human D1A and D1B subtypes (Jackson *et al.* 2000) were used as templates to construct two chimeric IL4N receptors. Likewise, we used wild-type receptors and readily available chimeras (D1A-IL4B and D1B-IL4A) in which the entire IL4 was swapped between the human D1A and D1B receptors (Tumova and Tiberi, unpublished data) as templates to construct two IL4C chimeric receptors (Fig. 6).

2.1. The D1A-IL4NB chimera

The first PCR round gave rise to two fragments: the first fragment encoding the N-terminus of the D1A receptor up to IL4N of the D1B subtype (using the D1A-CTB chimera as template), and the second fragment encoding the CT of the D1A receptor downstream of IL4NA (using the wild-type D1A receptor as template) (Fig. 7A). The primers used for amplifying the two PCR fragments are as follows: 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GCACCCCAGCAGCTGGGCAAA-3' (reverse primer) for the first fragment; 5'-CAGCTGCTGGGGTGCTACAGACTTTGCCCT-3' (forward primer) and 5'-GCTCTAGAGAATTCTCAGGTTGGGTGCTG-3' (reverse primer) for the second fragment.

2.2. The D1B-IL4NA chimera

The first PCR round generated also two fragments: the first fragment encoding the N-terminus of the D1B receptor up to IL4N of the D1A subtype (using the D1B-CTA chimera as template), and the second fragment encoding the CT of the D1B receptor downstream of IL4NB (using the wild-type D1B receptor as template) (Fig. 7B). The primers used for amplifying these two fragments are as follows: 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GCATCCTAAGAGGGTTGAAAA-3' (reverse primer) for

the first fragment; 5'-ACCCTCTTAGGATGCAGCCACTTCTGCTCC-3' (forward primer) and 5'-GCATTGGTTGGCCCAAGCTTGAATTCTTAATGGAATCCATT-3' (reverse primer) for the second fragment.

2.3. The D1A-IL4CB chimera

The first PCR round gave rise to two fragments: the first fragment encoding the N-terminus of the D1A receptor up to IL4NA (using the wild-type D1A receptor as template), and the second fragment encoding the IL4C of the D1B receptor followed by the D1A sequence located downstream of IL4C up to the end of CT (using the D1A-IL4B chimera as template) (Fig. 7C). The first fragment was amplified using 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GCAGCCTAAGAGGGTTGAAAA-3' (reverse primer). The second fragment was amplified using 5'-ACCCTCTTAGGCTGCAGCCACTTCTGCCCT-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3' (reverse primer).

2.4. The D1B-IL4CA chimera

The first PCR round generated two fragments: the first fragment encoding the N-terminus of the D1B receptor up to IL4NB (using the wild-type D1B receptor as template), and the second fragment encoding the IL4C of the D1A receptor followed by the D1B sequence, found downstream of IL4C up to the end of CT (using the D1B-IL4A chimera as template) (Fig. 7D). The first fragment was amplified using primers 5'-TACGGTGGGAGG-3' (forward primer) and 5'-GCACCCCAGCAGCTGGGCAAA-3' (reverse primer). The second fragment was amplified using 5'-CAGCTGCTGGGGTGCTATCGATTATGCTCC-3' (forward primer) and 5'-TGCAACTTAATTTTATTA-3' (reverse primer).

2.5. Silent mutations

To distinguish wild-type and chimeric receptors from each others, a silent mutation containing a restriction endonuclease site was introduced to individual construct (Fig. 5). The chimeric receptors and their corresponding restriction endonuclease sites were as follows. For the D1A-IL4NB chimera, a *MluI* site was engineered in nucleotide sequence corresponding for amino acids Tyr^{331}, Ala^{332} and Phe^{333} (5'-TA**T**GC**C**TTT-3')→(5'-TA**C**GC**G**TTT-3'). For the D1B-IL4NA receptor, a *HindIII* site was introduced in nucleotide sequence encoding amino acids Lys^{367}, Ala^{368} and Phe^{369} (5'-AA**G**GC**A**TTT-3')→(AA**A**GC**T**TTT-3'). For the D1A-IL4CB receptor, a *PstI* site was engineered in the nucleotide sequence corresponding to amino acids Gly^{346}, Cys^{347} and Ser^{348} (5'-GG**A**TGCAGC-3')→(5'-GG**C**TGCAGC-3'). For the D1B-IL4CA receptor, a *ClaI* site was introduced in the nucleotide sequence encoding amino acids Tyr^{376}, Arg^{377} and Leu^{378} (5'-TA**CA**GA**C**T**T**-3')→(5'-TA**TC**GA**T**T**A**-3') (Fig. 5).

2.6. PCR conditions

For the first PCR round, 27 cycling reactions were performed to generate DNA products used in the second PCR round (overlapping PCR). Typically, one cycle included three steps: *1)* denaturation of DNA template; *2)* annealing of single DNA strands with their corresponding primers; *3)* new DNA strand synthesis by Taq polymerase (Invitrogen, Burlington, Ontario, Canada) or extension step. The last PCR cycle was only composed of annealing and extension steps. The PCR conditions for each cycle were as follows. Cycle 1: denaturation at 94°C for 3 minutes, annealing at 42°C for 1 minute, and extension at 72°C for 3 minutes. Cycle 2—26: denaturation at 94°C for 45 seconds,

annealing at 42°C for 1 minute, and extension at 72°C for 1 minute. Cycle 27 (the last cycle): annealing at 42°C for 1 minute and extension at 72°C for 8 minutes. For all chimeric receptors, paired PCR fragments were generated in first PCR round and separated on a 1% agarose gel. Appropriate bands were excised and purified by QIAEX II gel extraction method (Qiagen, Valencia, CA, USA). Diluted aliquots of paired fragments were combined and subjected to the second PCR round (overlapping PCR) using appropriate 5' and 3' flanking primers and 22 cycling reactions. Cycle 1 includes three steps: *1)* denaturation of paired DNA fragments at 94°C for 3 minutes; *2)* annealing of overlapping sequences of paired fragments at 42°C for 1 minute; and *3)* extension of spliced DNA strands at 72°C for 10 minutes. At the end of cycle 1, samples were removed from thermocycler, 5' and 3' flanking primers added to reaction mixture and PCR products amplified for 21 cycles using the following conditions. Cycle 2-21: denaturation at 94°C for 45 seconds, annealing at 42°C for 1 minute, and extension at 72°C for 1 minute. Cycle 22 (the last cycle): annealing at 42°C for 1 minute and extension at 72°C for 1 minute.

2.7. Subcloning and sequencing

The resulting PCR products were then subcloned in pCMV5, a mammalian expression vector (Andersson *et al.* 1989). The pCMV5 vector includes the promoter-enhancer region of the major immediate early gene of the human cytomegalovirus, a synthetic polylinker region containing unique cutting sites for 11 restriction enzymes (*EcoR I, Bgl II, Kpn I, Mlu I, Cla I, Hind III, Pst I, Sal I, Xbal I, BamH I, and Sma I*), the transcription termination and polyadenylation region of the bovine growth hormone gene, and the SV40 virus DNA replication origin and early region enhancer from plasmid pcD-X

(Andersson *et al.* 1989). The nucleotide sequences of PCR products were confirmed by dideoxy sequencing using sequenase version 2.0 from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada).

3. Cell Culture and Transfection

Human embryonic kidney 293 (HEK293) 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% (v/v) heat-inactivated fetal bovine serum (FBS) and gentamicin (10 μg/ml) (Invitrogen, Burlington, Ontario, Canada). Cells were seeded into 100-mm dishes (2.5×10^6 cells/dish), followed by transient DNA transfection using a modified calcium phosphate precipitation procedure as described (Didsbury *et al.* 1991). Specifically, 100 μl of 2.5M $CaCl_2$ was added into a falcon tube containing 10 μg of DNA in 900 μl of sterile milli-Q-water. Subsequently, 1000 μl of 2×HEPES-buffered saline (HBS), pH 7.1 (0.28 M NaCl, 0.05 M HEPES, pH 7.0, 1.5 mM Na_3PO_4, pH7.1) was added dropwise to the DNA-calcium solution, mixed gently and used to transfect two 100-mm dishes at a time (5μg DNA/dish). For radioligand binding studies and whole cell cAMP assays assessing basal activity at maximal receptor expression, HEK293 cells were transfected with wild-type or chimeric receptors using 5μg DNA per dish. For whole cell cAMP assays assessing DA potency and DA-mediated maximal activation of AC, HEK293 cells were transfected with wild-type or chimeric receptors using the following amounts (in μg) of DNA per dish: 0.0125 (D1A), 0.0125 (D1B), 5 (D1A-IL4B), 0.025 (D1B-IL4A), 5 (D1A-IL4NB), 0.0125 (D1A-IL4CB), 0.0125 (D1B-IL4NA), 0.025 (D1B-IL4CA). When less than 5μg receptor DNA per dish 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 50 passages.

4. Crude Membrane Preparations for Radioligand Binding Studies

Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Transfected HEK293 cells were then washed with 10 ml of cold PBS, scraped from the dish in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5mM EDTA) and centrifuged twice at 40,000×g for 20 min at 4°C. The crude membrane pellet was resuspended in lysis buffer using a Brinkmann Polytron (17,000 rpm for 15s). The membranes were either used immediately (saturation studies) or aliquoted in 1.5ml centrifuge tubes, frozen in liquid nitrogen and stored at -80°C until required (competition studies).

5. Radioligand Binding Assays

Binding assays were performed with 100 μl of membrane preparations in a total volume of 500 μl using [^{3}H]-SCH as radioligand. Saturation studies were performed on fresh membranes using increasing concentrations (6) of [^{3}H]-SCH, ranging from 0.01 to 6 nM. For each concentration, duplicate determinations were done for both total binding and non-specific binding. Non-specific binding was determined by adding 50 μl of 100 μM FLU to assay tubes, whereas for total binding 50 μl of milli-Q-water was used. For competition studies, frozen membranes were thawed on ice and resuspended in binding buffer (55.6 mM Tris-HCl, pH 7.4, 133.3 mM NaCl, 5.6 mM KCl, 4.4 mM $MgCl_2$, 1.7

mM $CaCl_2$, 1.1 mM EDTA) using a Brinkmann Polytron. Then, membranes were incubated with a constant concentration of [^{3}H]-SCH (~0.6 nM) and increasing concentrations of competing ligand under study. The competing ligands tested were DA (agonist), FLU (inverse agonist), BUTA (inverse agonist) and SCH (partial agonist). DA solutions were made in 0.1 mM ascorbic acid to prevent its oxidation, while other ligand solutions were made in milli-Q-water. Binding assays were incubated for 90 min at room temperature and terminated by 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 of membrane preparations were measured using the Bio-Rad assay kit with bovine serum albumin as standard.

6. Whole Cell cAMP Assays

Regulation of AC activity by wild-type and chimeric receptors was assessed using a whole cell cAMP assay as described previously (Tiberi and Caron 1994). Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12- well dishes. The next day, the growth medium was replaced with labeling medium containing 5% (v/v) FBS, 2 μCi/ml of [^{3}H]-adenine and 10 μg/ml gentamicin and cells were incubated for 16-18 h at 37°C. At the end of the labeling period, [^{3}H]-adenine labeling medium was replaced with 20 mM HEPES-buffered MEM containing 1mM IBMX (phosphodiesterase inhibitor to prevent cAMP degradation) and HEK293 cells were incubated in the presence or absence of DA for 30 min at 37°C. At

the end of the incubation period, the medium was aspirated and each well was filled with 1 ml of ice-cold lysis solution (2.5% (v/v) perchloric acid, 0.1 mM cAMP and [^{14}C]-cAMP (0.25-0.5 nCi, ~5,000-10,000 cpm) and cells lysed for 30 min at 4°C. Cell lysates were then transferred to tubes containing 0.1 ml of 4.2 M KOH (neutralizing solution), vortex briefly, and precipitates were sedimented by a low speed centrifugation (1,500 rpm for 10 min) at 4°C. The amount of intracellular [^{3}H]-cAMP was determined from supernatants purified by sequential chromatography using Dowex and alumina columns as previously described (Johnson and Salomon *et al.* 1991). The amount of [^{3}H]-cAMP (CA) formed over the total amount of intracellular [^{3}H]-adenine (TU) times 1000 was calculated to determine the relative AC activity (CA/TU×1000). Two types of experiments were performed. One set of experiments was done in 6-well dishes to assess basal activity (agonist-independent activity or constitutive activity) at maximal expression of wild-type and chimeric receptors in HEK293 cells. Another set of experiments was performed in 12-well dishes to assess DA potency and DA-mediated maximal activation of AC at comparable lower expression of wild-type and chimeric receptors using dose-response curves. Receptor expression was determined using a saturating concentration (~6 nM) of [^{3}H]-SCH.

7. Data Analysis and Statistics

Non-linear curve-fitting of binding isotherms was performed using GraphPad Prism version 4.0 for windows (GraphPad software, San Diego, CA, USA, www.graphpad.com) to determine, equilibrium dissociation constant (Kd) and maximal binding capacity (Bmax) values of [^{3}H]-SCH (saturation studies), and inhibitory constant

(Ki) values of unlabeled ligand (competition studies). Dose-response curves to DA were analyzed by GraphPad Prism version 4.0 using a four-parameter logistic equation to determine best fitted values of bottom or basal activity, top or maximal stimulation, effective concentration that elicits 50% of maximal response or EC_{50} (index of potency), slope factor. A global fitting of dose-response curves was done to establish whether differences observed between best-fit values for EC_{50} were statistically different i.e. whether constraining all dose-response curves to share the same EC_{50} value worsens the goodness of fit. Kd and Ki values are reported as geometric mean ± S.E. as described (De Léan *et al.* 1982). All other data are expressed as arithmetic mean ± S.E. unless stated otherwise. Homogeneity of variance was determined using Fmax or Bartlett tests prior to statistical analyses (Rohlf and Sokal 1981). When variances were not equal, data were transformed into logarithms and homogeneity of variances reassessed. One sample t-test and analysis of variance (one-way ANOVA or two-way ANOVA) with Bonferroni multiple comparison test were performed using GraphPad Prism version 4.0. The level of significance was established at $p<0.05$.

Figure 5: Alignment of IL4 primary sequences and flanking TM7 and CT sequences

The amino acids from human D1A receptor are in red; the amino acids from human D1B receptor are in blue. The IL4N and IL4C are delimited with thick lines above the amino acid sequences. The left part of the panel illustrates the IL4 amino acid sequences of wild-type human D1A and D1B receptors. The right part of the panel shows the IL4 amino acid sequences of the four chimeric receptors. For each chimeric receptor, a silent mutation was introduced in the original nucleotide sequences (shown in black) by engineering a new endonuclease restriction site (shown in green). The modified nucleotides are underlined.

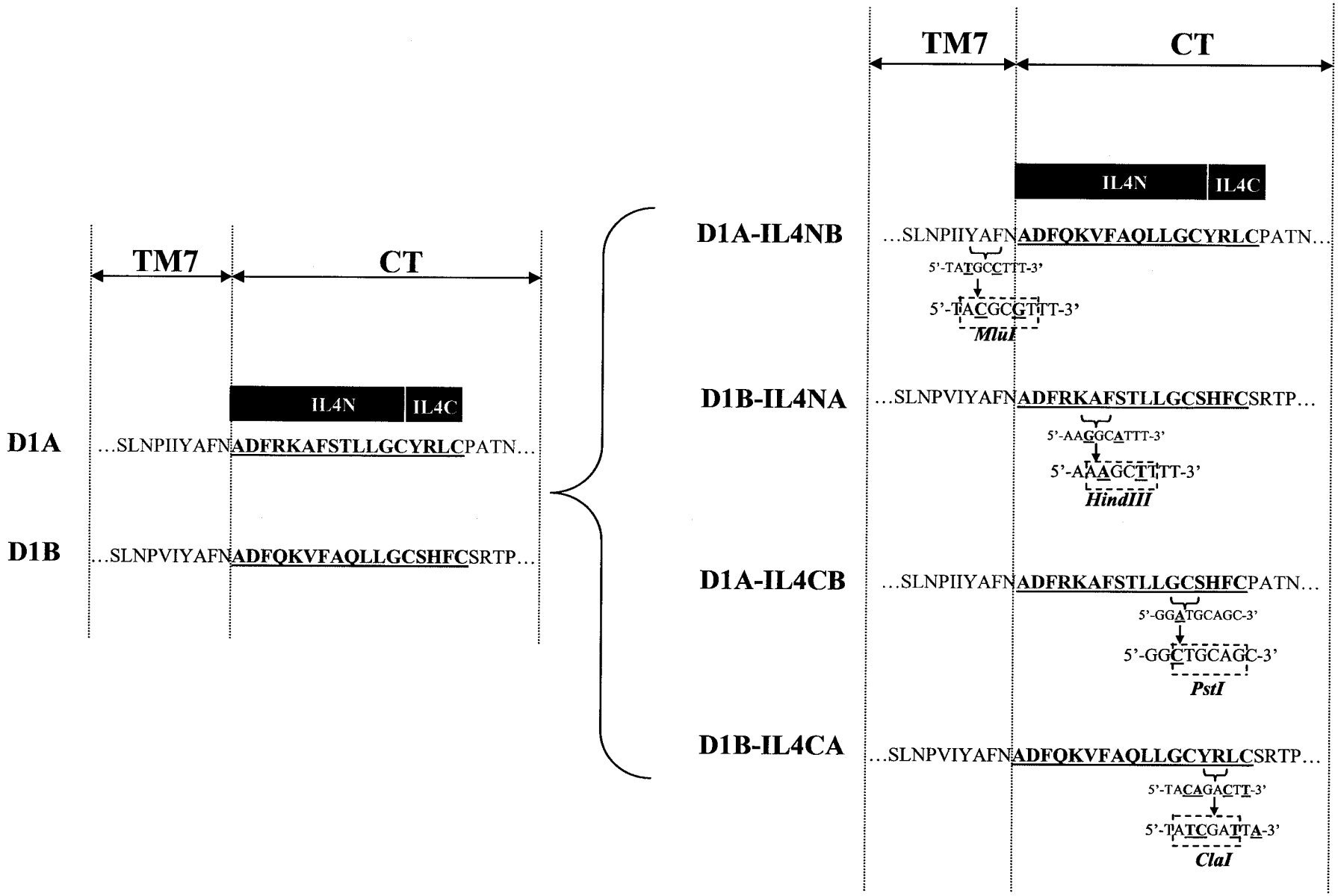
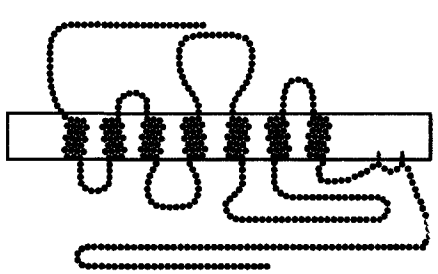
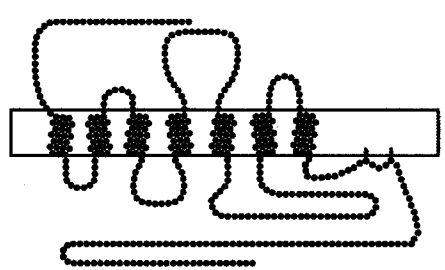

TM7
CT
IL4N
IL4C
D1A
...SLNPIIYAFNADFRKAFSTLLGCYRLCPATN...
D1B
...SLNPVIYAFNADFQKVFAQLLGCSHFCSRTP...
D1A-IL4NB
...SLNPIIYAFNADFQKVFAQLLGCYRLCPATN...
5'-TATGCCTTT-3'
5'-TACGCGTTT-3'
MluI
D1B-IL4NA
...SLNPVIYAFNADFRKAFSTLLGCSHFCSRTP...
5'-AAGGCATTT-3'
5'-AAAGCTTTT-3'
HindIII
D1A-IL4CB
...SLNPIIYAFNADFRKAFSTLLGCSHFCPATN...
5'-GGATGCAGC-3'
5'-GGCTGCAGC-3'
PstI
D1B-IL4CA
...SLNPVIYAFNADFQKVFAQLLGCYRLCSRTP...
5'-TACAGACTT-3'
5'-TATCGATTA-3'
ClaI

Figure 6: Schematic representation of wild-type and chimeric D1-like receptors

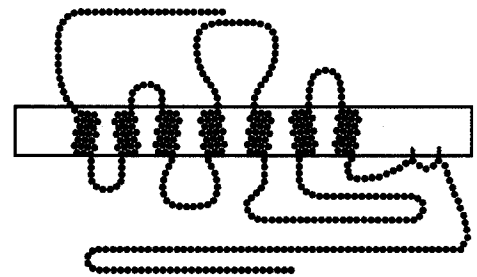
Red circles represent the amino acids of the D1A receptor; Blue circles represent the amino acids of the D1B receptor.



D1A



D1A-IL4B



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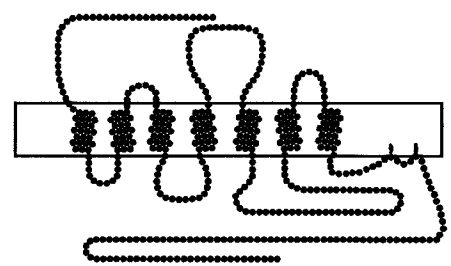

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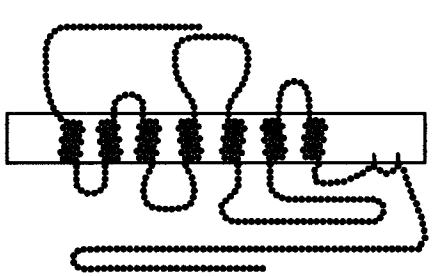
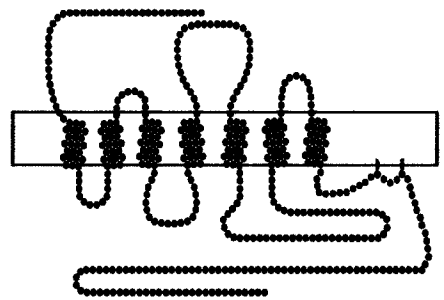
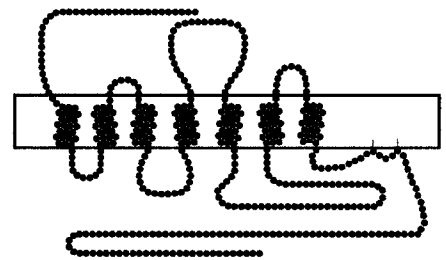


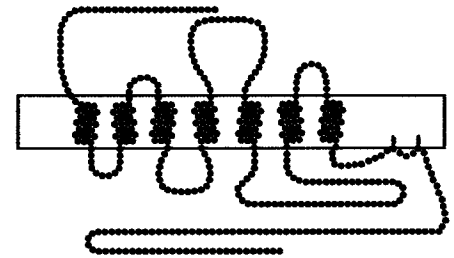
D1B



D1B-IL4A



D1B-IL4NA

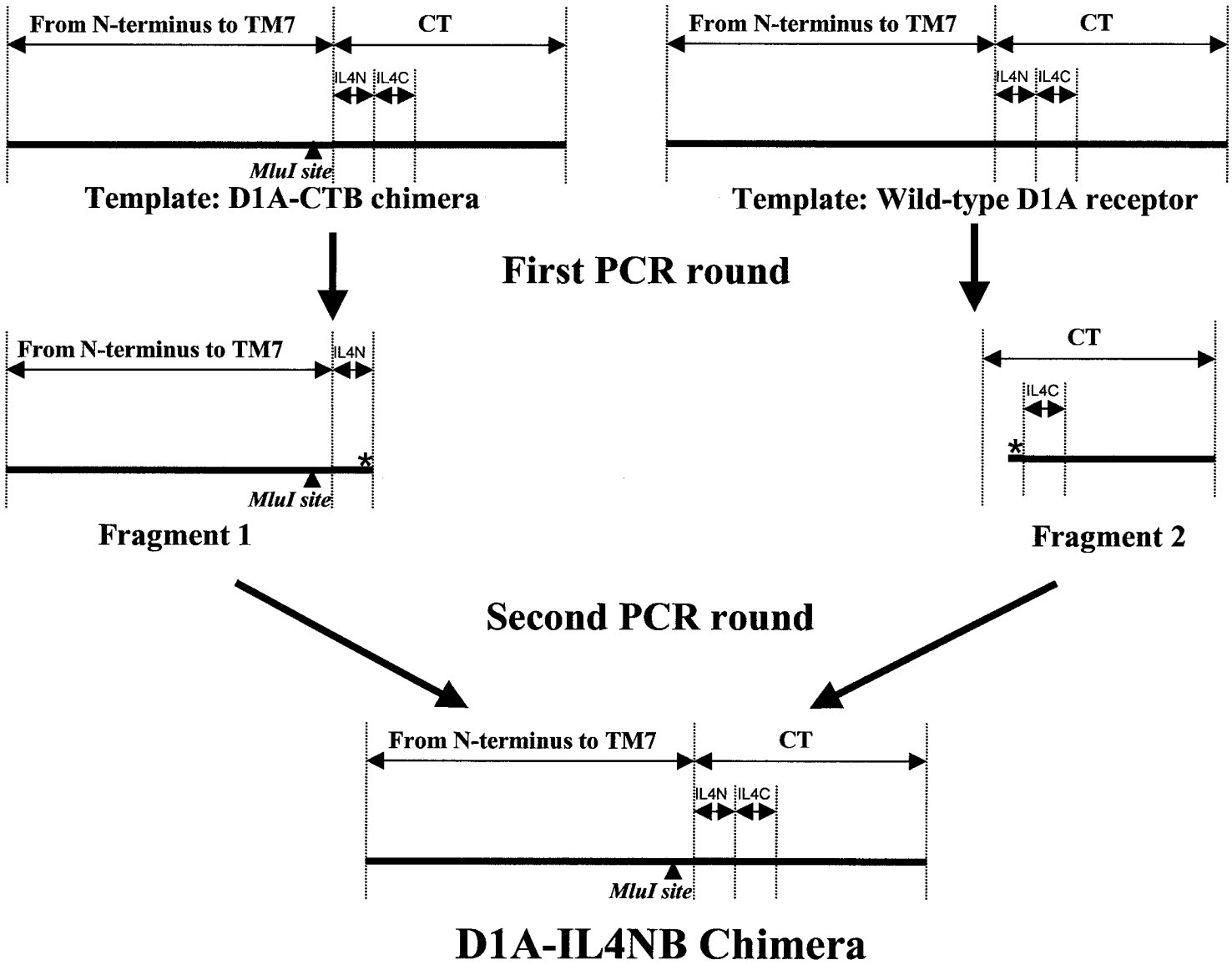


D1B-IL4CA

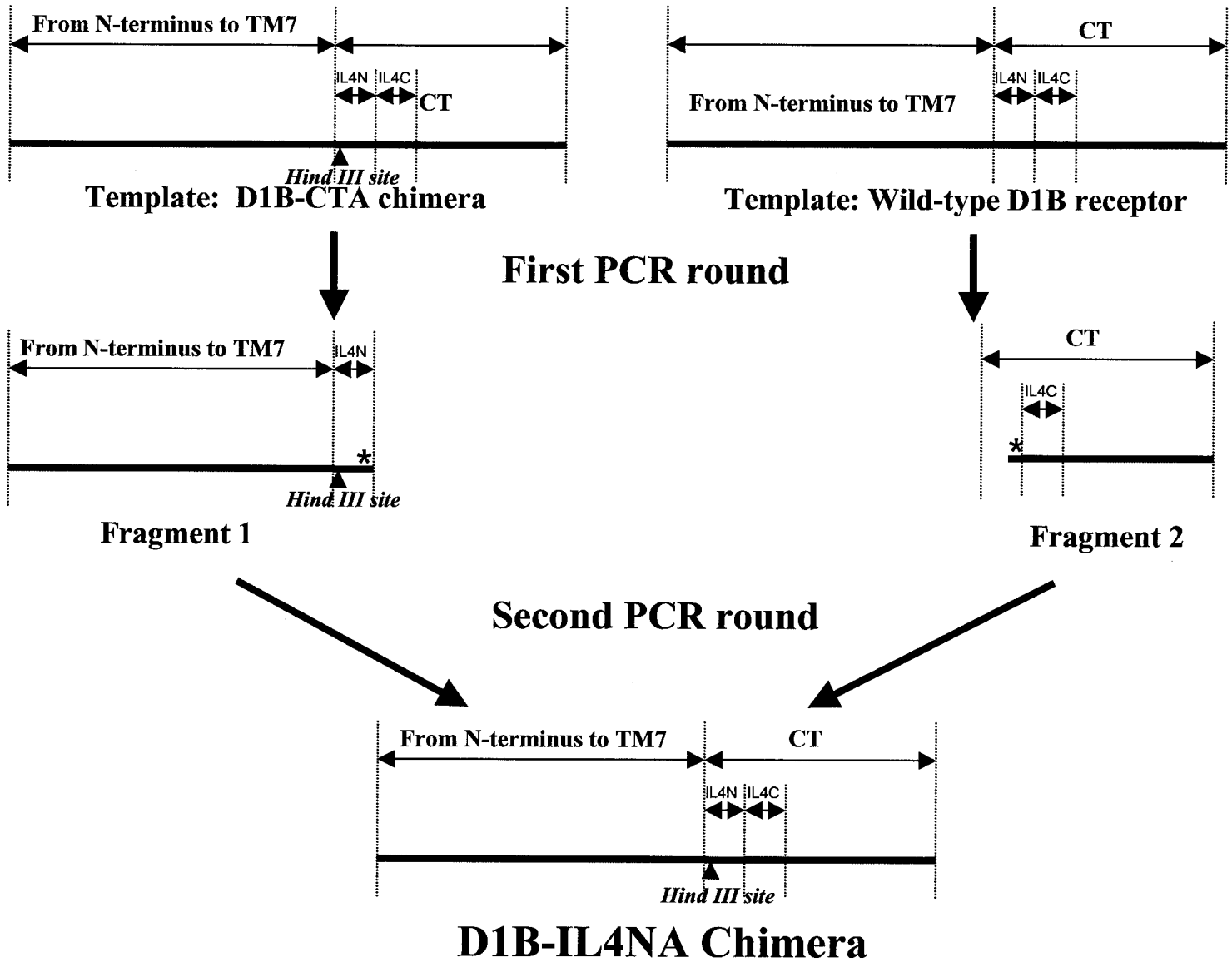
Figure 7: Schematic representation of the construction of chimeric receptors

The red lines represent the nucleotide sequences from the D1A receptor and the blue lines depict nucleotide sequences from the D1B receptor. The first PCR round generated two fragments by using either the wild-type receptors or the readily available chimeric receptors (D1A-CTB, D1B-CTA, D1A-IL4B and D1B-IL4A) as templates. The asterisk represents overlapping sequences of the two PCR fragments. The green triangle represents engineered endoclease restriction sites. In the second PCR round, a PCR-based overlap extension approach was used to splice the two fragments by annealing their overlapping sequences. This approach gave rise to the nucleotide sequence encoding the final chimeric receptors: ***A***, Construction of D1A-IL4NB chimera. ***B***, Construction of D1B-IL4NA chimera. ***C***, Construction of D1A-IL4CB chimera. ***D***, Construction of D1B-IL4CA chimera.

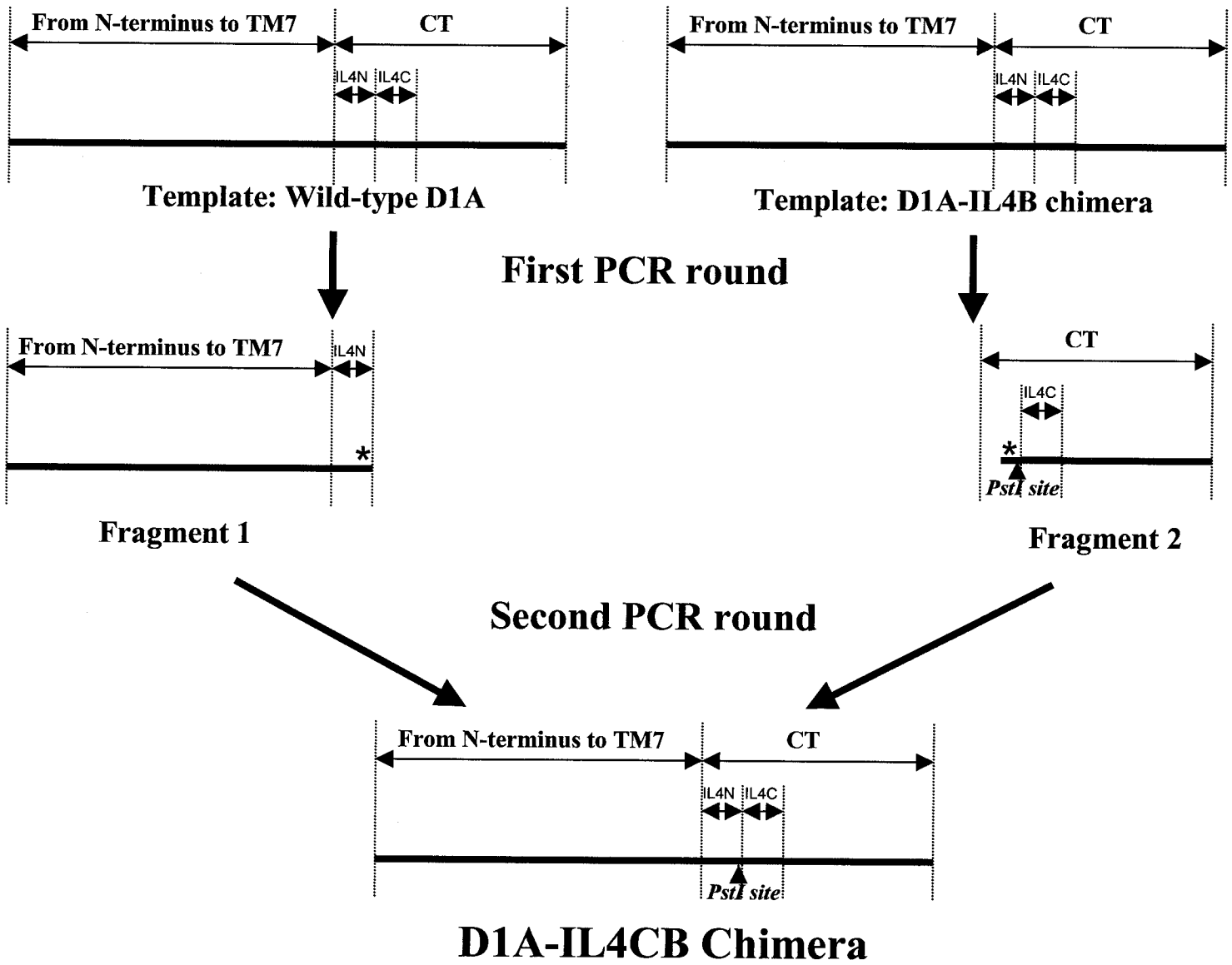
A


From N-terminus to TM7
CT
IL4N
IL4C
MluI site
Template: D1A-CTB chimera
From N-terminus to TM7
CT
IL4N
IL4C
Template: Wild-type D1A receptor
First PCR round
From N-terminus to TM7
IL4N
*
MluI site
Fragment 1
CT
IL4C
*
Fragment 2
Second PCR round
From N-terminus to TM7
CT
IL4N
IL4C
MluI site
D1A-IL4NB Chimera

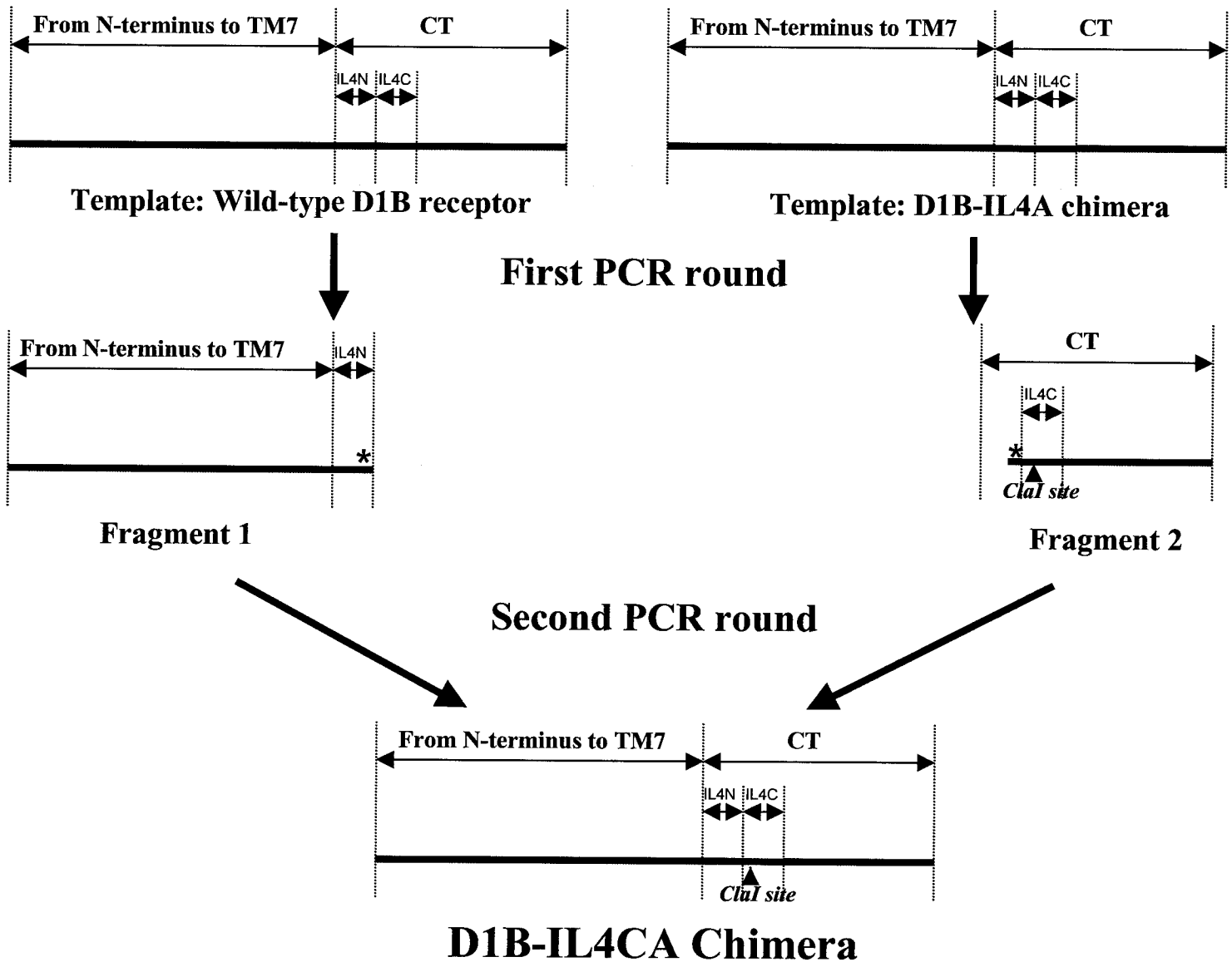
B


From N-terminus to TM7
IL4N
IL4C
CT
Hind III site
Template: D1B-CTA chimera
From N-terminus to TM7
CT
IL4N
IL4C
Template: Wild-type D1B receptor
First PCR round
From N-terminus to TM7
IL4N
*
Hind III site
Fragment 1
CT
IL4C
*
Fragment 2
Second PCR round
From N-terminus to TM7
CT
IL4N
IL4C
Hind III site
D1B-IL4NA Chimera

C


From N-terminus to TM7
CT
IL4N
IL4C
Template: Wild-type D1A
From N-terminus to TM7
CT
IL4N
IL4C
Template: D1A-IL4B chimera
First PCR round
From N-terminus to TM7
IL4N
*
Fragment 1
CT
IL4C
*
PstI site
Fragment 2
Second PCR round
From N-terminus to TM7
CT
IL4N
IL4C
PstI site
D1A-IL4CB Chimera

D


From N-terminus to TM7
CT
IL4N
IL4C
Template: Wild-type D1B receptor
From N-terminus to TM7
CT
IL4N
IL4C
Template: D1B-IL4A chimera
First PCR round
From N-terminus to TM7
IL4N
*
Fragment 1
CT
IL4C
*
ClaI site
Fragment 2
Second PCR round
From N-terminus to TM7
CT
IL4N
IL4C
ClaI site
D1B-IL4CA Chimera

CHAPTER THREE – RESULTS

1. Ligand Binding Properties of Wild-type and Chimeric D1-Like Receptors

1.1. The SCH binding affinities of wild-type and chimeric receptors

SCH is a prototype antagonist with high affinity for D1-like receptors (Billard *et al.* 1984). However, SCH displays partial agonism in HEK293 cells or Cos-7 cells expressing D1A or D1B receptors (Tiberi and Caron 1994; Cho *et al.* 1996; Sugamori *et al.* 1998). In the present study, SCH affinities of wild-type and chimeric D1-like receptors were indexed with equilibrium dissociation constant (Kd) values obtained from saturation studies using tritiated form of SCH as well as with inhibitory constant (Ki) values derived from competition studies using non-tritiated form of SCH. Kd and Ki values of SCH were not statistically different using a two-way ANOVA with Bonferroni multiple comparison test. Therefore, for the sake of simplicity, only Ki values of wild-type and chimeric D1-like receptors are reported in Table 2 as index of SCH affinity. The Ki values of wild-type receptors obtained in the present study confirm previous observations showing that D1B receptors displayed a lower affinity for SCH as compared with D1A receptors (Tiberi and Caron 1994; Iwasiow *et al.* 1996; Jackson *et al.* 2000). Importantly, the similar Ki values of chimeric receptors as compared with their wild-type counterparts indicate that these chimeras retain the ability to bind SCH with high affinity.

These results suggest that reciprocal swapping of structurally homologous IL4 regions does not interfere significantly with protein folding essential for receptor binding to SCH.

1.2. The contributions of IL4N and IL4C segments of D1-like receptors in regulating DA binding to D1A and D1B subtypes

Competition studies were also conducted to investigate whether IL4 region contains structural determinants regulating DA affinity at D1-like receptors. Table 2 shows the Ki values of DA binding to wild-type and chimeric D1-like receptors. In agreement with previous reports (Sunahara *et al.* 1991; Tiberi *et al.* 1991; Tiberi and Caron 1994), the D1B receptor exhibited higher affinity for DA (~ 10-fold) in comparison with the D1A receptor. Chimeric D1A receptors harboring the entire IL4 region of D1B (D1A-IL4B) displayed an increased DA affinity, which is indistinguishable from that of wild-type D1B receptors (Table 2). However, chimeric D1B receptors carrying the entire IL4 region of D1A (D1B-IL4A) exhibited a partial decrease of DA affinity in comparison with wild-type D1B receptors (Table 2). To further dissect the structural determinants within IL4 responsible for the modulation of DA binding, we investigated the potential roles of the N-terminal and C-terminal segments of IL4 using four additional chimeras (D1A-IL4NB, D1A-IL4CB, D1B-IL4NA, D1B-IL4CA). With respect to the new chimeric D1A receptors, our studies show that D1A-IL4NB chimera displayed an increased DA affinity as compared with D1A receptor. Interestingly, DA affinity of D1A-IL4NB chimera was similar to that exhibited by wild-type D1B and D1A-IL4B chimeric receptors (Table 2). Moreover, DA affinity exhibited by D1A-IL4CB chimera was slightly increased but not statistically different in comparison with wild-type D1A

receptor (Table 2). Chimeric D1B receptors bearing the IL4NA segment displayed a significantly decreased DA affinity when compared with wild-type D1B and D1B-IL4A chimeric receptor (Table 2). However, in an opposite manner, chimeric D1B receptors harboring IL4CA segment bound DA with a significantly increased affinity in comparison with the wild-type D1B receptor (Table 2).

1.3. The contributions of IL4N and IL4C receptor regions in regulating inverse agonist binding to D1A and D1B subtypes

FLU and BUTA are two antipsychotic drugs displaying inverse agonism at D1-like receptors. In agreement with previous studies (Tiberi and Caron 1994), binding results reported in Table 3 indicate that FLU and BUTA bind to D1A receptors with higher affinity than to D1B receptor. Previous mutagenesis studies have shown that swapping of entire CT or IL4 between D1A and D1B receptors did not switch inverse agonist affinity (Demchyshyn 2000; Jackson *et al.* 2000; Tumova and Tiberi, unpublished data). Interestingly, however, the present study shows that chimeric D1A receptors harboring the IL4N segment of D1B subtype displayed an increased affinity for binding of FLU and BUTA in comparison with wild-type D1A receptors (Table 3). In contrast, the chimeric D1A receptors carrying IL4C segment of D1B subtype exhibited a similar binding affinity for FLU and BUTA as compared with wild-type D1A receptors (Table 3). In addition, the insertion of either IL4N or IL4C segment of D1A subtype into D1B receptor did not alter significantly binding affinities for the two inverse agonists as compared with wild-type D1B receptor (Table 3).

1.4. The contributions of IL4N and IL4C regions in regulating maximal binding capacity (Bmax) values

The wild-type D1A and D1B receptors display similar receptor expression in HEK293 cells as indicated by the Bmax values of [^{3}H]-SCH (Tiberi and Caron 1994; Iwasiow *et al.* 1996; Jackson *et al.* 2000). However, a significant reduction was observed in Bmax values of D1A-IL4B and D1A-IL4NB chimeric receptors as shown in figure 8A. This finding agrees with a previous report from our group showing that HEK293 cells expressing chimeric D1A receptors bearing the entire CT of D1B subtype displayed a significant decrease in the Bmax for [^{3}H]-SCH (Jackson *et al.* 2000). Conversely, chimeric D1B receptors harboring either IL4N or IL4C segment of D1A subtype displayed similar Bmax values of [^{3}H]-SCH as compared with wild-type D1B receptors (Fig. 8B).

2. G Protein Coupling Properties of Wild-type and Chimeric D1-Like Receptors

2.1. Agonist-independent activity of the wild-type and chimeric D1-like receptors

The agonist-independent activity (constitutive activity) of the wild-type and chimeric receptors was assessed using a whole cell cAMP assay in HEK293 cells transfected with 5μg DNA, which lead to maximal expression of the various receptor constructs. A representative example using raw data is shown in Figure 9A. As demonstrated previously (Tiberi and Caron 1994), the wild-type D1B receptor exhibited a significantly higher agonist-independent activity as compared with the D1A receptor (Fig. 9). Upon reciprocal exchange of IL4 of D1-like receptors, the agonist-independent activity of D1B-IL4A chimera decreased to a level similar to that of the D1A receptor (Fig. 9B). The

agonist-independent activity of D1A-IL4B receptor was significantly decreased in comparison with wild-type D1A and D1B receptors (Fig. 9B).

In attempt to pinpoint further the structural determinants within IL4 responsible for the agonist-independent activity of D1-like receptors, we investigated the potential roles of IL4N and IL4C segments in the regulation of agonist-independent activity. In agreement with results obtained with the exchange of the entire IL4, a similar trend was observed with the swap of IL4N region. More specifically, as shown in figure 9B, the D1B-IL4NA chimera displayed a decreased agonist-independent activity in comparison with wild-type D1B subtype (Fig. 9B). Interestingly, the agonist-independent activity of D1B-IL4NA chimera was reminiscent of that exhibited by wild-type D1A receptor (Fig. 9B). Moreover, the D1A-IL4NB chimera displayed a decreased agonist-independent activity, which was statistically different from wild-type D1A and D1B receptors (Fig. 9B). The agonist-independent activity of the IL4C chimeras remained unchanged when compared with their respective parent receptors (Fig. 9B).

2.2. DA-mediated maximal activation of AC in HEK293 cells expressing wild-type and chimeric D1-like receptors

DA-mediated maximal activation of AC was obtained from dose-response curves performed on HEK293 cells transfected with each of wild-type or chimeric D1-like receptors expressed at comparable lower Bmax values (~0.5-1 pmol/mg) and a representative example is shown in figure 10A. In agreement with previous studies (Tiberi and Caron 1994; Iwasiow *et al.* 1996; Jackson *et al.* 2000), the D1A receptor displayed higher DA-mediated maximal activation of AC than the D1B receptor (Fig.

10B). Upon the exchange of entire IL4 region, chimeric D1A-IL4B receptors showed a dramatically increased DA-mediated maximal activation of AC as compared with wild-type D1A receptors (Fig. 10B). In a similar fashion to the D1A-IL4B chimera, chimeric D1A receptors harboring either IL4NB or IL4CB segment also displayed significantly increased DA-mediated maximal activation of AC in comparison with the wild-type D1A receptor (Fig. 10B). The magnitude of DA-mediated maximal activation elicited by wild-type and chimeric D1A receptors are as follows: D1A-IL4NB>D1A-IL4B>D1A-IL4CB>D1A. Surprisingly, however, DA-mediated maximal activation of AC in cells expressing individual D1B chimeras was similar to that measured in cells expressing the wild-type D1B subtype (Fig. 10B).

2.3. DA potency in HEK293 cells expressing wild-type and chimeric D1-like receptors

The DA potency, as indicated by EC_{50} values, was assessed using dose-response curves. As shown in figure 11A, HEK293 cells expressing D1B receptors displayed a significantly higher (~14-fold) DA potency when compared with cells expressing D1A receptors, as previously reported (Tiberi and Caron 1994, Iwasiow *et al.* 1996; Jackson *et al.* 2000). Previous mutagenesis studies in our laboratory revealed that the exchange of CT led to a reduced DA potency in cells expressing the chimeric CT receptors. The present results showed that the chimeric D1A-IL4B and D1B-IL4A receptors exhibited a ~ 1.2 and ~ 35-fold decrease in DA potency, respectively, as compared with their cognate wild-type receptors (Fig. 11B). Furthermore, the potential contribution of IL4N and IL4C segments in regulating DA potency was investigated. We show that chimeric D1A

receptors harboring IL4NB displayed a ~1.8-fold increase in DA potency when compared with wild-type D1A receptors (Fig. 11C). In striking contrast, chimeric D1B receptors bearing IL4NA segment exhibited a significantly decreased DA potency when compared with wild-type D1B receptor (Fig.11C). Interestingly, the DA potency in cells expressing the D1B-IL4NA chimera was reminiscent of that in HEK293 cells expressing wild-type D1A receptor (Fig. 11C). The DA potency measured in HEK293 cells expressing IL4C chimeras was essentially unchanged in comparison with cells expressing their respective parent receptors (Fig. 11D).

Table 2: Inhibitory constants (Ki) for binding of partial (SCH) and full (DA) dopaminergic agonists to wild-type and chimeric D1-like receptors

Values for Ki in nanomolar (nM) were determined from competition studies of three to eight experiments done in duplicate determinations and expressed as geometric mean ± SE. Ki values were also calculated relative to Ki values of D1A receptor (shown in brackets). SCH, SCH23390; DA, dopamine.

*, $p<0.05$ when compared with D1A; #, $p<0.05$ when compared with D1B; §, $p<0.05$ when compared with D1B-IL4A.

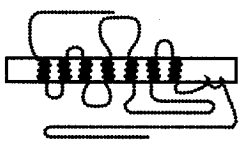
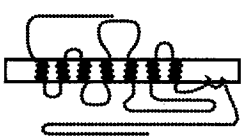
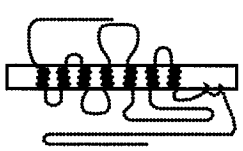
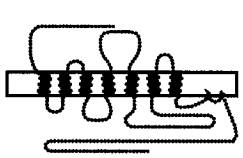
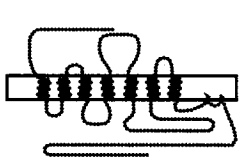
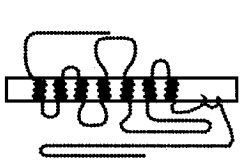
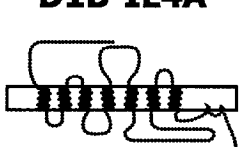
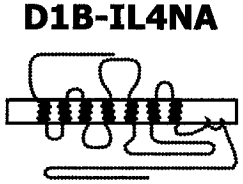
	Ki(nM)	
	SCH	**DA**
 **D1A**	0.68 ± 0.01 [1]	4817 ± 173 [1]
 **D1A-IL4B**	0.50 ± 0.01 [0.79 ± 0.04] (#)	458 ± 18 [0.11 ± 0.01] (*)
 **D1A-IL4NB**	0.54 ± 0.01 [0.79 ± 0.01] (#)	748 ± 11 [0.16 ± 0.01] (*)
 **D1A-IL4CB**	0.62 ± 0.02 [0.90± 0.02] (#)	3769 ± 109 [0.78 ± 0.04] (#)
 **D1B**	1.07 ± 0.02 [1.57 ± 0.05] (*)	576 ± 25 [0.12 ± 0.01] (*)
 **D1B-IL4A**	0.89 ± 0.03 [1.41 ± 0.08] (*)	1197 ± 25 [0.30 ± 0.02] (*#)
 **D1B-IL4NA**	0.95 ± 0.04 [1.42 ± 0.05] (*)	3172 ± 115 [0.66 ± 0.02] (*#§)
 **D1B-IL4CA**	0.93 ± 0.02 [1.36 ± 0.03] (*)	288 ± 16 [0.06 ± 0.01] (*#)

Table 3: Inhibitory constants (Ki) for binding of dopaminergic inverse agonists to wild-type and chimeric receptors

Values for Ki in nanomolar (nM) were obtained from competition studies of three to four experiments done in duplicate determinations and expressed as geometric mean ± SE. Ki values were also calculated relative to Ki values of D1A receptor (shown in brackets). FLU, flupentixol; BUTA, (+)-butaclamol.

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

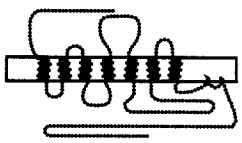
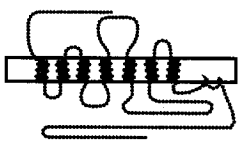
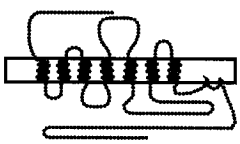
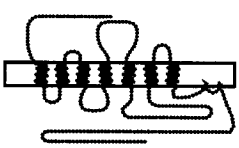
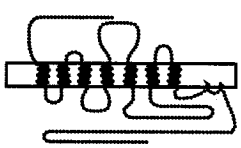
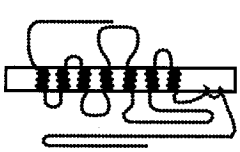
	Ki(nM)	
	FLU	**BUTA**
 **D1A**	14.9 ± 1.78 [1]	9.82 ± 0.93 [1]
 **D1A-IL4NB**	10.2 ± 1.00 [0.73 ± 0.01] (*#)	6.06 ± 0.61 [0.62 ± 0.02] (*#)
 **D1A-IL4CB**	14.9 ± 1.58 [1.01 ± 0.04] (#)	8.27 ± 0.55 [0.84 ± 0.02] (#)
 **D1B**	26.9 ± 2.68 [1.88 ± 0.06] (*)	53.1 ± 2.88 [5.41 ± 0.32] (*)
 **D1B-IL4NA**	19.8 ± 1.17 [1.65 ± 0.08] (*)	40.2 ± 1.36 [4.09 ± 0.25] (*)
 **D1B-IL4CA**	23.7 ± 2.20 [1.67 ± 0.04] (*)	43.6 ± 2.38 [4.44 ± 0.18] (*)

Figure 8: Maximal binding capacity (Bmax) values of N-[methyl-^{3}H]-SCH23390 for wild-type and chimeric D1-like receptors

Values for Bmax in picomole per mg of membrane proteins (pmole/mg prot.) were obtained from saturation studies. Data are expressed as arithmetic mean ± S.E. of three to seven experiments done in duplicate determinations. ***A***, Bmax values of wild-type D1A and chimeric D1A receptors; ***B***, Bmax values of wild-type D1B and chimeric D1B receptors.

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

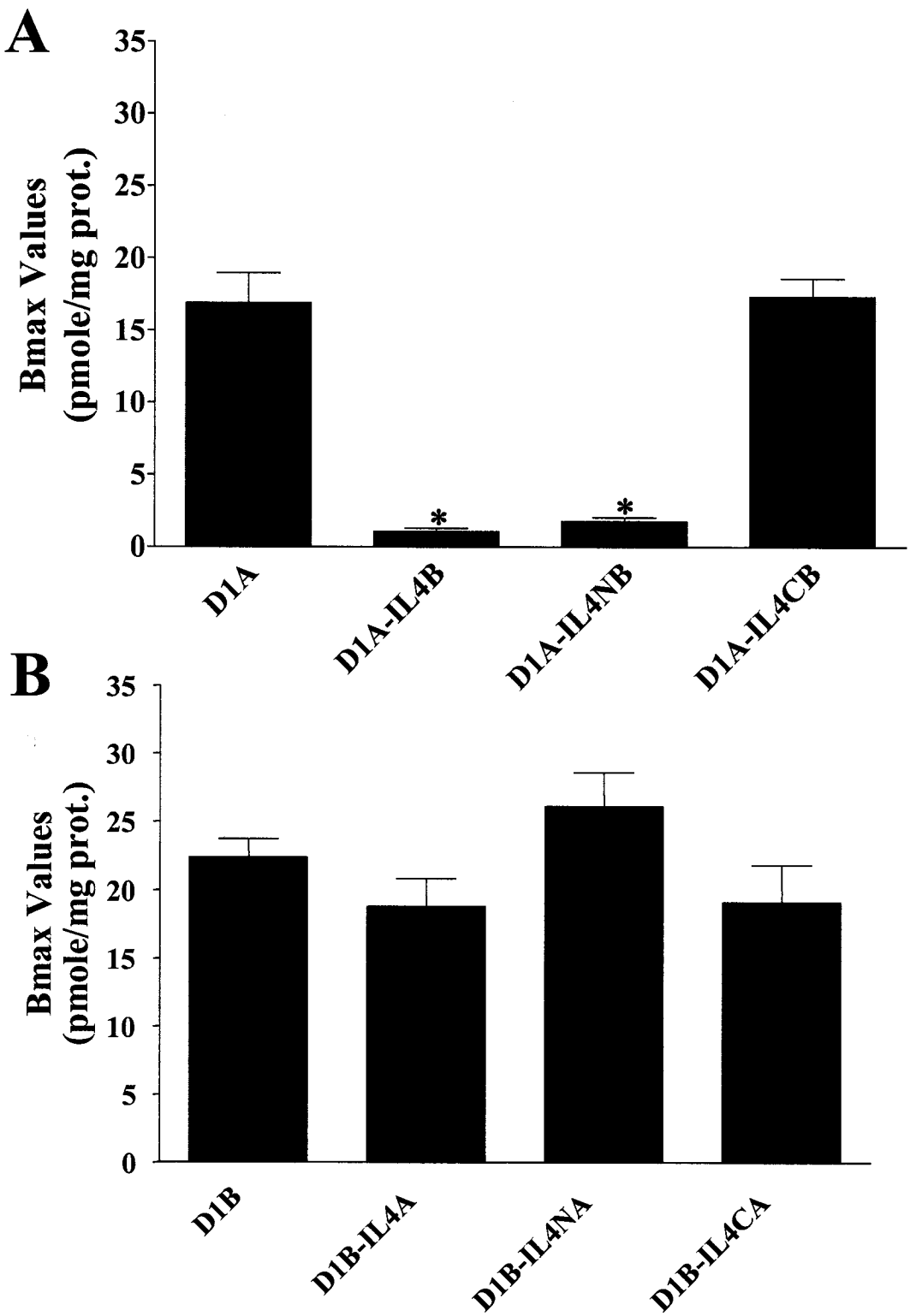

A
Bmax Values (pmole/mg prot.)
35
30
25
20
15
10
5
0
*
*
D1A
D1A-IL4B
D1A-IL4NB
D1A-IL4CB
B
Bmax Values (pmole/mg prot.)
35
30
25
20
15
10
5
0
D1B
D1B-IL4A
D1B-IL4NA
D1B-IL4CA

Figure 9: Agonist-independent activity of wild-type and chimeric D1-like receptors expressed in HEK293 cells

Basal levels of AC activity were determined in single wells of a 6-well dish using whole cell cAMP assays in the absence of DA. Data are expressed as arithmetic mean ± S. E. of five experiments done in triplicate determinations and calculated relative to agonist-independent activity of D1A receptor. ***A***, A representative example of agonist-independent activity of wild-type and chimeric D1-like receptors using raw data expressed as [^{3}H]-cAMP (CA) over the total amount of [^{3}H]-adenine uptake (TU) X 1000. ***B***, Agonist-independent activity of wild-type and chimeric D1-like receptors expressed relative to agonist-independent activity of wild-type D1A receptor. The Bmax values in picomole per mg of membrane proteins (expressed as the geometric mean ± S. E.) are 17.3 ± 0.62 (D1A), 16.7 ± 0.31 (D1B), 1.31 ± 0.02 (D1A-IL4B), 14.5 ± 0.69 (D1B-IL4A), 1.86 ± 0.05 (D1A-IL4NB), 16.0 ± 0.68 (D1A-IL4CB), 17.1 ± 0.49 (D1B-IL4NA), 12.8 ± 0.27 (D1B-IL4CA).

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

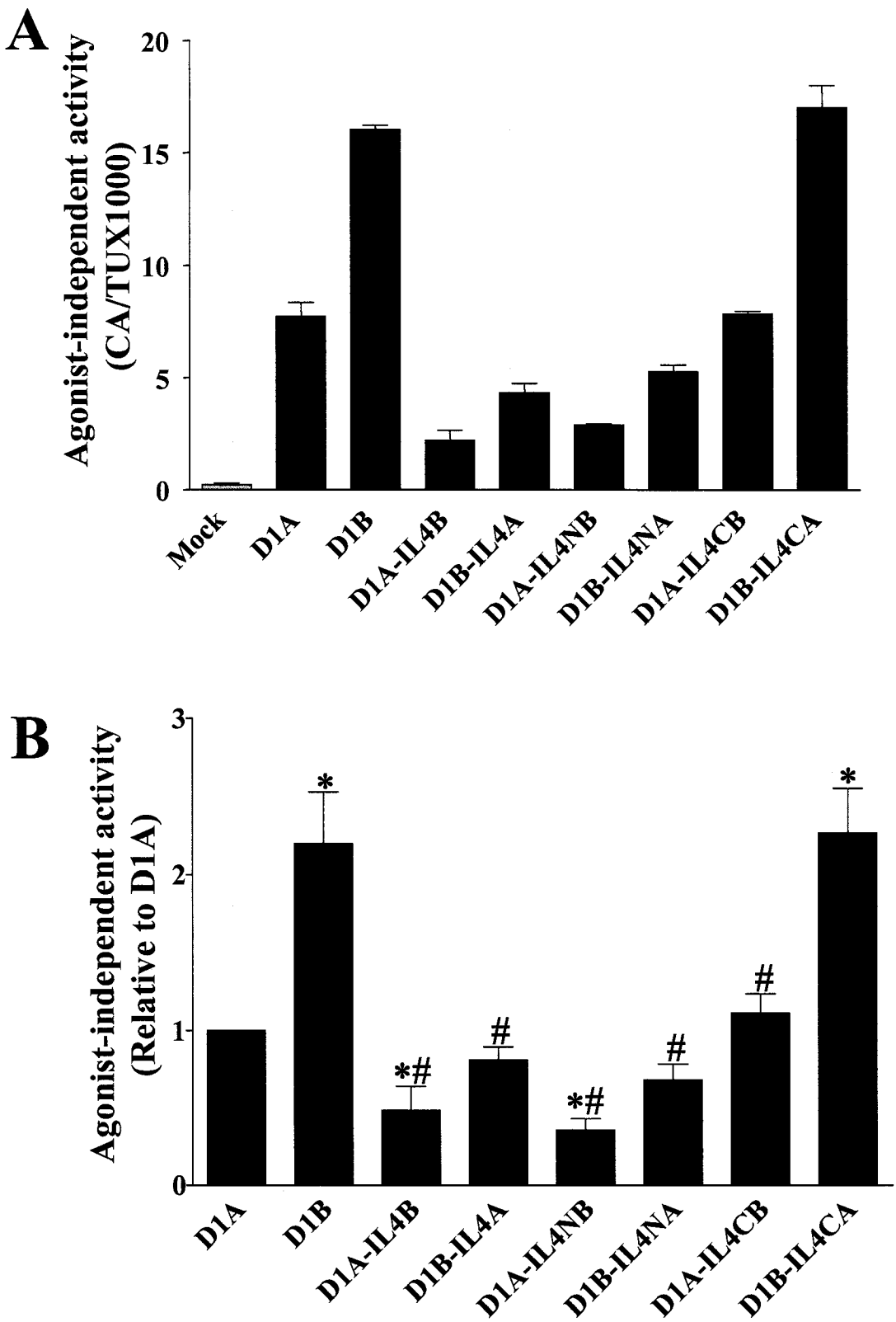
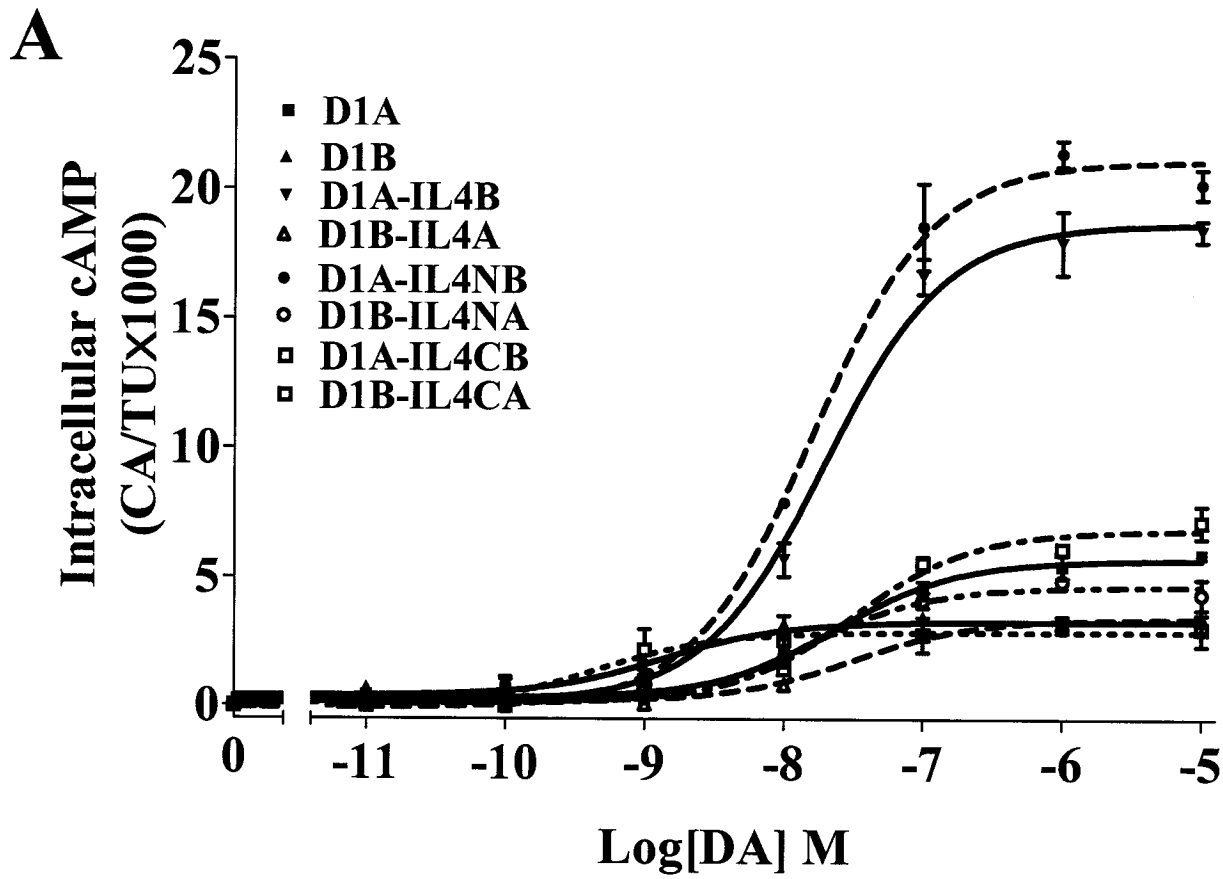

A
Agonist-independent activity
(CA/TUX1000)
20
15
10
5
0
Mock
D1A
D1B
D1A-IL4B
D1B-IL4A
D1A-IL4NB
D1B-IL4NA
D1A-IL4CB
D1B-IL4CA
B
Agonist-independent activity
(Relative to D1A)
3
2
1
0
*
*#
#
*#
#
#
*
D1A
D1B
D1A-IL4B
D1B-IL4A
D1A-IL4NB
D1B-IL4NA
D1A-IL4CB
D1B-IL4CA

Figure 10: DA-mediated maximal activation of AC in HEK293 cells

Intracellular cAMP levels were measured in single wells of a 12-well dish in absence or presence of increasing concentrations of DA and plotted as a function of log of DA concentrations. Net values for DA-mediated maximal activation of AC activity elicited by wild-type and chimeric D1-like receptors were obtained with dose-response curves using best-fit parameters for basal activity and maximal activation. Net values were calculated by subtraction of basal activity from maximal activation. ***A***, A representative example of dose-response curves of wild-type and chimeric D1-like receptors expressed as [^{3}H]-cAMP (CA) over the total amount of [^{3}H]-adenine uptake (TU) X 1000. ***B***, DA-mediated maximal activation of AC elicited by wild-type and chimeric D1-like receptors expressed relative to DA-mediated maximal activation produced by wild-type D1A receptor. Data for DA-mediated maximal activation are expressed as geometric mean ± S. E. of five experiments done in triplicate determinations and calculated relative to DA-mediated maximal activation elicited by D1A receptor. The Bmax values in pmole per mg of membrane proteins (expressed as the arithmetic mean ± S. E.) are 0.63 ± 0.06 (D1A), 0.62 ± 0.12 (D1B), 0.66 ± 0.08 (D1A-IL4B), 0.42 ± 0.07 (D1B-IL4A), 1.10 ± 0.17 (D1A-IL4NB), 0.83 ± 0.07 (D1A-IL4CB), 0.90 ± 0.13 (D1B-IL4NA), 0.48 ± 0.10 (D1B-IL4CA).

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


A
Intracellular cAMP
(CA/TUX1000)
25
20
15
10
5
0
0
-11
-10
-9
-8
-7
-6
-5
Log[DA] M
D1A
D1B
D1A-IL4B
D1B-IL4A
D1A-IL4NB
D1B-IL4NA
D1A-IL4CB
D1B-IL4CA

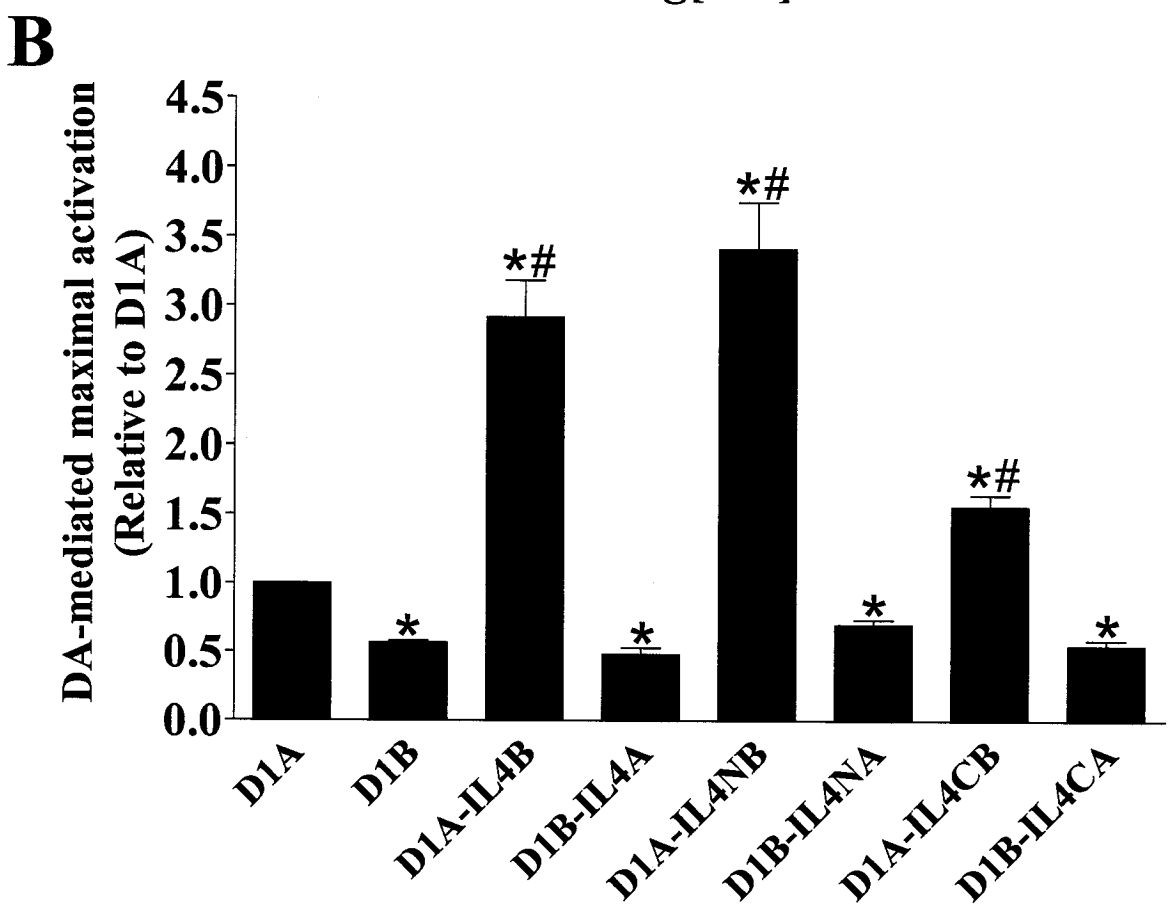
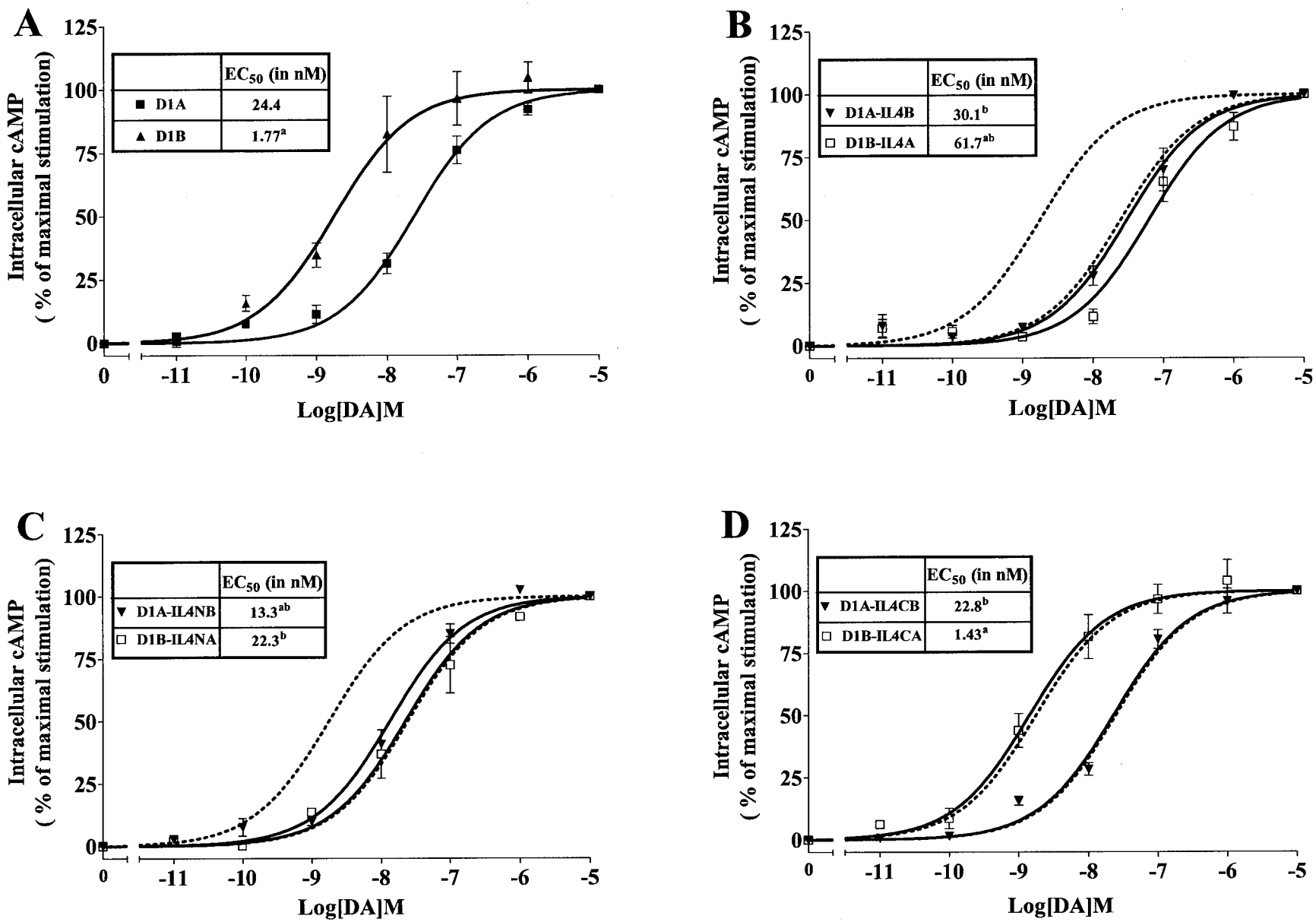

B
DA-mediated maximal activation
(Relative to D1A)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*#
*#
*#
*
*
*
*
D1A
D1B
D1A-IL4B
D1B-IL4A
D1A-IL4NB
D1B-IL4NA
D1A-IL4CB
D1B-IL4CA

Figure 11: Normalized dose-response curve of DA for AC stimulation by wild-type and chimeric receptors expressed in HEK 293 cells

Each point is the arithmetic mean of ± S. E. from five experiments done in triplicate determinations and expressed as the percentage of maximal stimulation obtained with respective wild-type or chimeric receptor after subtracting the basal value. Normalized curves for DA were analyzed by a four-parameter logistic equation using GraphPad Prism version 4.0. Statistical significance was determined using global fitting. ***A***, Normalized dose-response curves of DA for AC stimulation by wild-type receptors. ***B***, Normalized dose-response curves of DA for AC stimulation by chimeric IL4 receptors. As a reference, curves from wild-type receptors (broken lines) are shown. ***C***, Normalized dose-response curves of DA for AC stimulation by chimeric IL4N receptors. As a reference, curves from wild-type receptors (broken lines) are shown. ***D***, Normalized dose-response curves of DA for AC stimulation by chimeric IL4C receptors. As a reference, curves from wild-type receptors (broken lines) are shown. The $logEC_{50}$ values (in logM ± approximate logS.E. as obtained with GraphPad Prism) are as follows: -7.61 ± 0.02 (D1A), -8.75 ± 0.02 (D1B), -7.52 ± 0.03 (D1A-IL4B), -7.21 ± 0.03 (D1B-IL4A), -7.88 ± 0.03 (D1A-IL4NB), -7.64 ± 0.03 (D1A-IL4CB), -7.65 ± 0.02 (D1B-IL4NA), -8.84 ± 0.02 (D1B-IL4CA). The Bmax values in pmole per mg of membrane proteins (expressed as the arithmetic mean ± S. E.) are 0.63 ± 0.06 (D1A), 0.62 ± 0.15 (D1B), 0.66 ± 0.08 (D1A-IL4B), 0.42 ± 0.07 (D1B-IL4A), 1.10 ± 0.17 (D1A-IL4NB), 0.83 ± 0.07 (D1A-IL4CB), 0.90 ± 0.13 (D1B-IL4NA), 0.48 ± 0.10 (D1B-IL4CA).

a, $p<0.05$ when compared with D1A; b, $p<0.05$ when compared with D1B.


A
Intracellular cAMP
(% of maximal stimulation)
125
100
75
50
25
0
0
-11
-10
-9
-8
-7
-6
-5
Log[DA]M
EC50 (in nM)
D1A
24.4
D1B
1.77a
B
Intracellular cAMP
(% of maximal stimulation)
Log[DA]M
EC50 (in nM)
D1A-IL4B
30.1b
D1B-IL4A
61.7ab
C
Intracellular cAMP
(% of maximal stimulation)
Log[DA]M
EC50 (in nM)
D1A-IL4NB
13.3ab
D1B-IL4NA
22.3b
D
Intracellular cAMP
(% of maximal stimulation)
Log[DA]M
EC50 (in nM)
D1A-IL4CB
22.8b
D1B-IL4CA
1.43a

CHAPTER FOUR – DISCUSSION

Notwithstanding the high degree of overall homology in their primary sequences, D1A and D1B receptors display distinct ligand binding, agonist-independent and dependent G protein coupling properties in heterologous cells. The molecular basis underlying these differences must be addressed to gain insight into how D1-like subtype-specific functional properties regulate DA-mediated physiological roles in normal and pathological states. In addition, it is believed that a better understanding of the molecular basis would lead to improved therapeutic approaches for the treatment of human diseases displaying impaired D1-like receptor signal transduction (e.g. schizophrenia, Parkinson's disease and hypertension). Although the crystal structure of D1-like receptors has not been obtained, D1A and D1B receptors are believed to adopt various degrees of "constrained" receptor conformations (Tiberi and Caron 1994; Tumova *et al.* 2003). Specifically, a "constrained" conformation is defined as a receptor state that shows decreased affinities for agonist binding and low propensity for G protein coupling in the absence or presence of agonists (Samama *et al.* 1993; Scheer and Cotecchia 1997). From this point of view, experimental evidence suggests that the D1B receptor adopts a less "constrained" receptor conformation in comparison with D1A receptor, as evidenced by its significantly higher DA affinity, agonist-independent activity (constitutive activity) as well as DA potency (Tiberi and Caron 1994). Previous mutational studies have suggested

that the CT is an important structural domain, which bestows D1-like subtype-specific functional properties (Demchyshyn *et al.* 2000; Jackson *et al.* 2000; Chaar *et al.* 2001). Recently, work from our lab (Tumova and Tiberi, unpublished data) has identified a region in D1A and D1B receptors beginning at the exit of TM7 region and ending at conserved cysteine residues of the CT as the structural determinant responsible for the CT-conferred D1-like subtype-specific signaling properties (Jackson *et al.* 2000). This CT region has been implicated in the formation of a fourth intracellular loop (IL4) in rhodopsin, β_2-adrenergic receptor and other few of the family A GPCRs (Konig *et al.* 1989; O'Dowd *et al.* 1989; Kennedy and Limbird 1993; Schulein *et al.* 1996; Soskic *et al.* 1999). Interestingly, the crystal structure of rhodopsin has revealed that this region is forming a helical region referred to as H8 (Palczewski *et al.* 2000). In the present study, I narrow down the structural determinants within IL4 region of D1-like receptors responsible for regulating distinct functional properties of D1A and D1B receptors. The IL4 region of D1-like receptors can be divided into two segments (N- and C-terminal segments) that are defined by highly conserved cysteines (Fig. 4). The aim of this master project was to investigate the potential role of N- and C-terminal segments of IL4 (IL4N and IL4C) in regulating the functional properties of D1-like receptors as well as to study the potential contributions of the two segments to the overall D1-like subtype-specific receptor conformations.

1. The Roles of IL4N and IL4C Segments of D1-Like Receptors in Ligand Binding

1.1. The role of IL4N and IL4C segments in regulating DA binding affinity

Ligand binding studies showed that chimeric D1A receptor harboring the entire IL4 region of D1B (D1A-IL4B) displayed an increased DA affinity, which is indistinguishable from that of wild-type D1B receptors (Table 2). Chimeric D1A receptors carrying the IL4CB segment (D1A-IL4CB) exhibited a small enhanced but not statistically different DA affinity as compared with D1A receptor. Moreover, chimeric D1A receptor harboring IL4NB segment (D1A-IL4NB) displayed a significantly increased DA affinity in comparison with D1A receptors. Interestingly, DA affinity of D1A-IL4NB chimera was similar to that exhibited by wild-type D1B and chimeric D1A-IL4B receptors (Table 2). Based upon these results, I propose that the N-segment of IL4B region is mainly responsible for shaping DA receptor binding domain in a less "constrained" conformation. Additionally, results obtained with chimeric D1A receptors (D1A-IL4B, D1A-IL4NB and D1A-IL4CB) suggest that N- and C- segments of IL4 exert potentially additive effects on intramolecular interactions regulating a "less" constrained conformation for DA binding.

With respect to DA binding to chimeric D1B receptors, the insertion of the entire IL4A region into D1B receptor led to a decreased DA affinity as compared with wild-type D1B receptor. However, this decrease failed to recapitulate the DA affinity observed in cells expressing wild-type D1A receptors, suggesting that IL4A region may not be sufficient to confer to the D1B subtype a more "constrained" receptor conformation. Meanwhile, results obtained with the chimeric D1B receptors harboring only IL4NA segment exhibit a significantly decreased DA affinity when compared to wild-type D1B

and chimeric D1B-IL4A receptors. Importantly, while results suggest that IL4NA can impart to D1B subtype a more "constrained" conformation, this D1B-IL4NA chimera failed to reproduce fully the DA affinity measured at wild-type D1A receptors. This can be explained in part by results showing that D1B receptors bearing the IL4CA segment display a significant increase in DA affinity in comparison with both wild-type receptors (Table 2). These results suggest that the IL4CA segment induces a less "constrained" receptor conformation. Moreover, data obtained with the D1B chimeras (D1B-IL4A, D1B-IL4NA and D1B-IL4CA) imply that interfering intramolecular interactions elicited by N- and C- segments of IL4A region shape the receptor conformation for DA binding. Overall, the DA binding results highlight a distinct molecular interplay between the N- and C-segments of IL4 of D1-like receptors.

Interestingly, the affinity of the partial agonist SCH at chimeric D1-like receptors was essentially unchanged in comparison with their cognate wild-type receptors. Therefore, IL4 sequences may not be involved in regulating binding of partial agonists to D1-like receptors.

1.2. The role of IL4N segment of D1B receptor in inverse agonist binding

Studies in our lab using chimeric D1-like receptors, generated from a full swap of IL4 between D1A and D1B receptors (Fig. 6), indicated that the affinity of inverse agonists was not modulated by this CT region (Tumova and Tiberi, unpublished data). However, results showing a potential interplay between N- and C-terminal segments of IL4 in regulating DA affinity (Table 2) suggest that a role of the IL4 segments in inverse agonist binding may be adequately tested using more refined IL4 chimeras (IL4N and IL4C). In

fact, investigation of the potential role of IL4N and IL4C segments in inverse agonist binding indicates that the D1A-IL4NB chimera displayed an increased affinity for FLU and BUTA binding as compared with its cognate wild-type D1A receptor (Table 3). However, inverse agonist affinity of D1A-IL4CB, D1B-IL4NA and D1B-IL4CA was essentially unchanged in comparison with their wild-type parent receptors (Table 3). These results suggest that receptor conformational changes elicited by IL4N segment of D1B receptor may control inverse agonist binding to this D1-like subtype. Overall, our studies suggest that inverse agonist binding to D1A and D1B subtypes may require distinct and subtype-specific structural determinants.

2. The Contributions of IL4N and IL4C Segments in G protein Coupling Properties of D1-Like Receptors

2.1. The involvement of IL4N segment in regulating agonist-independent activity

As mentioned previously, the D1B receptor has an increased ability to generate intracellular signals in the absence of agonist as compared with the D1A receptor (Tiberi and Caron 1994). The present study shows that upon reciprocal exchange of entire IL4 region between the two D1-like receptors, agonist-independent activity of chimeric D1B receptors carrying IL4 of D1A subtype decreased to a level similar to wild-type D1A receptors. However, chimeric D1A receptor harboring IL4 of D1B subtype did not display increased agonist-independent activity. These results may be potentially explained by the lower expression of D1A-IL4B chimera in comparison with its wild-type parent receptor (1 vs 17 pmole/mg prot.) in HEK293 cells. Indeed, a linear relationship between agonist-independent activity and receptor density has been

demonstrated (Samama *et al.* 1993; Tiberi and Caron 1994, Scheer *et al* 1996, Jackson *et al.* 2000). Accordingly, it is anticipated that chimeric D1A-IL4B receptors would display enhanced agonist-independent activity in comparison with wild-type D1A receptors at comparable levels of receptor expression. Therefore, our studies suggest that the IL4 region of D1-like receptors is a crucial structural domain that confers D1-like subtype-specific agonist-independent activity. In an attempt to pinpoint structural determinants within IL4 responsible for the regulation of agonist-independent activity, we investigated the potential role of IL4N and IL4C segments. Interestingly, as seen with the exchange of entire IL4 region, a similar trend was observed with the swap of IL4N segment. Meanwhile, the agonist-independent activity of IL4C chimeras remained essentially unchanged in comparison with their wild-type parent receptors. Specifically, chimeric D1B receptor carrying IL4N segment of D1A subtype displayed decreased agonist-independent activity, which is similar to that of the D1A receptor. These data suggest that the IL4NA segment may induce a more "constrained" receptor conformation for G protein binding and activation. However, in a similar fashion to D1A-IL4B chimera, agonist-independent activity of chimeric D1A-IL4NB receptor was diminished as compared with wild-type D1A and D1B receptors, presumably due to the significantly lower receptor expression. Therefore, as stated above, I believe that D1A-IL4NB chimera would display enhanced agonist-independent activity in comparison with wild-type D1A receptors at comparable levels of receptor expression. Consequently, IL4NB segment may promote a less "constrained" receptor conformation for G protein coupling. Overall, these results suggest that IL4N imparts different degrees of "constrained" conformations to D1-like receptors (i.e. more "constrained" conformation to D1A and less "constrained"

conformation to D1B) for G protein coupling and activation in the absence of agonists. Meanwhile, an intriguing finding of my master project is that IL4C segment does not regulate the extent of agonist-independent activity, while modulating DA affinity. These findings provide further support to the notion that the signaling properties of D1-like receptors (e.g. agonist binding, G protein coupling) are regulated by discrete regions and can be separated by mutations (Iwasiow *et al.* 1999; Jackson *et al.* 2000; Tumova *et al.* 2003). Moreover, my results strongly imply that IL4C segment has no role in regulating the agonist-independent activity of D1-like receptors. Meanwhile, mutagenesis studies have suggested that agonist-independent and dependent G protein coupling may rely on different receptor conformations (Kjelsberg *et al.* 1992; Tumova *et al.* 2003; Kenakin 2003). Thus, IL4C may be important for controlling agonist-dependent G protein coupling properties of D1-like receptors. Indeed, our binding studies have shown that IL4C is able to modulate DA affinity, suggesting that this segment may be involved in regulating receptor conformations critical for binding to and activation of G protein in the presence of agonists. This was tested further by measuring agonist-dependent G protein coupling properties, notably efficacy (as indexed by maximal activation of AC) and potency (as indexed by EC_{50}) using dose-response curves to DA in cells expressing similar levels of wild-type and chimeric receptors. This issue will be discussed further in 2.3. and 2.4. sections. Another important observation made during my research project is the regulation of receptor number or Bmax values by IL4C segment.

2.2. The role of IL4N segment of D1-like receptors in controlling receptor number

I show that chimeric D1A receptors harboring IL4B displayed a significant decrease in Bmax values as compared with wild-type D1A receptors (Fig. 8A). Most importantly, these results were recapitulated in cells expressing D1A-IL4NB chimera (Fig. 8A). These findings addressed an unresolved issue by a previous study from our group showing that a chimeric D1A receptor bearing the full CT region of D1B subtype exhibited a similar drastic decrease in the Bmax value. Interestingly, in addition to significantly decreased Bmax values displayed by D1A-IL4B and D1A-IL4NB chimeras, both of the two receptors also exhibit potentially a constitutively active phenotype (i.e. increased agonist-independent activity). Importantly, studies have shown that constitutively active mutant GPCRs display also lower Bmax values in comparison with their wild-type counterparts due to a reduced structural stability. Therefore, a swap of IL4N segment of D1A receptor by that of D1B subtype may decrease the overall structural stability of D1A-IL4B and D1A-IL4NB chimeras because of an increased agonist-independent activity (constitutive activity). This issue reinforces the view that D1A-IL4B and D1A-IL4NB chimeras display a higher constitutive activity in comparison with wild-type D1A receptors. One should bear also in mind that the presence of IL4NB segment in D1A receptor may promote a conformational state of chimera that exposes intracellular domains regulating receptor internalization and targeting to degradation pathways.

2.3. The contribution of IL4N and IL4C segments in mediating DA-mediated maximal activation

The insertion of the N-segment of IL4B region into D1A subtype led to a chimeric receptor exhibiting a drastic increase in DA-mediated maximal activation. In a similar fashion but to a lesser extent, the addition of C-segment of IL4B region into D1A subtype resulted in a chimeric receptor displaying an enhanced DA-mediated maximal activation as compared with wild-type D1A receptor. However, surprisingly, the insertion of both N- and C-segments of IL4B into D1A receptor did not lead to a further increase of DA-mediated maximal activation. These results could be explained potentially by interfering intramolecular interactions elicited by N- and C-segments of IL4B region. Alternatively, studies have shown that agonist-induced phosphorylation, desensitization and internalization play an important role in diminishing GPCR responsiveness (as reviewed in Ferguson 2001). Accordingly, the lack of further increase in DA-mediated maximal activation displayed by D1A-IL4B chimera may also be explained potentially by an increased DA-induced phosphorylation, desensitization and internalization of the chimera and subsequently decreased receptor responsiveness. In striking contrast, cells expressing chimeric D1B receptors exhibited the same DA efficacy when compared with cells expressing wild-type D1B subtype. Overall, these results underscore the underlying molecular complexity that controls agonist-induced receptor conformations for G protein coupling and effector activation. Moreover, our results showing that cells expressing wild-type and chimeric D1B receptors exhibit essentially the same DA efficacy while displaying differences in DA affinity and potency (please refer to section 2.4.) strengthen

the notion that D1-like receptors can adopt multiple active states (Iwasiow *et al.* 1999; Jackson *et al.* 2000; Tumova *et al.* 2003).

2.4. The roles of IL4N and IL4C segments of D1-like receptors in DA potency

2.4.1. IL4N segment plays an important role in regulating DA potency

Our results have shown that the swap of IL4N segment of D1-like receptors resulted in a partial switch of DA potency. In particular, chimeric D1A receptor harboring IL4NB segment displayed a ~1.8 fold increase in DA potency as compared with wild-type D1A receptor. This finding is somewhat in agreement with enhanced DA binding affinity exhibited by D1A-IL4NB chimera, supporting the assumption that IL4NB segment is critical for inducing a less "constrained" receptor conformation. The discrepancy between the fold change observed in DA affinity and potency (1.8 vs 6 fold) in cells expressing D1A-IL4NB chimera suggests that IL4N segment of D1B receptor may exert its effect predominantly on the receptor conformation underlying DA binding. In contrast, chimeric D1B receptor carrying IL4NA segment showed a decreased DA potency, which is indistinguishable from wild-type D1A receptor. This result is in a line with a decreased DA affinity displayed by D1B-IL4NA chimera. Therefore, IL4NA segment may be responsible for conferring a more "constrained" receptor conformation, which displays lower affinity for DA and decreased coupling abilities to G protein upon agonist activation.

2.4.2. Interfering intramolecular interaction between IL4N and IL4C in regulating DA potency

Substitution of IL4C between D1A and D1B receptors did not lead to noticeable changes in DA potency (Fig. 11D). Most interestingly, when switching the entire IL4 region (IL4N and IL4C), both led to chimeras (D1A-IL4B and D1B-IL4A) displaying a loss of DA potency. These results strongly suggest that IL4C segment controls the extent of IL4N-mediated effects on DA potency through interfering intramolecular interactions. Additionally, the DA potency results obtained with IL4 chimeric receptors are of importance since they provide insight into the molecular basis for loss of DA potency previously observed in cells expressing chimeric D1-like receptors in which the entire CT region was swapped (Jackson *et al.* 2000). We believed that the loss of DA potency observed with the CT chimeras can be explained by the presence of IL4C sequences. Interestingly, our resent studies have shown that the aberrant loss of DA potency observed in cells expressing these CT chimeras was "rescued" by exchanging the EL3 region (Tumova *et al.* 2003). Most importantly, these EL3/CT chimeras exhibit the same DA potency when compared with their respective cognate wild-type D1-like receptors, suggesting that a molecular interplay between EL3 and CT was involved in conferring the D1-like subtype-specific DA potency (Tumova *et al.* 2003). Most likely, my studies suggest that the C-terminal segment of IL4 is the structural determinant involved in the molecular interplay between EL3 and CT (Tumova *et al.* 2003). Further studies would be required to support this assertion.

3. Conclusions

In the present study, I have investigated the role of IL4 and more specifically IL4N and IL4C segments of D1A and D1B subtype in regulating D1-like subtype-specific conformations and signaling properties. Importantly, studies investigating structure-function relationships of IL4 or H8 of rhodopsin may provide insight into how IL4 region regulates D1-like receptor function. IL4 of rhodopsin is thought to be involved in regulating a conformational change critical for rhodopsin activation (Krishna *et al.* 2002). Upon rhodopsin activation by light the H8/IL4 region undergoes a conformational change, switching from a helical to looplike structure (Krishna *et al.* 2002). Furthermore, the N-terminal tripeptide (Asn^{310}-Lys^{311}-Gln^{312}) of IL4 of rhodopsin has been shown to be critical for inducing the helical structure, which corresponds to the inactive conformational state of IL4 (Krishna *et al.* 2002). Truncating or mutating this tripeptide disrupted IL4 helical conformation (Krishna *et al.* 2002). With respect to D1-like receptors, the IL4 N-terminal tripeptides of D1A and D1B receptors are identical, whereas the more distal sequences of IL4 N-terminal segment of D1-like receptors (Ala^{335} — Cys^{347} in D1A; Ala^{363} — Cys^{375} in D1B) are distinct (Fig. 4). Whether IL4N segment of D1-like receptors is critical for holding IL4 in an inactive conformation remains to be addressed further. However, data obtained from my DA binding and G protein coupling studies illustrate that IL4N segment may be involved in shaping the D1A and D1B receptor into more and less "constrained" conformational states, respectively. Additionally, a study on rhodopsin revealed that the N-terminal tripeptide of IL4 serves as the binding site for α and γ subunits of transducin (retinal G protein) (Ernst *et al.* 2000). Accordingly, due to their distinct primary structures, IL4N segments of D1-

like receptors may also serve as the binding sites for different G protein subunits. In fact, a study using ribozyme strategy suppressing the expression of the G protein γ_7 subunit has shown that D1A but not D1B receptor couples to a heterotrimeric Gs protein containing the γ_7 subunit to activate AC in HEK293 cells (Wang *et al.* 2001). In terms of the contribution of IL4C segment to distinct functional properties of D1-like receptors, this segment may not be involved in shaping D1-like subtype-specific conformations regulating G protein coupling in the absence of agonists. However, ligand binding results suggest that the IL4C segment of D1A receptors plays an important role in regulating the D1-like subtype-specific conformational state for DA binding. Furthermore, G protein coupling data reveal that IL4C segment of D1-like receptors may be also involved in regulating IL4N-mediated effects on DA potency through interfering intramolecular interactions.

Another important fact regarding the structure-function relationship studies of IL4 region is the potential contribution of palmitoylation to the regulation of GPCR conformation and function. As mentioned earlier, IL4 region of GPCRs in family A contains one or more highly conserved cysteines, which can be modified by the fatty acid palmitate through thioester linkage (Qanbar and Bouvier 2003). Due to its reversible property, palmitoylation/depalmitoylation cycles may change the receptor conformation and this process may have important functional implications (Moffett *et al.* 1996; Krishna *et al.* 2002; Qanbar and Bouvier 2003). More specifically, Krishna and collaborators have shown that IL4 of rhodopsin adopts helical conformation only in the membrane lipid environment (Krishna *et al.* 2002). On the one hand, this finding along with the fact that palmitoylation is a dynamic lipid modification, which helps the CT to associate with

plasma membrane, raises the possibility that alteration of palmitoylation status (palmitoylation/depalmitoylation cycles) may play a role in regulating helical conformation and proper orientation of IL4 region (Krishna *et al.* 2002). On the other hand, palmitoylation may also regulate GPCR responsiveness since palmitoylation/depalmitoylation cycles also lead to receptor conformational changes that mask or unmask certain CT domains essential for phosphorylation and desensitization (Moffett *et al.* 1996; Qanbar and Bouvier 2003). However, we should bear in mind that the potential contribution of palmitoylation to the regulation of receptor conformation and function may vary according to specific GPCRs. For instance, elimination of palmitoylation of β_2-adrenergic receptor significantly enhanced receptor phosphorylation and desensitization (Moffett *et al.* 1996), whereas removal of palmitoylation of D1A receptor did not affect receptor normal ligand binding and G protein coupling properties (Jin *et al.* 1997). In terms of D1B receptor, two cysteine residues in IL4B are also found in locations similar to those in IL4A (Fig. 4). However, palmitoylation of the two cysteines in D1B receptor remains to be explored. It is possible that the D1A and D1B receptor conformations are differently regulated by palmitoylation and swapping of IL4N or/and IL4C segments between D1A and D1B receptors may change receptor palmitoylation status. As a result, alteration of palmitoylation may underlie potential distinct desensitization properties of chimeric D1A receptors (please refer to section 2.3.). Overall, palmitoylation status may underlie D1-like subtype-specific functional properties. However, studies aimed at the investigation of the palmitoylation of D1B receptor are required to assess whether this post-translational modification regulates D1B subtype signaling features.

Taken together, results obtained during my master project suggest crucial roles of IL4N and IL4C in mediating the distinct ligand binding and G protein coupling properties of D1-like receptors. Finally, these findings expand our knowledge on structure-function relationships and signal transductions of D1-like receptors. Hopefully, these findings will prove useful in developing D1-like subtype-specific ligands, which are critical for the treatment and management of human diseases displaying impaired D1-like receptor functions.

CHAPTER FIVE – REFERENCES

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