Mutational Analysis to Define the Functional Role of the Third Intracellular Loop of D1-Class Dopaminergic Receptors

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Abstract

The third intracellular loop (IL3) and cytoplasmic tail (CT), which are the most divergent regions between human D1-class dopaminergic receptors (hD1R and hD5R), have been implicated in modulating their subtype-specific functional phenotypes. The importance of the IL3 for Guanine nucleotide-binding protein (G-protein) coupling and specificity has long been acknowledged in the G-protein-coupled receptor (GPCR) field. However, the exact role the central region of the IL3, notably the N- and C-terminal moieties, plays in GPCR receptor functionality remains unclear. Studies in our laboratory indicated that the IL3/N-terminal moiety of hD1-class receptors appears to be critical for facilitating agonist-independent and dependent activation of hD1R and hD5R. Furthermore, the IL3/C-terminal portion of hD1-class receptors constrains the receptor in the inactive state and reduces receptor affinity for agonists and G-protein coupling. I put forward the following hypothesis: 1. The functional properties of hD1-class receptors are regulated via a molecular micro-switch present within the IL3 central region modulating the functional properties of the receptor distinctly, 2. The functional differences between D1R and D5R require structural elements from both N- and C-terminal halves of the IL3 central region, and 3. The molecular interplay between the N- and C-terminal halves of the IL3 central region is dependent on the amino acid chain length and content. Herein, I have employed site-directed mutagenesis, and alanine replacement approaches to analyze comprehensively the structural determinants within the N- and C-terminal moieties of the IL3 central region that regulate ligand binding and G-protein coupling properties of hD1-class receptors. Moreover, my Ph.D. research aimed to pinpoint whether the IL3 length and/or structural motif(s) regulate ligand binding and activation properties of hD1R and

hD5R. The results of my study highlight the importance of structural elements from both the proximal and distal segments of the IL3/central region of hD1-class receptors for the ligand binding and receptor activation status. Additionally, my results underline the significance of preserving the length of the IL3 regardless of the amino acid content. This study also shows the pivotal role played by a phenylalanine residue, $F264^{6.27}$, in the signaling properties of hD1R. Notably, mutating $F264^{6.27}$ leads to a mutant hD1R with characteristics resembling those of constitutively active mutant GPCRs. Unraveling the amino acid/amino acids constraining the receptor in the inactive state will perhaps provide an attractive target for drug design. Future work aims at developing drugs that particularly bind to the intracellular face of hD1R and improving selectivity towards hD1R may prove useful in limiting the side effects associated with the conventional therapy of brain disorders such as in the case of L-DOPA induced dyskinesia (LID) seen in individuals suffering from Parkinson's disease.

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List of Abbreviations

A_{2A}: Adenosine 2A receptor

AC: Adenylyl cyclase

Ach: Acetylcholine

AP-2: Adapter protein-2

AP: Action potential

AT1R: Angiotensin II type 1 receptors

AT2R: Angiotensin II type 2 receptors

ATP: Adenosine triphosphate

B_{max}: Maximal binding Capacity

BSA: Bovine serum albumin

Ca^{2+}: Calcium

CAM: Constitutively active mutant

cAMP: Cyclic adenosine monophosphate

CDK5: Cyclin-dependent kinase 5

CK: Casein kinases

CNS: Central nervous system

COMT: Catechol-O-methyl transferase

CRE: cAMP response element

CREB: cAMP response element-binding protein

CT: The carboxyl-terminus

CXCR4: Chemokine receptors type 4

Cys347: Cysteine 347

Cys351: Cysteine 51

D1R: D1 dopaminergic receptor

D2R: D2 dopaminergic receptor

D3R: D3 dopaminergic receptor

D4R: D4 dopaminergic receptor

D5R: D5 dopaminergic receptor

DA: Dopamine

DAG: Diacylglycerol

DARPP-32: Dopamine- and cAMP-regulated phosphoprotein, 32 kDa

DARs: Dopamine receptors

DAT: Dopamine transporter

DRY: Aspartate-arginine-tyrosine motif

E: Effector

EC50: Half maximal effective concentration

EL: Extracellular loop

EL1: Extracellular loop 1

EL2: Extracellular loop 2

EL3: Extracellular loop 3

ELISA: Enzyme linked immunosorbent assay

ER: Endoplasmic reticulum

ERK: Extracellular signal regulated kinase

FBS: Fetal bovine serum

FSH: Follicle stimulating hormone

G protein: Guanine nucleotide-binding protein

GABA: gamma-amino butyric acid

GDP: Guanosine diphosphate

GIPs: GPCR interacting proteins

GPCRs: G-protein coupled receptors

GRKs: GPCR kinases

GTP: Guanosine triphosphate

hD1R: Human D1R

hD5R: Human D5R

HEK293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IBMX: Isobutyl-1-methylxanthine

IL1: Intracellular loop 1

IL2: Intracellular loop 2

IL3: Intracellular loop 3

IL4: Intracellular loop 4

IP3: Inositol triphosphate

K_D: Equilibrium dissociation constant

K_I: Inhibitory dissociation constant

KO: Knock out

L-DOPA: L-3,4-dihydroxyphenylalanine

LH: Luteinizing hormone

LID: L-DOPA induced dyskinesia

LTP: Long-term potentiation

M5R: Muscarinic receptor type 5

MAO: Monoamine oxidase

MC4R: Melanocortin 4 receptor

MEM: Minimal essential media

mGluRs: Metabotropic glutamate receptors

MSNs: Medium spiny neurons

NA: Nucleus accumbens

NPY: Neuropeptide Y

NT: Amino-terminus

OPD: O-phenylenediamine dihydrochloride

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PFC: prefrontal cortex

PIP2: Phosphatidylinositol biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PP1: Protein phosphatase 1

PSD-95: Postsynaptic density protein 95

PTH: Parathyroid hormone

RAMPs: Receptor activity modifying proteins

TCM: The ternary complex model

TH: Tyrosine hydroxylase

TIDA: Tuberoinfundibular pathway

TM: Transmembrane

TRL: Terminal receptor locus

TSH: Thyroid stimulating hormone

V1R: Vasopressin 1 receptor

VMAT2: Vesicular monoamine transporter type 2

VTA: Ventral tegmental area

WT: Wild type

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This doctoral thesis would not have been possible without the support and the encouragement of the kind people around me.

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Chapter 1

INTRODUCTION

1. Plasma membrane receptors

Transmission of extracellular signals from outside to inside the cells requires the presence of transmembrane receptors, the so-called, "cell surface receptors". These receptors transmit extracellular stimuli into the cells interior through activation of multiple intracellular signaling pathways and responses. Cell surface receptors are categorized into three groups according to their biological characteristics, structures, and ligands: enzyme-linked receptors, ligand-gated ion channels, and G-protein coupled-receptors (Wess, 1998, Hermans, 2003).

1.1 G-protein coupled receptors (GPCRs)

GPCRs are seven transmembrane receptors and comprise the largest group of cell surface receptors, with more than 800 receptors identified in the human genome (Hoffmann et al., 2008, Luttrell, 2008, Rajagopal et al., 2010). They are stimulated by different endogenous ligands including hormones, biogenic amines, lipids, and enzymes and extracellular stimuli like light, odors, and pheromones (Gether, 2000, Latek et al., 2012).

Signaling impairment of GPCRs has been linked to many endocrine, cardiovascular, and central nervous system (CNS) diseases. In fact, several genetic disorders have been associated with activating and inactivating mutations in different GPCRs genes (Hermans, 2003, Karnik et al., 2003, Schöneberg et al., 2004, Johnston and Siderovski, 2007, Trzaskowski et al., 2012). Therefore, it is not surprising that drugs targeting GPCRs account for about 50-60 % of the currently marketed drugs (Karnik et al., 2003, Trzaskowski et al., 2012).

1.2 GPCR structure

GPCRs share a common architecture of seven hydrophobic transmembrane alpha-helices (TM1-TM7) connected by three extracellular loops (EL1-3) and three intracellular loops (IL1-3). The external region of the receptor is called the amino-terminus (NT) while the internal area of the receptor is known as the carboxyl-terminus (CT) **(Fig. 1)**.

The NT contains N-glycosylation sites, which aid in cell surface expression. A disulfide bridge between EL1 and EL2 is often present in GPCRs and is crucial for proper protein folding conformation. The TM domains are barrel-shaped; arranged in anticlockwise fashion and they sit perpendicular to the cell membrane. They have the highest degree of sequence conservation in contrast to other domains of the receptors, which vary markedly in size and amino acids sequence. The hydrophobic TM domains are responsible for the ligand binding while the ILs and the CT are necessary for interaction with heterotrimeric G-proteins and different intracellular partners. The CT can contain cysteine residues serving as palmitoylation sites, which were believed to help in the formation of a fourth intracellular loop (IL4) until this putative loop was shown in crystallized GPCRs to be an alpha-helix region now referred to as helix 8 (Wess, 1998, Luttrell, 2008, Latek et al., 2012). GPCRs owe their name to their ability to regulate and interact with different intracellular heterotrimeric G-proteins (Gether, 2000).

1.3 Heterotrimeric G-proteins and GPCR activation dynamics

Heterotrimeric G-proteins are the main molecular switches for GPCRs, which connect receptors to multiple downstream effectors like enzymes and proteins to convey different cellular response. G-proteins composed of three tightly connected subunits: Gα,

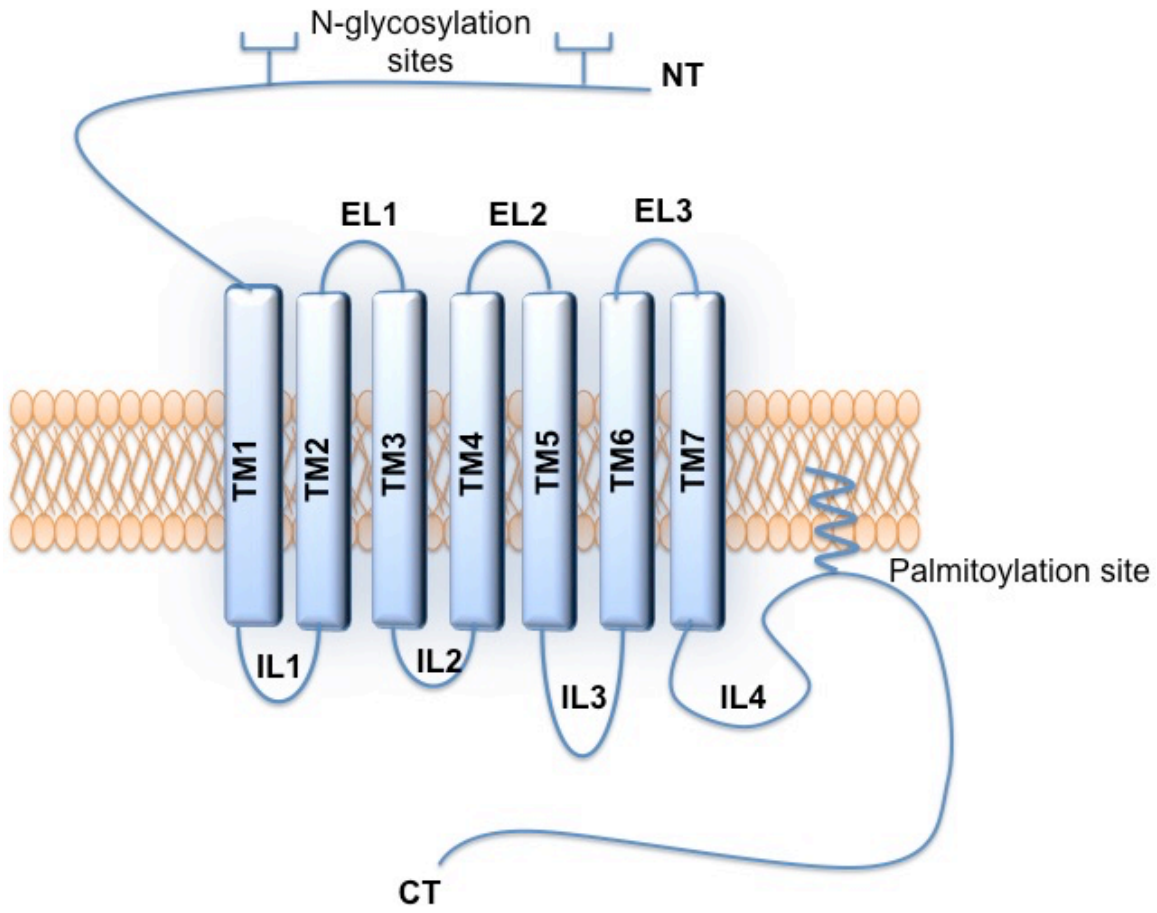


Figure 1. Schematic diagram of a general GPCR structure.
Seven TM spanning domains are shown and connected by three ELs and three ILs. The external end of the receptor is the NT, which contains N-glycosylation sites. The internal end of the receptor is the CT, which contains cysteine residues serving as palmitoylation sites, forming the IL4 (GPCR crystal structures suggest that this region is α-helical and termed the H8 region). TM domains form the ligand-binding site while the three ILs and the CT region are sites for interaction with G-protein.

Gβ, and Gγ. There are 16 different isoforms of Gα protein varying in size between 39-52 kDa. They are classified according to their sequence similarity into four groups: $G\alpha_{s/olf}$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (McCudden et al., 2005).

The Gα-subunit is composed of two domains: GTPase domain and α-helical like domain. The nucleotide-binding site [Guanosine diphosphate (GDP)/Guanosine triphosphate-binding site (GTP)-binding site] is located in between these two domains. There are 6 different isoforms of Gβ and 12 different subunits of Gγ yielding about 72 possible combinations between different Gβ and Gγ isoforms. The Gβ- subunit has a size of approximately 35-39 kDa and forms a non-dissociable functional heterodimer with Gγ-subunit, which is 7-8 kDa in size (Clapham and Neer, 1997, Oldham and Hamm, 2006).

In the inactive state, Gα is bound to Gβγ subunits forming the heterotrimeric G-protein complex. Ligand binding to GPCRs produces conformational changes in the intracellular domains of the receptors. These changes include binding of the ligand-occupied receptor to different G-proteins, which then triggers the exchange of GDP for GTP; followed by dissociation of the Gα subunit from the Gβγ complex. Then both the Gα and the Gβγ complex will be able to interact with different downstream signaling and effector partners. The GTPase activity of Gα-subunit will then hydrolyze the GTP into GDP and reunion of G-protein subunits again to form the non-functional Gαβγ complex (Hermans, 2003, McCudden et al., 2005) **(Fig. 2)**. The type of response produced and the signaling pathway activated upon receptor activation differ based on the ligand, the Gα protein, and which intracellular partners have been activated.

1.4 GPCR classification

GPCRs are classified according to their physiological and sequence identity using the GRAFS classification system, into five main families: glutamate (G, 15 members), rhodopsin (R, 701 members), adhesion (A, 24), frizzled/taste2 (F, 24), and secretin (S, 15) (Fredriksson et al., 2003).

The rhodopsin family is the largest subfamily of GPCRs; it includes about 700 receptors and by far the most studied. They are classified into four main groups: α (prostaglandin receptors, biogenic amine receptors including dopamine receptors (DARs), serotonin receptors, opsins, melatonin receptors, and MECA receptors, which include melanocortin receptors and cannabinoid receptors), β [includes receptors that bind peptides such as the neurotensin and the neuropeptide Y (NPY) receptors], γ (e.g. somatostatin receptors and chemokine receptors); and δ (which has four main branches: the MAS-oncogene-related receptor cluster, the glycoprotein receptor cluster, the purine receptor cluster, and the olfactory receptor cluster) (Fredriksson et al., 2003).

One of the most important structural features of this group is the presence of about 20 highly conserved amino acids in the cytoplasmic half of the TMs. It has been suggested that these highly conserved residues are critical for receptor stability and proper protein folding upon receptor activation. Members of this group share a conserved aspartate-arginine-tyrosine motif (DRY) located at the cytoplasmic side of TM3. This motif is necessary for maintaining the receptor in the inactive state (Moreira, 2014).

Although the TM domains are highly conserved among the rhodopsin family, the intracellular loops are variable in length and amino acid contents. The IL2 is the most conserved region (20 ± 2 amino acids) while the IL3 is the most disordered and divergent

region (41 ± 43 amino-acids). The NT contains about 62 ± 98 amino-acids and the CT contains approximately 53 ± 36 amino-acids (Moreira, 2014). The TM domains of the rhodopsin family share a common structural characteristic regardless of the differences in their amino acids content and length. They include the (D/E)RY motif in TM3 in which $D/E^{3.49}$ interacts with $D/E^{6.30}$ to stabilize the receptor in the inactive state, the NPxxYxF motif in which $Y^{7.53}$ interacts with $F^{7.60}$ to stabilize the receptor in the inactive state (Fredriksson et al., 2003, Schwartz et al., 2006, Rosenbaum et al., 2009, Moreira, 2014).

The glutamate family is comprised of GPCRs such as metabotropic glutamate receptors (mGluRs), gamma-amino butyric acid (GABA) receptors, and calcium sensing receptors. This group is characterized by having a very long NT, which is involved in ligand binding.

The secretin group includes receptors for glucagon, secretin, calcitonin, and other peptidic hormones. They share about 20 conserved amino acids and have a relatively long NT domain containing six conserved cysteine residues (Schiöth and Fredriksson, 2005).

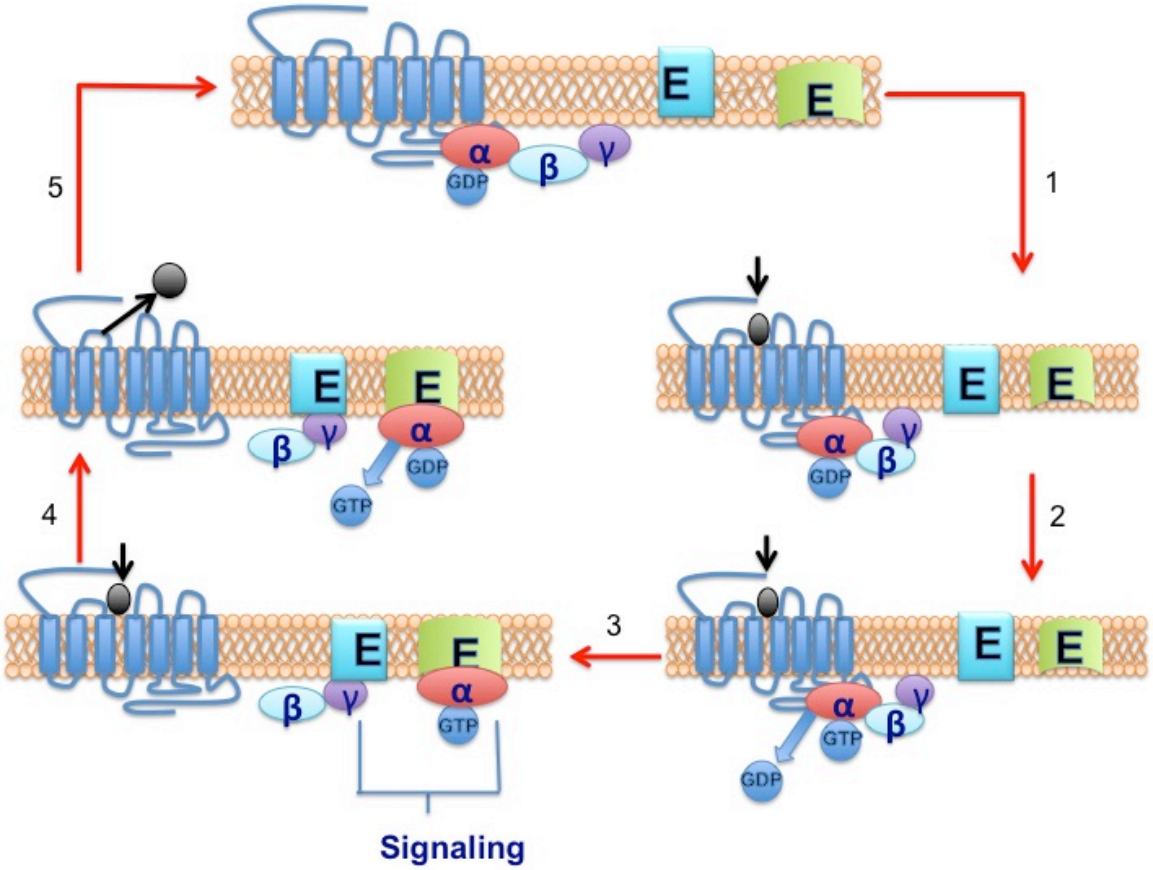


Figure 2. Basic cycle of GPCR activation dynamics.
In the inactive state, GDP-bound Gα associates with Gβγ subunits forming the heterotrimeric G-protein complex. **1.** Ligand binding to GPCRs produces conformational changes in the intracellular domains of the receptors; **2.** These changes trigger the exchange of GDP for GTP; **3.** Dissociation of the Gα subunit from the Gβγ complex, and both Gα and Gβγ can interact with different downstream signaling and effector partners; **4.** The intrinsic GTPase activity of Gα-subunit will then hydrolyze the GTP into GDP; **5.** G-protein subunits reunite again to form a non-functional Gαβγ complex and to return back to the resting state.

1.5 Canonical GPCR signaling pathways

As revealed from various GPCR crystallographic and biophysical studies, ligand binding to GPCRs allows the rearrangement of TM domains and the outward movement of the intracellular end of TM6, making a crevice inside the receptor where G-protein can bind (Moreira, 2014).

Activation of $G\alpha_s$ stimulates adenylyl cyclase (AC) while stimulation of $G\alpha_i$ inhibits AC. AC catalyzes the conversion of adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP), which then activates cAMP-dependent protein kinase A (PKA). On the other hand, activation of $G\alpha_q$ leads to stimulation of phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 increases the release of calcium (Ca^{2+}) through activation of IP3 receptors on the endoplasmic reticulum (ER). Ca^{2+} and DAG then activate the conventional protein kinase C (PKC) in contrast to novel PKC isoforms, which are Ca^{2+} insensitive and only require DAG for activation (Asaoka et al., Newton, 1995, Wettschureck and Offermanns, 2005). In addition to the classical model of GPCRs coupling to G proteins, studies suggest that GPCRs can signal through coupling to non G-protein effectors like β-arrestins (Miller and Lefkowitz, 2001, Perry and Lefkowitz, 2002). In recent years, it became apparent that β-arrestins could act as a signal terminator as well as a signal transducer. β-arrestins have the ability to behave as scaffolding proteins, which allow interactions with several intracellular signaling partners and connect GPCRs to diverse signaling pathways such as mitogen activated protein kinase (MAPK) cascades and proto-oncogene tyrosine kinase Src family, mediating G-protein

independent signaling (Luttrell and Lefkowitz, 2002, Reiter and Lefkowitz, 2006, Ma and Pei, 2007, Luttrell, 2008). This led to the development of what is called "biased ligand" or functional selective ligand, which favours the activation/inhibition of one pathway over the other, i.e. G-protein dependent signaling over β-arrestin-dependent signaling or vice versa (Shonberg et al., 2014, Luttrell et al., 2015, Hodavance et al., 2016, Rankovic et al., 2016).

1.5.1 Ternary complex model

The ternary complex model (TCM) was proposed in 1980 to explain the connection between the receptor, agonist, and the G-protein (De Lean et al., 1980). In this model, GPCRs are in a state of equilibrium between two inter-convertible states (active/inactive) in the absence of G-protein and can bind agonists with different affinities. Coupling of the receptor to its G-protein results in an active state of the receptor which can bind agonists with high affinity while uncoupling results in the inactive state with a low affinity for agonist binding (Park et al., 2008b). However, this theory cannot accommodate constitutive activity observed for some GPCRs, which led to the proposal of a new model called, the extended TCM.

According to the extended TCM, the receptor exists in two states, an inactive state (R) which bind agonists with low affinity and an active state (R*) that binds agonists with high affinity where G-protein merely binds to the R*. According to this model, agonists promote changes in the inactive receptor conformation. Theses changes allow G-protein binding and receptor activation preferentially by stabilizing the receptor in the R* state, which results in a maximal response. Whereas, the inverse agonists shift the equilibrium toward the R state and maintain the receptor in the inactive conformation. On the other

hand, antagonists (neutral ligands, no efficacy), produce their action by blocking the binding of the receptor to other ligands and have an equal affinity for both the R and the R* states **(Fig. 3)** (Samama et al., 1993, Park et al., 2008b).

While the two-state model helps to explain some of the behaviors produced by ligand, receptor, and G-protein interaction, it does not provide sufficient proof to account for the mode of response produced by other ligands like partial agonists.

Pharmacologically, the responses produced by full agonists and partial agonists are not the same and cannot be explained by the two-state model proposed. The existence of a multi-states model has been supported by studies done with rhodopsin and adrenergic receptors. In the multi-state model, different agonists adopt different receptor conformations depending on their efficacies (Nygaard et al., 2013, Manglik et al., 2015). Interestingly, an alternative (modified) TCM has been proposed for M2 muscarinic receptors. The modified TCM connects the agonist, the receptor, and the β-arrestin together instead of agonist, receptor, and G-protein (Gurevich et al., 1997, Kenakin, 2002, 2003, Swaminath et al., 2004).

1.5.2 Conformational changes associated with GPCRs activation

A universal numbering scheme is required to facilitate the comparison of homologous residues within the same GPCRs class or between different classes. The most common and reliable system currently in use is the Ballesteros-Weinstein numbering scheme. This system relies on giving the most conserved residue in each helix the number 50, which is used as a reference point for other residues preceding it or following it. The TM domains have been given a whole number (TM1-7). For example, 4.45 indicates a residue in TM4 located five residues preceding the conserved residue in

TM4, 4.50; 4.53 indicates a residue in TM4 located three residues ascending the 4.50 conserved residue (Ballesteros and Weinstein, 1995).

Conformational changes associated with GPCRs activation have been examined using a number of mutagenesis, biophysical, and spectroscopic studies. The prediction of conformation changes was based on the rhodopsin family of GPCRs. These studies have suggested that there are sequential structural changes upon binding of ligands to their cognate receptors (Swaminath et al., 2004, Swaminath et al., 2005, Granier and Kobilka, 2012).

In summary, these changes include rearrangement of the cytoplasmic side of TMs during receptor activation, most importantly the movement of TM6, to expose receptor to the intracellular signaling molecules such as G-proteins and arrestins. TM1 to TM4 seems to be more rigid and remains stable upon activation (Hubbell et al., 2003, Trzaskowski et al., 2012). The intracellular segment of TM6 moves away from the center of the receptor, away from TM3 toward TM5 **(Fig. 4)** (Oldham et al., 2007, Rasmussen et al., 2011a). The large movement of TM6 seems to be caused by the IL3 flexibility, which connects TM5 and TM6. In fact, it was shown that the IL3 is necessary for receptor G-protein interaction (Hubbell et al., 2003). Intriguingly, blocking the movement of TM6 in crosslinking studies was shown to inhibit G-protein activation for some members of Rhodopsin family (Sheikh et al., 1999).

Also, the $Y^{7.53}$ in the highly conserved motif NPXXY motif located at the end of TM7 interacts with water molecules to stabilize the inactive state of the receptor. Upon receptor activation, TM7 appears to move outward away from the center of the protein.

TM7 intracellular segment is positioned in a way that prevents TM6 from moving back toward TM3 stabilizing the receptor in the active state (Schwartz et al., 2006, Scheerer et al., 2008, Rosenbaum et al., 2009). Moreover, the ionic lock between $R^{3.50}$ of the highly conserved DRY motif in TM3 and $E^{6.30}$ in TM6 breaks upon receptor activation, liberating the receptor constrains and allowing TMs movement (Park et al., 2008a, Scheerer et al., 2008, Rosenbaum et al., 2009). The inactive state of rhodopsin, adrenoceptors, and rhodopsin-like GPCRs stability was shown to depend on the presence of this intact ionic lock (Dror et al., 2009, Hofmann et al., 2009). Moreover, in the active state, it was predicted from the crystal structure of β_2R-Gαs complex that H5 of Gαs is the main contact interface with IL2, TM5, and TM6 of β_2R (Rasmussen et al., 2011b, Flock et al., 2015) **(Fig. 5)**.

Agonist-bound GPCRs does not only promote receptor activation but also subsequently initiates different GPCRs regulatory processes to suppress receptor activity to avoid prolonged receptor activation. It also activates different G-protein independent signaling pathways, such as the β-arrestin dependent signaling (Hodavance et al., 2016).

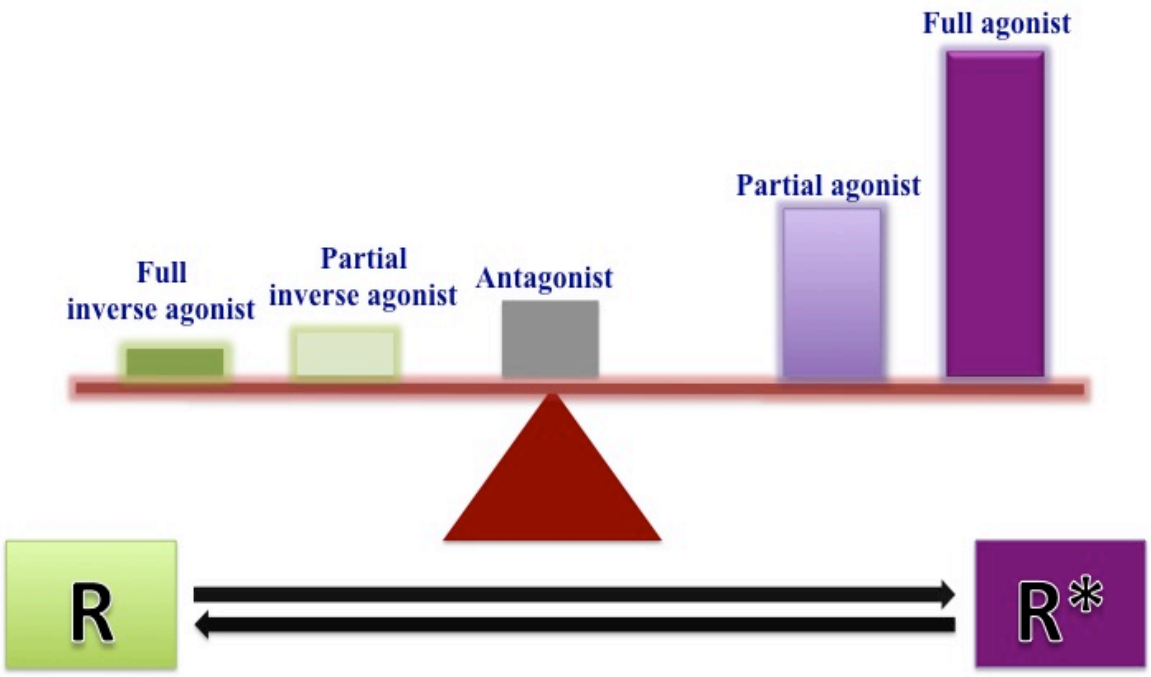


Figure 3. Dynamic nature of GPCRs, the two-state model.
GPCRs isomerize between the inactive (R) and the active state (R*). The full agonist stabilizes the receptor in the active state (R*) whereas the full inverse agonist stabilizes the receptor in the inactive state (R). The antagonist is neutral, i.e it does not have any preference for one state over the other and it blocks the binding of agonists and inverse agonists to GPCRs. The partial agonist and partial inverse agonist promote intermediate activation states between full state of receptor activation and full state of receptor inhibition, respectively.

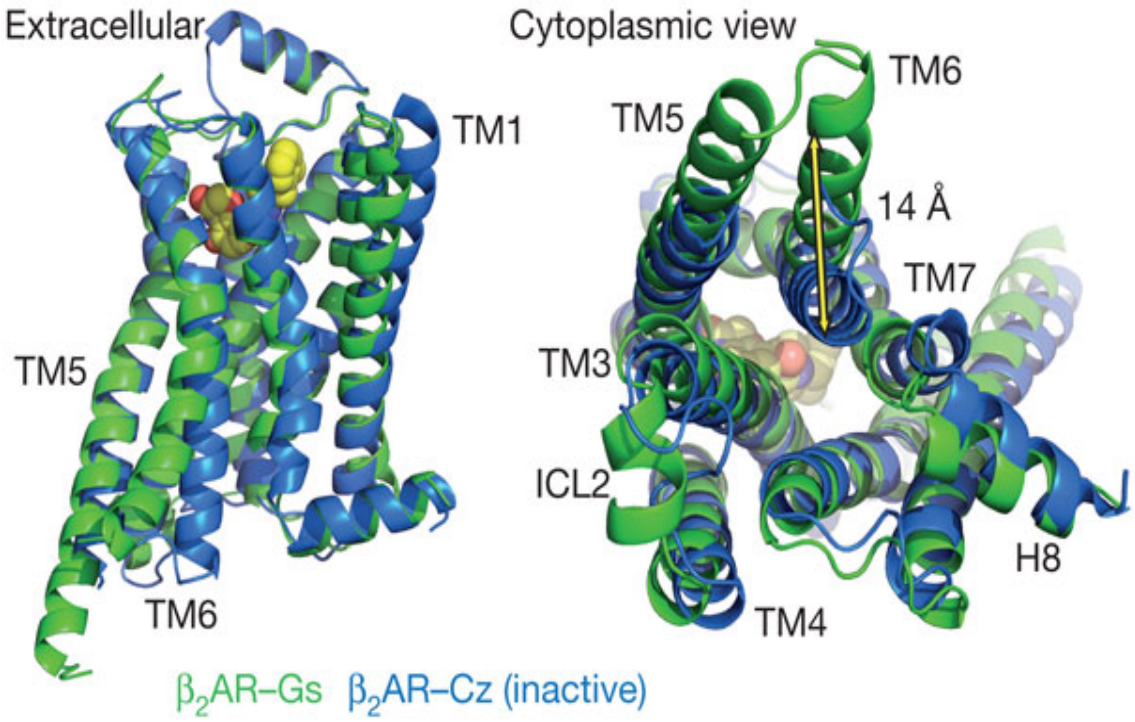


Figure 4. Predicted changes of GPCR conformation upon receptor activation based on the β_2-adrenergic receptor model.

Extracellular and cytoplasmic views of the different β_2AR crystal structures are shown. The carazolol-bound β_2AR structure (inactive state-blue) is compared to the β_2AR–Gs structure (active state-green). Agonist binding induces rearrangement of the cytoplasmic side of TMs, TM6 is moved outward and the TM5 is extended by two helical turns (Rasmussen et al., 2011b).

A. **B.**

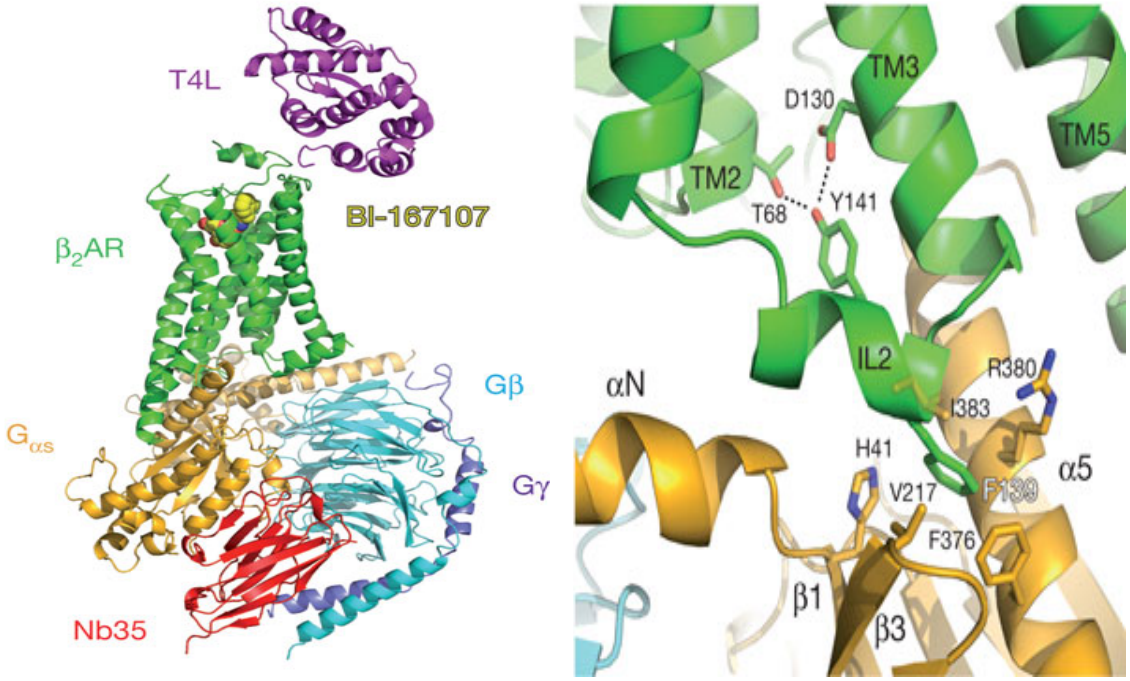


Figure 5. Structural organization of β_2AR-Gαs complex.
A. The overall structural assembly the β_2AR (green) bound to an agonist (BI-167107, yellow spheres) interacting with Gαs (orange). Gαs, Gβ (cyan), and Gγ (purple) constitute the heterotrimeric Gs protein. The nanobody, Nb35 (red) and T4 lysozyme (magenta) facilitated the crystallization process. **B.** β_2AR residues interaction interface with α5 (H5) of Gαs. H5 is the main contact interface with residues from IL2, TM5, and TM6 of β2R (Rasmussen et al., 2011b).

1.6 Regulation of GPCRs signaling

1.6.1 Negative regulation of GPCRs

The dampening of GPCR signaling is an imperative step for regulation of the receptors responsiveness. This process is, in fact, a sequential process including receptor desensitization (homologous and heterologous desensitization), internalization, recycling, and down regulation of the receptors. Eventually, it leads to uncoupling of the receptor from its G-protein, removal of the receptor from the plasma membrane, recycling, or directing the receptor to the degradation pathway (Luttrell, 2008).

1.6.1.1 Homologous desensitization

The desensitization process occurs directly after receptor activation upon agonist binding. Agonist-bound receptors recruit the GPCRs kinases (GRKs), which phosphorylate the receptors on serine and threonine residues present in their IL3 and their CT. The phosphorylated receptors will then have a high affinity for β-arrestin binding. There are two isoforms of β-arrestins. β-arrestin 1 and β-arrestin 2 are the most commonly involved in GPCR regulation (Luttrell, 2008).

The rhodopsin subfamily of GPCRs, including DARs, has higher affinity for binding to β-arrestin 2 than β-arrestin 1 (Kohout et al., 2001). The C-terminal tail of β-arrestin contains a binding motif for clathrin and β2-adaptin subunit of the adaptor protein-2 (AP-2) (Goodman et al., 1996, Laporte et al., 1999). This allows β-arrestin to target GPCRs to clathrin-coated pits for endocytosis and removal from the plasma membrane (Luttrell, 2008). The GPCRs are then directed to the endosomes for sorting either to the lysosomes for degradation or dephosphorylation, release of arrestin,

resensitization and then recycling back to the cell surface (Kohout et al., 2001, Luttrell, 2008) **(Fig. 6)**.

It is worth mentioning here that GRKs have no direct effect on receptor desensitization. Instead, they serve as catalysts to increase the affinity of the phosphorylated GPCRs to arrestins, which are the main player in the homologous desensitization process. It was shown that β_2-adrenergic receptor phosphorylation by GRK2 increases its affinity to β-arrestin 1 by about 10- to 30- fold (Muller et al., 1993, Luttrell, 2008). Conversely, in some cases, GRKs act as the main modulator of receptor inactivation, independent from receptor phosphorylation. For example, it has been shown that the dampening of mGluR1a signaling is dependent on binding of the regulator of G-protein signaling (RGS) homology (RH) domain of GRK2 (RH has no kinase activity) to $G\alpha_{q/11}$, which interrupts mGluR1a/$G\alpha_{q/11}$ complex. Noteworthy, this process does not involve the GRK2 kinase domain (Dhami et al., 2004).

1.6.1.2 Heterologous desensitization

This desensitization process does not require the receptor to be activated by agonist binding. Instead, it is modulated via activation of a nearby receptor, which activates the second messenger-dependent protein kinases PKA and PKC. They phosphorylate the receptor on its IL3 and CT, which directly impairs the coupling of G-protein to the receptor (Luttrell, 2008).

1.6.2 Positive regulation of GPCRs

GPCR ligand binding, signaling, coupling to G-proteins, and subcellular localization have been shown to be positively modulated by different regulatory processes via protein-protein interaction. Protein-protein interactions include GPCR

homodimerization (same receptors interacting with themselves), or GPCR heterodimerization (two distinct receptors interacting with each other) (Luttrell, 2008). GPCR dimerization is sometimes necessary for receptor trafficking to the plasma membrane to form a functional unit, such as in the case of GABA-B receptors (Jones et al., 1998, Kaupmann et al., 1998, Robbins et al., 2001, Kniazeff et al., 2002).

Another positive modulatory mechanism for GPCRs includes the interaction of GPCRs with several other proteins, including receptor activity modifying proteins (RAMPs), GPCR-associated sorting protein (GASP), and several Post-Synaptic Density of 95 kDa (PSD-95)-Disc large-Zona occludens (PDZ) domain-containing proteins such as the Na+/H+ exchanger regulatory factor/ezrin binding protein 50 (NHERF/EBP50) and SH3 multiple ankyrin domain-containing protein (Shank), regulating the receptor signaling, trafficking, and localization specificity. For instance, RAMPs and PSD-95 were shown to be important for receptor trafficking and function, and to modulate ligand specificity of the receptor (Brady and Limbird, 2002, Luttrell, 2008, Dunn and Ferguson, 2015).

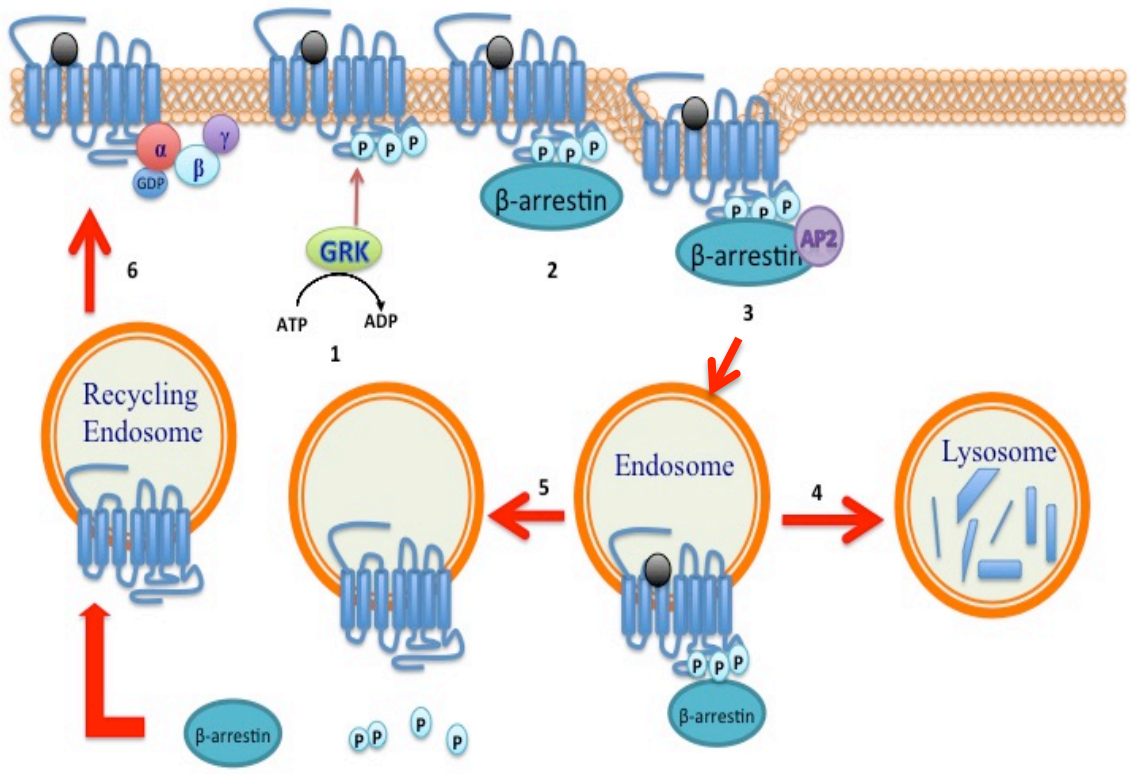


Figure 6. Desensitization and trafficking of GPCRs.
Upon ligand binding, GPCRs traditionally signal via G proteins. After receptor activation and signaling, GRKs then phosphorylate GPCRs on their CT and ICLs (**1).** β-arrestins is recruited to the phosphorylated GPCRs and prevent further coupling of G proteins **(2)**. The phosphorylated GPCRs-β-arrestins complex is targeted for endocytosis via clathrin-coated pits **(3)**. Internalized GPCRs are subsequently sorted to lysosomes for degradation (**4)** or recycling endosomes for dephosphorylation (**5),** the release of β-arrestins allows recycling back to the cell surface (**6)**.

2. Dopaminergic system

DA is one of the principal neurotransmitters in the CNS. Dopaminergic neurotransmission is the most abundant in the brain, and it regulates many central and peripheral functions. It has a major role in modulating several brain functions such as voluntary movement, attention, memory, reward, sleep, feeding, and learning. DA also regulates peripheral functions including hormonal release, cardiovascular and renal functions, and sympathetic activity.

Dysregulation of dopaminergic functions has been linked to many human diseases such as Parkinson's disease, schizophrenia, Huntington's disease, depression, Tourette's disorder, hyperprolactinemia (Missale et al., 1998, Lebel et al., 2007, Beaulieu and Gainetdinov, 2011).

2.1 Dopamine synthesis

DA synthesis process starts in the presynaptic neurons and passes through different sequential enzymatic modulations of DA-precursor, L-tyrosine. It is hydroxylated by tyrosine hydroxylase to 3,4-dihydroxy-L-phenylalanine (L-dopa). Tyrosine hydroxylase (TH) is the rate-limiting enzyme during the process of DA synthesis. L-dopa is then decarboxylated by the aromatic L-amino acid decarboxylase (DOPA decarboxylase) to DA. After that, DA is packed in the storage vesicles by the vesicular monoamine transporter type 2 (VMAT2) and the free DA in the presynaptic terminal is degraded by monoamine oxidase (MAO). Neuronal stimulation by action potential provokes the increase in the Ca^{2+} influx in the nerve terminal causing fusion of DA-storing vesicles with the presynaptic terminals leading to the release of DA in the synaptic cleft. Once released into the synaptic cleft, DA binds to different dopaminergic

autoreceptors located presynaptically or binds to DARs located postsynaptically leading to activation of different signaling pathways **(Fig. 7)**. DARs signaling can be blocked by the action of various antipsychotic drugs. Excess DA in the postsynaptic cleft is then exposed to degradation by catechol-O-methyl transferase (COMT). DA activity in the presynaptic terminal can also be terminated via an active reuptake process by dopamine transporters (DAT), which can be blocked by cocaine and amphetamine (Verheij and Cools, 2008).

2.2 Major central dopaminergic pathways

Four major dopaminergic pathways have been identified to regulate DA activity in the brain: the nigrostriatal pathway, the mesolimbic and mesocortical pathways (often referred to as the mesocorticolimic pathway), and the tuberoinfundibular pathway (Beaulieu and Gainetdinov, 2011) **(Fig. 8)**. The nigrostriatal pathway connects the substantia nigra to the dorsal striatum, which is part of the basal ganglia. This pathway is responsible for movement control through maintaining the balance between the direct and indirect dopaminergic pathways in the basal ganglia. Loss of dopaminergic neurons in the substantia nigra leads to Parkinson's disease (Civelli et al., 1993, Missale et al., 1998, Iversen and Iversen, 2007).

The mesocortical pathway connects the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and regulates memory, emotions, and cognitive function while the mesolimbic pathway links the VTA of the brain to the nucleus accumbens (NA) and is involved in motivation and reward. Dopamine hypoactivity in the PFC pathway is thought to be responsible for the negative symptoms of schizophrenia, including flat emotions, lack of desire, and motivation.

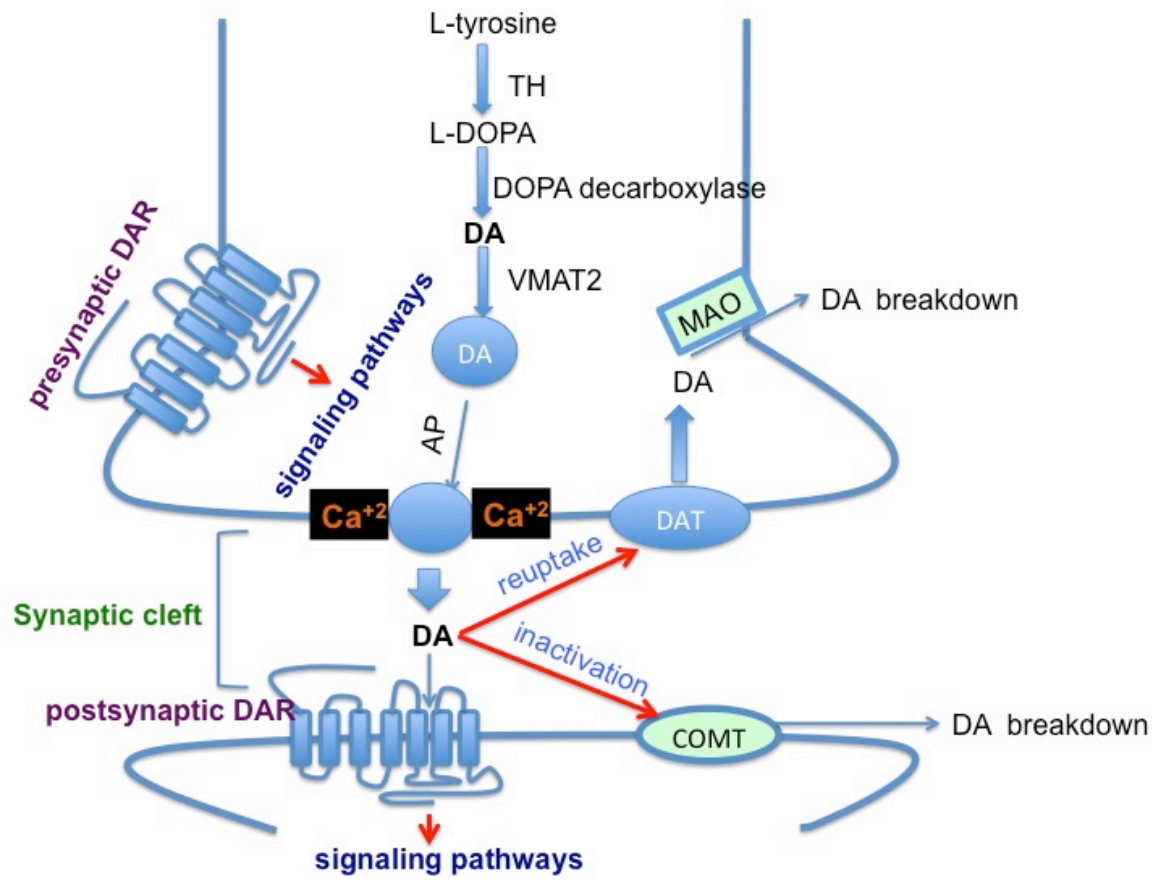


Figure 7. Schematic representation of dopamine synthesis.
DA synthesis process starts in the presynaptic neurons. TH is the rate-limiting enzyme during the process of DA synthesis TH converts L-tyrosine to L-dopa, which is then decarboxylated by DOPA-decarboxylase to DA. After that, DA is packed in the storage vesicles by VMAT2 and MAO then degrades the free DA in the presynaptic terminal. Neuronal stimulation by action potential provokes the increase in the Ca^{2+} influx in the nerve terminal causing fusion of DA-storing vesicles with the presynaptic terminals leading to the release of DA into the synaptic cleft. Once released in the synaptic cleft, DA binds to different dopaminergic autoreceptors located presynaptically or binds to postsynaptic DARs leading to activation of various signaling pathways. Excess DA in the postsynaptic cleft is then degraded by COMT. DA activity in the presynaptic terminal can also be terminated via an active reuptake process by DAT.

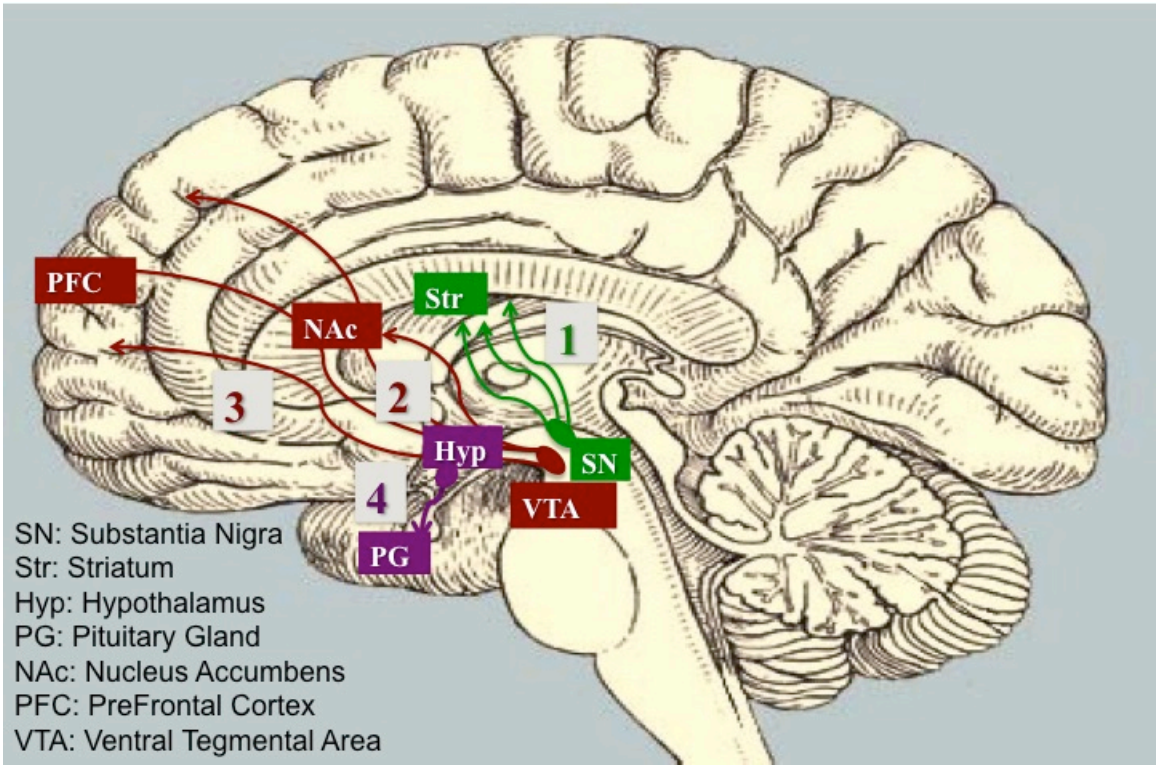


Figure 8. Dopaminergic neuronal pathways.
There are four dopaminergic neuronal pathways: 1. Nigrostriatal pathway; projecting from the substantia nigra to dorsal striatum. 2. Mesolimbic pathway; projecting from the ventral tegmental area to the nucleus accumbens. 3. Mesocortical pathway; projecting from the ventral tegmental area to the prefrontal cortex. 4. Tuberoinfundibular pathway; projecting from the hypothalamus to the pituitary gland. Modified from news.bioscholar.com

In contrast, dopamine hyperactivity in the mesolimbic pathway is thought to cause the positive symptoms of schizophrenia: hallucination, euphoria, and delusion (Civelli et al., 1993, Missale et al., 1998, Kienast and Heinz, 2006, Iversen and Iversen, 2007).

The fourth dopaminergic pathway is the tuberoinfundibular pathway (TIDA), which connects the hypothalamus to the pituitary gland. This pathway regulates prolactin secretion. Disruption of the neuronal regulation of this pathway leads to hyperprolactinemia, which is often treated with the D2R agonist, bromocriptine (Civelli et al., 1993, Missale et al., 1998).

2.3 Dopamine receptors

Kebabian and Calne first introduced the concept of classifying DARs as D1- and D2- class receptors in the late 1970's (Kebabian and Calne, 1979). Nowadays, D1-class includes D1R and D5R, and D2-class includes D2R, D3R and D4R subtypes. DARs are classified based on their amino acid sequences and their ability to stimulate (D1-class) or inhibit (D2-class) AC through coupling to heterotrimeric $G\alpha_{s/olf}$ and $G\alpha_{i/o}$ proteins, respectively (Missale et al., 1998). There are some differences between D1-class and D2-class receptors in regard to their localization and their structure **(Fig. 9A, Fig. 9B)**.

D1-class receptors are solely expressed postsynaptically while D2-class receptors are expressed pre- and postsynaptically (De Mei et al., 2009, Beaulieu and Gainetdinov, 2011). In addition, D2-class receptors (in contrast to D1-class subtypes) have introns in their coding sequences, which allow the generation of spliced variants. D2R exists in two isoforms, D2-short (D2S) and D2-long (D2L). The latter has an additional 29 amino acids in the IL3 (Usiello et al., 2000).

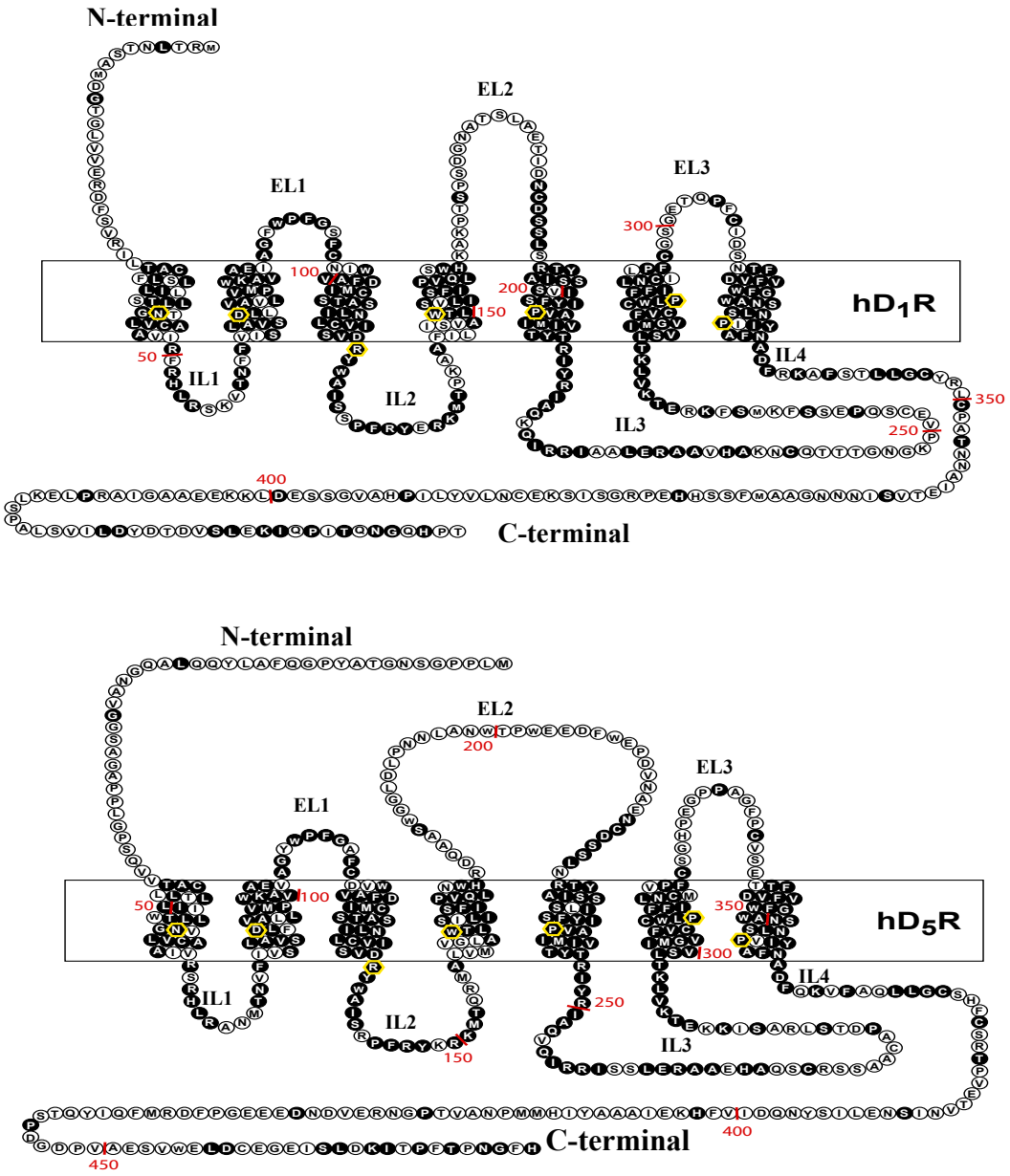


Figure 9A. Secondary structure of human D1-class receptor (D1R and D5R).
Open circles represent identical residues between the two subtypes, whereas black circles represent divergent residues between human D1 and D5 receptors. The most conserved residue in each TM is marked with a yellow hexagon. EL1: extracellular loop 1; EL2: extracellular loop 2; EL3: extracellular loop 3; IL1: intracellular loop 1; IL2: intracellular loop 2; IL3: intracellular loop 3 [Modified from (Zhang et al., 2014)].

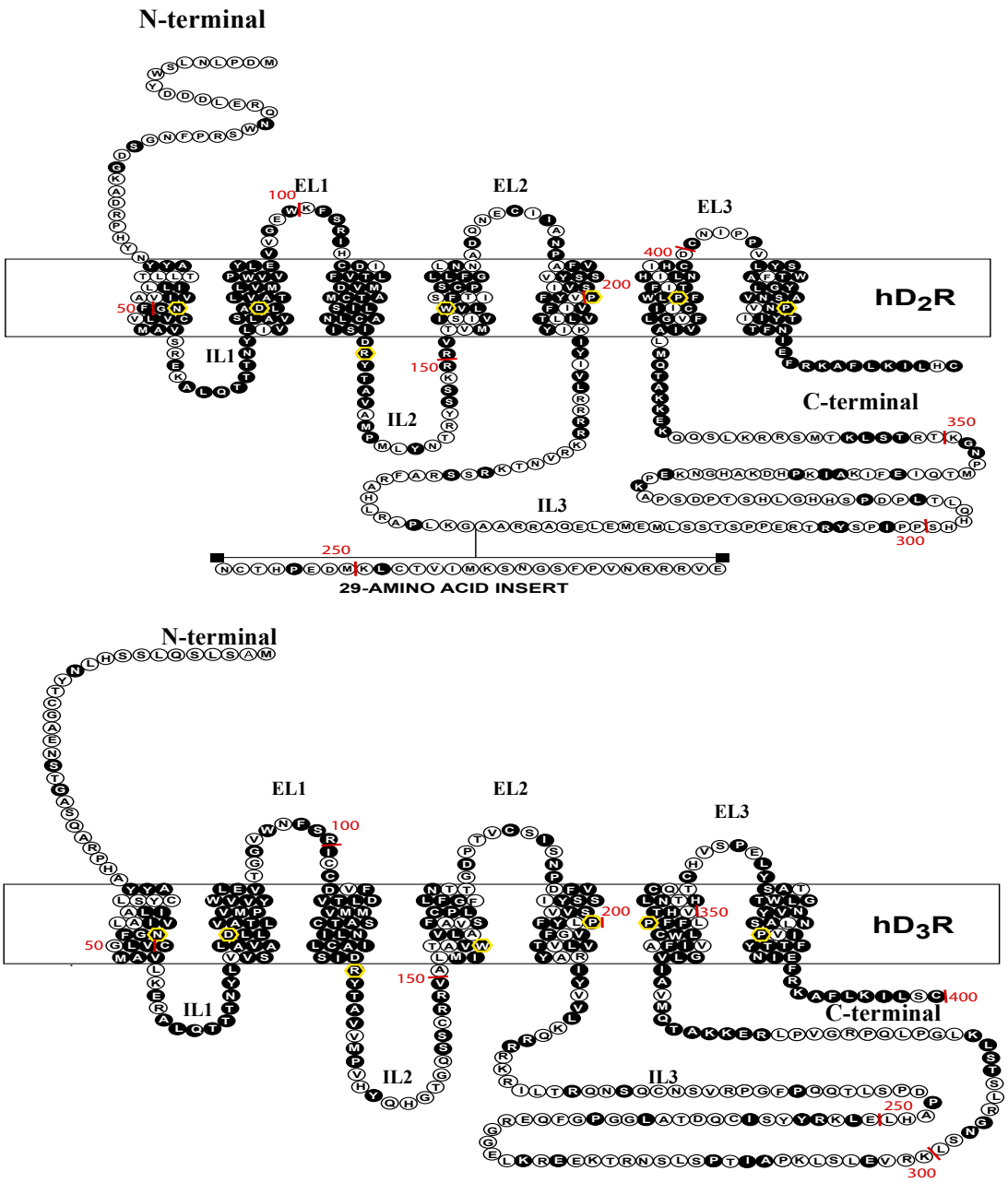


Figure 9B. Secondary structure of human D2-class receptor (D2R and D3R).
Open circles represent identical residues between the two subtypes, whereas black circles represent divergent residues between human D2 and D3 receptors. The most conserved residue in each TM is marked with a yellow hexagon. The hD2R-long and hD2R-short differ by a 29 amino acid insertion within IL3. EL1: extracellular loop 1; EL2: extracellular loop 2; EL3: extracellular loop 3; IL1: intracellular loop 1; IL2: intracellular loop 2; IL3: intracellular loop 3 [Modified from (Zhang et al., 2014)].

Although D1-class receptors are intronless, it was found that D5R has two related pseudogenes, with 98% identical to each other and 95% identical to D5R, but they exist in non-functional forms (Grandy et al., 1991, Weinshank et al., 1991). Members of the same class of DARs share a high level of identity but differ in their physiological properties and functions, which might be related to their level of expression in the brain and where they are expressed (Missale et al., 1998).

2.3.1 Dopamine receptor expression in the brain

Considering the broad level of DAR expression in the brain, it is not surprising that DA is involved in regulating many central and peripheral functions. Centrally, D1R is the most abundant DARs in the brain (Dearry et al., 1990, Fremeau et al., 1991). D1R is highly expressed in the mesolimbic and mesocortical areas such as striatum, substantia nigra pars reticulata, olfactory bulb, amygdala, and frontal cortex. It is also expressed at low level in the hippocampus, cerebellum, and hypothalamic region (Beaulieu and Gainetdinov, 2011; Holmes et al., 2004; Missale et al., 1998). In contrast to D1R, D5R is expressed at a much lower level than D1R. D5R expression is more restricted and is found in the hypothalamus, the hippocampus, the substantia nigra, and the pyramidal neurons of the PFC. D5R mRNA expression is found in the cerebral cortex, striatum, and to a lesser extent, in the hypothalamus (Huntley et al., 1992, Rappaport et al., 1993).

D1R and D5R are more abundant postsynaptically. They are both coexpress in different brain regions, as in the striatum and the hippocampus. Ultrastructural studies suggested that D1R and D5R differ in their subcellular localization within individual pyramidal neurons. It has been shown that D1R is mainly expressed in the dendritic

spines while D5R is more abundant in the dendritic shafts (Bergson et al., 1995, Kruusmagi et al., 2009). Different localization and subcellular location patterns for these two closely related D1-class receptors suggest that they may play different roles in different neuronal circuits and exhibit different physiological roles.

D2R is highly expressed in the striatum, NA, and olfactory tubercle. Also, they have significant level of expression in the substantia nigra, VTA, hippocampus, hypothalamus, and cortical areas. The development of bacterial artificial chromosome transgenic mice expressing enhanced green fluorescent protein helps to differentiate between the localization of D1R and D2R in the medium spiny neurons (MSNs). It has been shown by several studies (in situ hybridization, immunohistochemistry, immunocytochemistry) that D1R and D2R are segregated in different neuronal populations and the percentage of MSNs coexpressing both D1R and D2R is very low (≤10%) (Hersch et al., 1995, Le Moine and Bloch, 1995, Yung et al., 1995, Surmeier et al., 1996, Deng et al., 2006). In situ hybridization studies have suggested that D1R is expressed in neurons originating from MSNs coexpressing substance P, projecting to the substantia nigra. On the other hand, D2Rs are found to be expressed in MSNs neurons coexpressing enkephalin, projecting to the globus pallidus (Gerfen et al., 1990, Le Moine et al., 1991, Surmeier et al., 1996, Niesen et al., 2011).

D3Rs have a confined level of expression to the limbic areas such as the shell of the NA and olfactory tubercles. They are also expressed at a much lower level in the striatum, VTA, and hippocampus. D4R expression pattern is the lowest among DARs. D4R has a considerable level of expression in the frontal cortex, amygdala, hippocampus, and hypothalamus (Jaber et al., 1996, Missale et al., 1998).

2.3.2 Dopamine receptor expression in the periphery

A high level of expression of D2R has been detected in the pituitary gland. D1R, D2R, and D4R are all expressed in the retina. All DAR subtypes are expressed in the adrenal glands, kidney, sympathetic ganglia, blood vessels, heart, and gastrointestinal tract at significantly different levels of expression (Beaulieu and Gainetdinov, 2011).

2.3.3 Dopamine receptor functions

Due to the broad level of expression of DARs in the body, it is not surprising that they are involved in regulating many physiological functions, centrally and peripherally. In the brain, it has been shown that locomotor activity is mainly regulated by D1R, D2R, and D3R. The extent of locomotor activation by the striatum depends on which DAR has been activated (Missale et al., 1998). The effect of D1R on locomotion is mainly mediated by the action of postsynaptic D1R while D2R effect on movement is regulated via both pre- and postsynaptic D2R leading to a decrease and an increase in locomotion, respectively. Although some studies have shown that D1R has a minimal effect on locomotor activity; however, there is clear evidence now that activation of D1R is required for D2R agonists to evoke a maximal locomotor activity (Jackson and Westlind-Danielsson, 1994, Missale et al., 1998). As stated above, D3R is expressed mainly in the NA. Agonistic effect of D3R on locomotion is mainly inhibitory while antagonizing the action of D3R on locomotion has a stimulatory effect (Waters et al., 1993).

Moreover, several studies have highlighted the importance of D1R and D2R involvement in reward and addiction mechanisms. DA in the mesocorticolimic is implicated in the reward and reinforcement mechanism, as administration of drugs of abuse has shown to increase the release of DA in the mesolimbic areas. Interestingly,

both D1R and D2R have been shown to be involved in the drugs of abuse-reinforcing properties, in which D1R has a permissive effect on D2R mediating the stimulant drug reinforcement effect. It has been suggested that stimulating D1R, in fact, can be a potential therapy for cocaine addiction (Di Chiara, 1995). In the last ten years, several studies have also shown that administration of different D3Rs agonists like 7-hydroxydipropylaminotetralin (7-OH-DPAT) and quinelorane modulate the effect of cocaine self-administration mainly through modulating the level of DA in the NA where D3R is expressed (Parsons et al., 1996).

In addition to the role of D1R and D2R on locomotion and rewards mechanisms, pharmacological studies have shown that activation of D1R and D2R in the hippocampus positively impacts retention of working memory tasks in rats (Packard and White, 1991). The effect on memory is likely to be modulated via D5R, which has higher level of expression in the hippocampus than D1R. Due to the lack of selectivity of the agonists used, it is difficult to ascribe this effect to one specific DAR as opposed to the others (Missale et al., 1998). Activation of D1R and D2R in the PFC has resulted in performance improvement of working memory in monkeys (Sawaguchi and Goldman-Rakic, 1994, Arnsten et al., 1995).

DARs also play a major role in psychosis and schizophrenia. The continuous efforts over the years to investigate the involvement of DA in the physiology of schizophrenia led to development of the reformulated DA hypothesis (Goto and Grace, 2007, Guillin et al., 2007). The old DA hypothesis was based on the fact that blocking the action of DA on D2R improves the symptoms of schizophrenia (Carlsson, 1974). However, the reformulated hypothesis is distinguished by the imbalance between the

action of D1R and D2R in two different brain regions. It involves hyperactivation of D2R in the cortical area, represented by the positive symptoms of schizophrenia, and hypoactivation of D1R in the PFC represented by the negative symptoms of schizophrenia (Goto and Grace, 2007, Guillin et al., 2007).

Furthermore, it was shown that D2Rs regulate prolactin secretion as they are expressed in the anterior and intermediate lobes of the pituitary gland. D2R-agonists, most commonly bromocriptine, are used for treatment of hyperprolactinemia (Missale et al., 1998). Neither can the effect of D3R on the TIDA neurons be neglected. It has been observed that DA acts on D3R to inhibit its release from TIDA neurons (Lin et al., 2000).

DARs are widely distributed in the periphery as aforementioned; therefore, they have other regulatory effects beyond their CNS regulatory functions; principally, the effect exerted by DARs on the kidney. Messenger RNA for all DAR isoforms was detected in the kidney, and it has been shown that all DARs have important roles in the regulation of renal function (Wang et al., 2008b, Zeng et al., 2008). Increase in renal blood flow and a direct inhibition of renal tubular sodium transport leading to the increase in sodium excretion are mainly modulated through D1-class receptors in the kidney most likely, D1R.

D1-class receptors have a contrasting effect on natriuresis compared to angiotensin receptors. D1-class receptor natriuresis is enhanced when angiotensin II secretion is reduced or when angiotensin II type 1 receptors (AT1R) are blocked but not that of AT2Rs (Chen and Lokhandwala, 1995, Salomone et al., 2007). Studies also showed that D2-class receptors have a natriuretic effect in the kidney, regulating DA

transporter activity, and renal DA production. In addition to the direct effect the D1-class receptors has on sodium excretion, D1R and D2R receptors seem to act synergistically to inhibit several sodium transporters such as Na^+-K^+ ATPase activity in renal tubules. D1-class receptors enhance the inhibitory effect of D2-class receptors, leading to the increase in sodium excretion (Eklof, 1997, Jose et al., 1998).

In vivo studies in both humans and animals demonstrated expression of D1-class and D2-class receptors in the adrenal medulla and adrenal cortex (Amenta et al., 1994). It has been observed that the plasma level of aldosterone was increased after the administration of D2R antagonist, which was blocked by DA administration. The observations from these studies suggested that there is a functional interaction between D2R and angiotensin II in regard to regulation of aldosterone secretion (Pivonello et al., 2007). DARs also seem to have a dual effect on catecholamine release. In stressful situations, for instance, DA has a stimulatory action mediated through D1R while D2R has a tonic inhibitory effect (Artalejo et al., 1990, Missale et al., 1998).

2.3.4 D1R-class knockout mice

The lack of pharmacological ligands with specific selectivity to one DAR over the others makes it difficult to distinguish between the physiological functions of different DARs. However, the ability to target specific gene changes with homologous recombination has provided a valuable tool to differentiate between DAR subtypes. The first DAR null mice were the D1R knockout (KO).

D1R KO mice have growth retardation and low survival rates due to the loss of fine motor control to pick up their food unless provided with easy access to palatable

food on the cage floor (Drago et al., 1994). The loss of fine motor control was in fact supported by different locomotor tests performed as these mice had a lower locomotor habituation in an open field test and impaired balance and motor coordination on the rotarod apparatus (Karasinska et al., 2000). Additionally, these mice exhibited reduced orofacial movements and reduced spontaneous grooming behaviors (Drago et al., 1999). These observations in D1R KO mice appear to confirm as a matter of fact the strong contribution of D1R in motor activity modulation. Also, the hyperactivity and hypoactivity effects of D1-class agonists and antagonists were attenuated in these KO mice. Furthermore, the hyperlocomotion effects of psychotomimetic, ketamine (NMDA-antagonist), were diminished in D1-KO mice (Miyamoto et al., 2001). Keeping in mind the importance of D1R for reward-related behaviors, hyperactivity, and phosphorylation of 32-kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) associated with acute and chronic psychostimulants administration was blunted in D1R-null mice (Becker et al., 2001, Svenningsson et al., 2003). The reinforcing properties of rewarding stimuli like ethanol consumption and sucrose also were compromised in these mice, which parallels the clinical effect of D1-class antagonists on cocaine addicts. Administration of D1-class antagonist abolished the euphoric effects associated with cocaine administration (El-Ghundi et al., 1998, Romach et al., 1999, El-Ghundi et al., 2003). D1Rs play essential roles in cognition, learning, and memory. These D1-null mice were reported to fail in performing such tasks as the Morris water maze test, which was correlated with the impairment of long-term potentiation (LTP) in the hippocampus and corticostriatal neurons lacking D1R (Matthies et al., 1997, Smith et al., 1998, Centonze et al., 2003). The LTP in the PFC was also compromised in D1 KO mice, represented in these mice by

deficits in fear extinction and reversal of learning in these animals (El-Ghundi et al., 2001, Huang et al., 2004).

In contrast to D1R, D5R has a very limited expression pattern confined mainly to the hippocampus. D5R KO mice appear to be healthy and develop normally with no motor abnormality, contrary to what was observed in D1R KO mice (Holmes et al., 2001, Hollon et al., 2002). However, these mice developed hypertension by three months of age, likely due to the CNS defect. This abnormality in blood pressure and increased sympathetic outflow are mediated via a defect in the interaction between D5R, oxytocin-dependent sensitization of vasopressin 1 receptors (V1R) and non-NMDA glutamatergic receptor-mediated pathways within the medulla (Hollon et al., 2002). Furthermore, a significant reduction in the extracellular level of Ach and a remarkable reduction in Ach release in response to D1-like agonist administration have been reported in these mice (Laplante et al., 2004). Lastly, D5R KO mice exhibited an antidepressant-like phenotype in a mouse model of behavioural despair (Holmes et al., 2001). This could be explained by the high level of expression of D5R in the hippocampus, which has a significant role in the pathophysiology of major depressive disorder (Ciliax et al., 2000, MacQueen et al., 2003).

2.3.5 D1-class receptor signaling

GPCR signaling is mediated mainly by coupling to different G-proteins. The various structural elements in each individual receptor determine their preferential G-protein coupling partner. For the purpose of this project, from here on, my focus will be on D1-class receptors, the principal element of my project. The classical scheme for D1-class receptor activation involves coupling to $G\alpha_{s/olf}$, production of the second messenger

cAMP upon activation of AC followed by activation of PKA (Kebabian and Calne, 1979). PKA is involved in regulation of gene expression and several downstream signaling pathways, such as phosphorylation of different PKA substrates including DARPP-32, cAMP response element-binding protein (CREB), and ion channels (Neve et al., 2004) **(Fig. 10)**.

2.3.5.1 D1-class receptor-induced regulation of DARPP32 activity

DARPP-32 is highly expressed in the MSNs and is known to modulate DARs signaling in the striatum. It is one of the main substrates activated by PKA. PKA phosphorylates DARPP-32 at Threonine 34 (Thr34), which activates the inhibitory function of DARPP-32 on protein phosphatase 1 (PP1). Inhibition of PP1 increases the phosphorylation of different neurotransmitter receptors such as glutamate receptors and ion channels, which aids in synaptic function and plasticity. The net result is that DARPP-32 participates in the amplification of PKA signaling and influences dopamine-mediated behaviours (Hemmings et al., 1984, Svenningsson et al., 2004, Fernandez et al., 2006, Agarwal et al., 2008).

In contrast, phosphorylation of DARPP-32 at Thr75 by cyclin-dependent kinase 5 (CDK5) prevents the inhibition of PP1 and dampens PKA activity (Bibb et al., 1999). DARPP-32 can also be phosphorylated by different casein kinases (CK). CK1 and CK2 phosphorylate DARPP-32 at serine 137 and serine 97, leading to reduction and enhancement of Thr34 phosphorylation by PKA, respectively. In contrast to the effect of CK2, PP2A dephosphorylates DARPP-32 at serine 97 resulting in the enhancement of gene expression in the striatum upon D1R stimulation (Stipanovich et al., 2008).

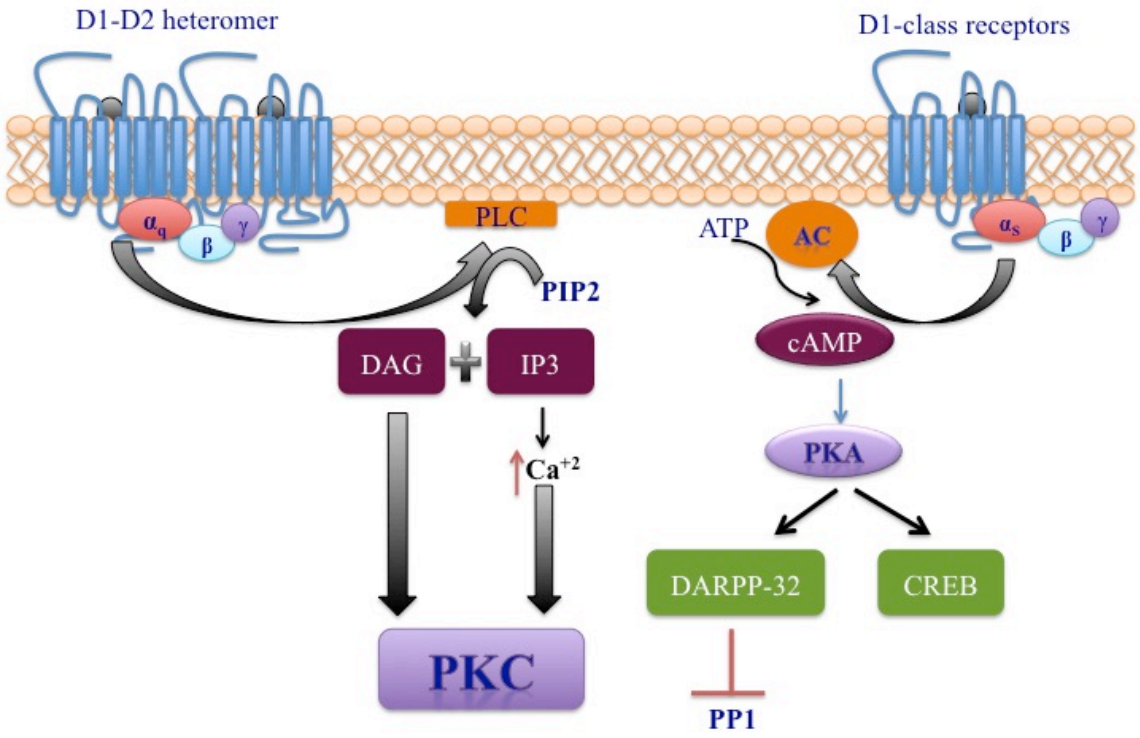


Figure 10. D1-class receptors signaling pathways.
Ligand binding activates D1-class receptors, which leads to coupling to $G\alpha_s$, followed by activation of AC and production of the second messenger cAMP. cAMP stimulates PKA; which phosphorylates DARPP-32; thus, activates the inhibitory function of DARPP-32 on PP1. PKA also phosphorylates CREB. Heterodimerization of D1R and D2R is another possible mechanism for D1R signaling. D1-D2 dimer regulates the coupling of D1R to $G\alpha_q$, which stimulates PLC. PLC catalyzes the hydrolysis of PIP_2 into DAG and IP_3. IP_3 increases the release of Ca^{2+} through activation of IP3 receptors on the ER. Ca^{2+} and DAG then activate the PKC.

2.3.5.2 D1-class receptor-induced modulation of ion channels

The activity of different voltage-gated and ligand-gated ion channels is regulated via activation of the PKA/DARPP-32 signaling pathway and numerous other signaling pathways. As previously mentioned, D1-class receptor activation leads to phosphorylation of DARPP-32, resulting in positive or negative regulation of various ion channels (Neve et al., 2004). In summary, D1-class receptors activation causes a reduction in the opening ability of the Na^{+2} channels; and therefore, provokes a decrease in the Na^{+2} current (Surmeier and Kitai, 1993, Cantrell et al., 1997). It also reduces K^{+} and Ca^{+2} currents through different types of K^{+} -inwardly rectifying channels and N, and P/Q type of calcium channels, respectively. In contrary, D1-class receptor activation increases the activity of L-type Ca^{+2} channels (Kitai and Surmeier, 1993, Surmeier and Kitai, 1993).

2.3.5.3 D1-class receptor regulation of CREB activity

CREB is another PKA substrate activated upon D1-class receptor activation. Activation of CREB follows stimulation of D1-class receptors and PKA (Konradi et al., 1994, Cole et al., 1995, Berke and Hyman, 2000, Johannessen et al., 2004). PKA-independent activation of CREB can also occur via activation of extracellular signal-regulated kinase (ERK) and activation of L-type Ca^{+2} channels (Liu and Graybiel, 1996, Brami-Cherrier et al., 2002, Johannessen et al., 2004). Phosphorylation-dependent activation of CREB through Ser133 regulates the interaction with gene CRE (cAMP response elements) and other transcriptional factors, both of which play a significant role in gene activation and synaptic plasticity (Berke and Hyman, 2000). The effect of CREB on gene expression in developing striatum has shown to be reversed by Ca^{+2}-sensitive

phosphatases like calcineurin (Liu and Graybiel, 1996).

2.3.5.4 Alternative D1-class receptor signaling pathway

Evidence from several studies suggested that D1-class receptors could couple to $G\alpha_q$ in addition to their classical coupling mechanism to $G\alpha_{s/olf}$ (Mahan et al., 1990, Lezcano and Bergson, 2002). However, the exact mechanism is not yet identified and is one of the most contentious signaling pathways for D1-class receptors. One possible mechanism is that the interaction of D1R with a protein called calcyon facilitates their coupling to $G\alpha_q$ but requires the cells priming with $G\alpha_q$ -linked GPCRs (Lidow et al., 2001, Lezcano and Bergson, 2002, Ali and Bergson, 2003, Bergson et al., 2003).

Heterodimerization of D1R and D2R is another possible mechanism, which regulates the coupling of D1R-class receptors to $G\alpha_q$. It has been shown that D1/D2 can form heterodimers in neurons and couple to $G\alpha_q$ leading to activation of PLC and increasing the release of intracellular Ca^{+2} (Ng et al., 2010) **(Fig. 10)**. Interestingly, in vitro expression of D1R in human embryonic kidney 293 (HEK293) cells did not induce any increase in the intracellular Ca^{+2} signaling even though a significant increase in Ca^{+2} signaling was seen when D5R expressed in the same cells. Some believe this effect can still be seen in D1-deficient mice but not in D5 KO mice. It was shown that the effect of PLC was completely abrogated in different brain tissues, hippocampus, and striatum of D5R-null mice, after treatment with DA and various D1-class selective agonists. This suggests that D5R might be the principal contributor to this signaling effect (Friedman et al., 1997, Sahu et al., 2009). In fact, it has been shown that D5/D2 dimer also exists, leading to coupling to $G\alpha_q$ followed by activation of PLC signaling pathway and increasing the Ca^{+2} release, although to a less extent than D1/D2 dimer (Hasbi et al.,

2010). However, controversy still exists regarding whether these receptors are coexpressed and in which neurons, as well as how Ca^{+2} release occurs.

2.3.6 D1-class receptor trafficking

D1-class receptor signaling and trafficking are regulated via the interaction with different cytoskeleton proteins, neurofilament-M (NF-M), gamma subunit of the coatomer protein complex (gamma COP), PSD-95, and different adapter/chaperone proteins such as the dopamine receptor interacting protein-78 (DRiP-78), calnexin, N-ethylmaleimide-sensitive factor (NSF) and sorting nexin-1 (SNX-1) (Wang et al., 2008a). For instance, NF-M and D1R were shown to coexpress in vivo, in the striatum, in the pyramidal cells, and in the interneurons within the frontal cortex. Notably, it was shown that NF-M reduces the expression of D1R at the cell surface, and increases its accumulation in the cytosol (Kim et al., 2002b). Furthermore, the interaction of the γ-subunit of gamma COP complex with the C-terminal of D1R in vitro led to D1R transport to the cell surface. Interestingly, trunctation made at the C-terminal of D1R has resulted in ER-retention of D1R (Bermak et al., 2002). Likewise, it has been shown that the D1R interacts with DRiP78, ER-membrane bound protein, through the carboxyl-terminal hydrophobic motif, $F(X)^3F(X)^3F$, which is present in D1R-class receptor and highly conserved among GPCRs (Bermak et al., 2001).

Calnexin is another chaperone protein that resides in the ER and modulates the trafficking and the quality control process for the newly synthesized receptors. Calnexin was shown to interact with D1R, regulating its delivery to the cell surface. Interestingly, a balanced level of DRiP-78 and calnexin seems to be required for optimal trafficking of D1R from the ER (Wang et al., 2008a). Moreover, NSF is involved in the post-endocytic

recycling of the receptor to the plasma mebrance and was shown to interact with the C-teminal of D1-class receptor while SNX-1 only interacts with D5R and is responsible for targeting the receptor to lysosomal degradation (Heydorn et al., 2004).

2.4 Structural characteristics and ligand-binding pocket of D1-class receptors

Structural analysis of different GPCRs pointed to some common similarities and differences between members of the same class and members of different GPCRs classes (Gingrich and Caron, 1993, Beaulieu and Gainetdinov, 2011). DARs have two cysteine residues in EL2 and EL3, which are believed to stabilize the receptor structure of GPCRs through the formation of an intramolecular disulphide bridge (Fraser, 1989, Missale et al., 1998). Ligand binding is likely to occur within the hydrophobic TM domains, as suggested by different mutagenesis studies and protein modeling of various catecholamine receptors like D2R and β2-adrenegic receptors. Several conserved residues inside the core of the ligand-binding pocket between TM2, TM3, TM5, and TM6 are potentially involved in ligand binding of catecholamines to their cognate receptors (Ulloa-Aguirre et al., 1999). Highly conserved aspartate residue in TM3 ($D^{3.32}$) is a critical residue for binding to the amine group of catecholamine side chain. Also, two conserved serine residues in TM5 ($S^{5.42}$, $S^{5.46}$) have been shown to form a hydrogen bond with the hydroxyl group of the catechol moiety for β2, α2, and D1R (Tomic et al., 1993, Kikkawa et al., 1997, Kristiansen, 2004). Additionally, phenylalanine residues in TM6 of D1R ($F^{6.51}$, $F^{6.52}$) have been shown to form a stable interaction with the aromatic moiety of catecholamines (Tomic et al., 1993, Floresca and Schetz, 2004). Aspartate, another conserved residue in TM2 ($D^{2.50}$), is also important for agonist binding and receptor

activation of β2, α2 adrenergic receptors, D1R, and D2R. These conserved residues are in fact conserved among catecholamine receptors and are known to be critical for ligand binding **(Fig 11)** (Ulloa-Aguirre et al., 1999).

Moreover, D1-class receptors are characterized by having a short IL3 and a very long CT regions, which is a common characteristic of receptors coupled to $G\alpha_s$ protein in contrast to D2-class receptors, which possess a very long IL3 and a short CT, and couple to $G\alpha_i$ protein. The CT region of D1-class receptors is about seven times longer than that of D2-class receptors (Strange, 1993). It is rich in serine and threonine residues and also contains cysteine residues, which constitute a common structural feature in all GPCRs (Missale et al., 1998). It has been shown that these cysteine residues seem to be palmitoylated in β-adrenergic receptors, which helps to anchor the CT to the membrane (O'Dowd et al., 1989). Additionally, D1R has shown to be palmitoylated at their CT at cysteine 347 (Cys347) and cysteine 351 (Cys351). Individual substitution of each of these two-cysteine residues to alanine reduced the level of D1R palmitoylation while dual substitution to alanine (A), Cys347---Cys351 to Ala347---Ala351, in fact, abolished the receptor palmitoylation completely (Ng et al., 1994, Jin et al., 1999)

2.5 D1-class receptor pharmacological properties and structurally related signaling differences

Although D1-class receptors share 80 % sequence identity in their TMs region, they exhibit different pharmacology profiles **(Fig. S1)**. D5Rs have ten times higher affinity for DA, than D1R, while antagonists show higher affinity at D1R than D5R (Sunahara et al., 1991, Tiberi et al., 1991). D1Rs have been shown to exhibit higher

affinity for non-benzazepine antagonists like flupenthixol and (+) butaclamol than D5R. Some antagonists have been shown to exhibit a more profound negative efficacy at D5R than at D1R, which could be explained by the high level of basal activation of D5R (Tiberi and Caron, 1994, Demchyshyn et al., 2000, Zhang et al., 2014).

Interestingly, it has been reported that D5R expression in different cell lines has a higher basal level of AC activation than cells expressing D1R, but agonists induced maximal activation was higher for D1R than D5R (Tiberi and Caron, 1994, Missale et al., 1998). Considering these distinctive features, D5R has been proposed to behave as a naturally occurring constitutively active mutant (CAM) form of the D1R similar to mutants obtained by site-directed mutagenesis of adrenergic receptors (Lefkowitz et al., 1993). IL1 and IL2 are highly conserved between D1R and D5R. Notwithstanding the fact that the IL3 and the CT of D1-class receptors are similar in size, their amino acid sequence is different. Interestingly, several mutagenesis studies of the IL3 and the CT have elucidated the importance of these domains for G-protein coupling and binding properties of GPCRs. Through the years, our lab and other labs have used a chimerical and site-directed mutagenesis approaches to delineate the structural determinants responsible for the distinctive coupling and pharmacological profile between the different DAR subtypes. As a matter of fact, it has been demonstrated that intramolecular interactions between different intracellular and extracellular regions of D1R and D5R are responsible for bestowing various functional aspects to these two subtypes (Zhang et al., 2014).

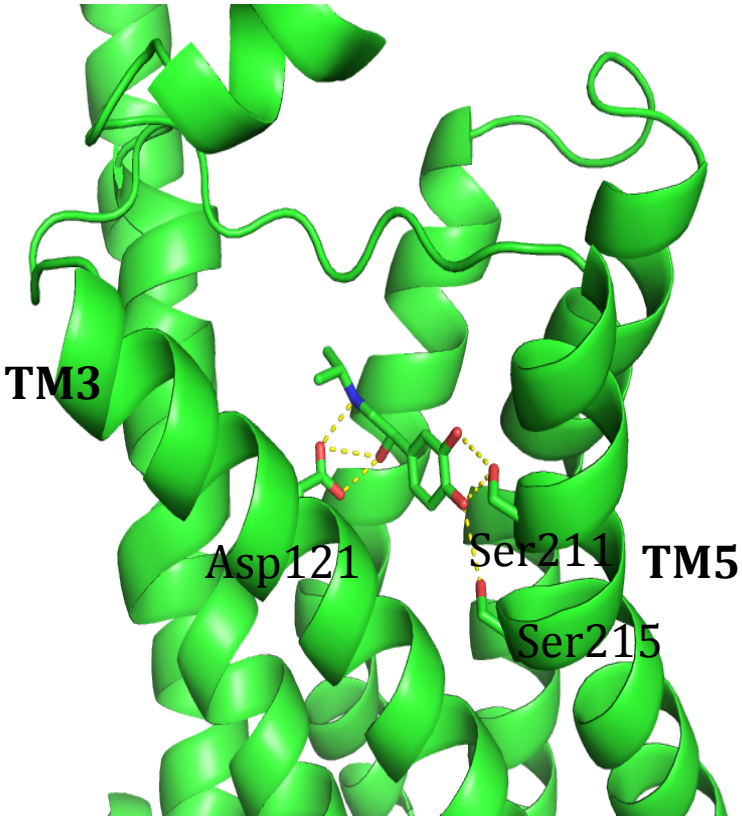


Figure 11. Crystal structure of turkey β1-adrenergic receptor bound to isoprenaline. Isoprenaline interacts with Asp121 in TM3, Ser 211 and Ser 215 in TM5.

Site-directed mutagenesis studies showed that mutations performed on the IL3 of β2 and α1- adrenergic receptors have resulted in constitutively active phenotypes, suggesting that the C-terminal end of the IL3 of adrenergic receptor acts to constrain the receptor in the inactive conformation in the absence of agonists (Cotecchia et al., 1990, Kjelsberg et al., 1992, Ren et al., 1993, Samama et al., 1993). By keeping that evidence in mind and comparing the C-terminal end of the IL3 of D1R with that of D5R, one observes that the only differences in this region between the two receptors are two amino acids residues located at the cytoplasmic end of TM6 of D1R (Phe264, Arg266) and D5R (Ile288, Lys290) **(Fig. S2**). To elucidate the role played by these residues, Charpentier et al. (1996) swapped these two residues between D1R and D5R using site-directed mutagenesis. Interestingly, swapping Phe264 and Ile288 between D1R and D5R resulted in a partial recapitulation of D1R and D5R phenotype in terms of receptor affinity to DA, agonist-independent (basal) activity, and DA potency. The partial swap in receptor functionality (basal activity, agonist affinity, and potency) between D1-class receptors has suggested the involvement of other receptor regions/residues in modulating these functions. On the other hand, Arg266/Lys290 replacement had no effect on either receptor (Charpentier et al., 1996).

In order to further investigate how various structural elements of the receptor could modulate the pharmacological properties of different receptor subtypes, our lab has constructed chimeras between the D1R and D5R exchanging a region referred to as terminal receptor locus (TRL). TRL includes the TM6, EL3, TM7 and CT regions. D1-TRL5 and D5-TRL1 chimeras showed identical phenotype to D5R and D1R, respectively. Only agonist-induced maximal activation of AC of D1-TRL5 and D5-TRL1

chimeras remained unchanged relative to the D1R and D5R wild type, respectively. To gain further insight, two additional chimeras were generated in which only EL3 was exchanged. Interestingly, EL3 chimeras moderately modulated the receptor basal activity but provoked a full swap in the agonist-induced maximal activation of AC contrary to the results seen with TRL chimeras (Iwasiow et al., 1999). The results of this study suggest that different behaviors adopted by various DARs are a product of the interplay between the intracellular and the extracellular domains of the receptors. As a follow-up to this study, our lab has investigated the role of the CT in D1R conformation and G-protein coupling. Swapping the CT of D1R with that of D5R resulted in a complete exchange of D1R binding profile to DA, recapitulating the effect seen with D5R, and vice versa (Jackson et al., 2000), which was confirmed by another group (Demchyshyn et al., 2000). Using the mutational analysis approach, this study pointed to a particular motif within the CT tail of D5R, Gln439, which is responsible for the receptor high agonist affinity without changing the receptor constitutive activity or intrinsic efficacy (Demchyshyn et al., 2000).

Furthermore, the role of the IL4 referred to as the cytoplasmic helix 8 connecting TM7 and CT tail of D1-class receptors has been investigated. Swapping the IL4 between D1R and D5R has resulted in a reverse phenotype recapitulating the parent receptor in regard to DA affinity and basal activation (Tumova et al., 2004). Although these studies help to shed some light on important structural domains regulating ligand binding, G-protein coupling, and selectivity, the role of the IL3 of D1-class receptors remains to be fully appreciated. In fact, it is uncertain if previous studies done with other GPCRs can be generalized to D1R and D5R mainly due to the considerable degree of diversity in the

IL3 structure among GPCRs.

3. The third intracellular loop

The importance of the IL3 for G-protein coupling and specificity has long been acknowledged; however, the ambiguity regarding its role and the involvement of specific motifs in regulating agonist affinity/potency, basal activity, and ligand-mediated maximal activation is still unclear. Studies on the role of IL3 have mainly focused on stretches of residues forming the cytoplasmic extension of TM5 and TM6 while less attention has been given to the IL3 central region. Herein, the IL3 central area is defined as the amino acid stretch joining the extension of TM5 and TM6 **(Fig. 12)**.

It is worth mentioning here that TM5 and TM6 cytoplasmic extensions are conserved (Couvineau et al., 2003, Kuniyeda et al., 2007, Bockenhauer et al., 2011, Zhou et al., 2013). However, the exact role played by the cytoplasmic extension of TM5 and TM6 in regard to G protein coupling, selectivity, and receptor activation is still controversial. On the one hand, several mutagenesis studies done with β_2 adrenergic receptor pointed to the cytoplasmic extension of TM5 as a key for G protein coupling while cytoplasmic extension of TM6 as a key for receptor activation (Dixon et al., 1987, Strader et al., 1987, Hausdorff et al., 1990, Liggett et al., 1991). Additionally, the $G\alpha_s$ coupling domain for α_{2A} adrenergic receptor was shown to localize adjacent to the TM5-cytoplasmic extension, within a stretch of eleven amino acids located at the far proximal side of the IL3 (Eason and Liggett, 1995). In concurrence with these results, a study using chimeras for angiotensin receptors, AT1R/AT2R suggested that the N-terminal end of the IL3 is essential for $G\alpha_q$ coupling and activation (Wang et al., 1995). Another study on the

rat AT1A receptor also suggested that the N-terminal end of the IL3, adjacent to TM5, is responsible for $G\alpha_q$ coupling, while the C-terminal end of the IL3, adjacent to TM6, is important for G-protein selectivity and activation (Franzoni et al., 1999). On the other hand, other mutagenesis studies claimed that TM5 and TM6 cytoplasmic extensions by themselves are not sufficient to mediate G-protein coupling and receptor activation, and other structural elements of the receptor are required for a full state of receptor activation and G-protein coupling to take place (Voss et al., 1993). Notably, other studies have suggested that the central region of the IL3 may have a permissive role in modulating the function of TM5 and TM6 extension (Green and Liggett, 1994, Franzoni et al., 1999, Kim et al., 2002a, Chaipatikul et al., 2003, Bongers et al., 2007, Chee et al., 2008). As a matter of fact, the role of the central region of the IL3 was shown to be essential for G protein coupling, selectivity, and receptor activation.

For instance, the IL3 has been shown to stabilize the NPY1 receptor in the inactive confirmation (Chee et al., 2008). Naturally occurring single point mutation P230L located in the middle of the IL3 of melanocortin 4 receptor, in the obese population, was shown to be responsible for the receptor constitutive activation (Kim et al., 2008). In contrast, others proposed that the IL3 has no role in modulation of G-protein coupling and activation, and suggested that it can even be substituted or completely deleted without having an impact on ligand binding or receptor activation (Wang et al., 1995, Nielsen et al., 1998).

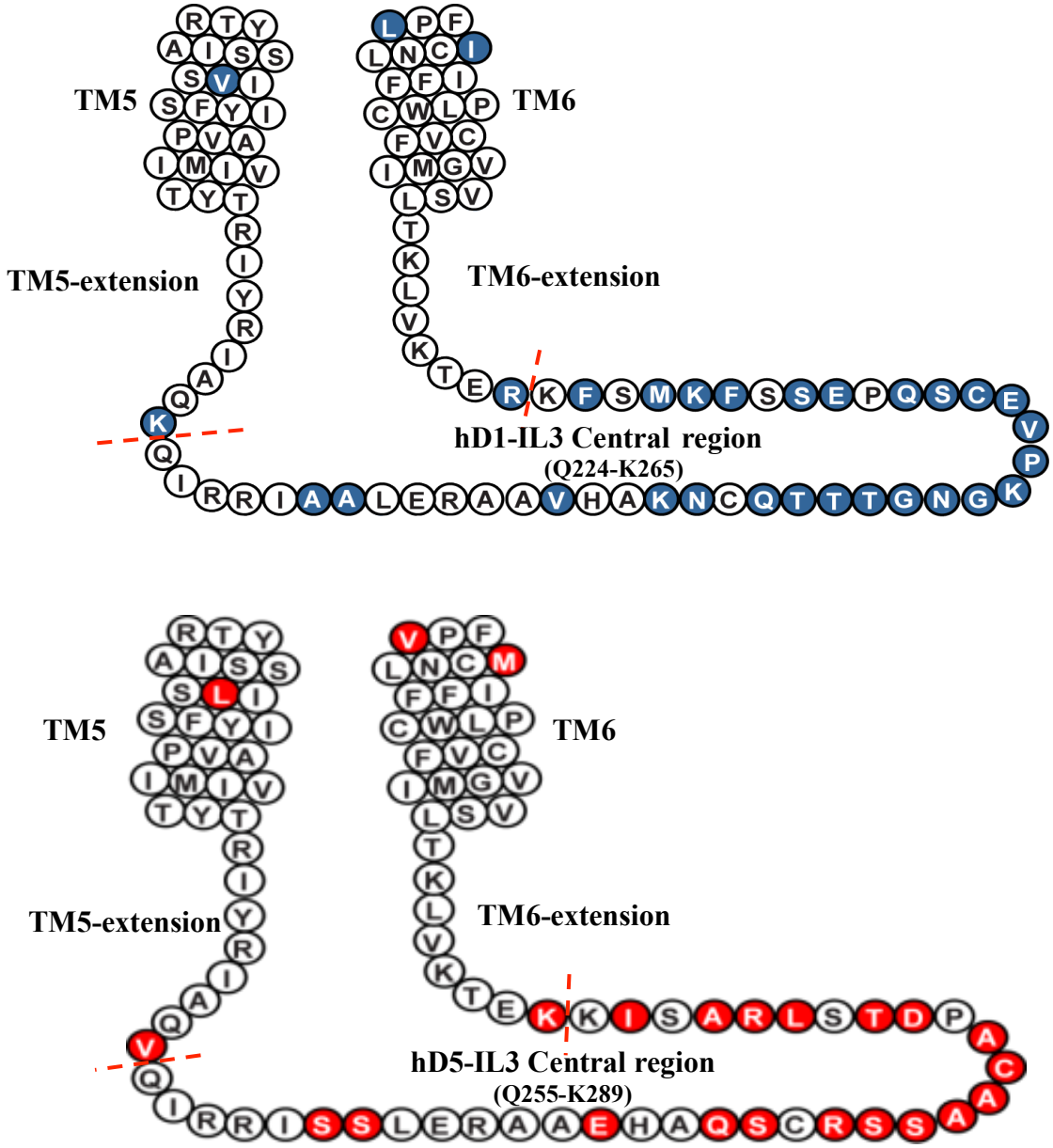


Figure 12. Schematic representation of the IL3-central region of hD1-class receptors. White circles represent conserved residues, and colored circles represent divergent residue. The IL3-central region is delimited by dashed lines.

Crystallization of GPCRs will likely facilitate the drug discovery process, notably by increasing the knowledge of how ligands bind to GPCRs and mediate signal transduction. However, it remains unclear how the central region of the IL3 can modulate G protein coupling property through the analysis of currently available crystal structures of GPCRs. Indeed, while crystallization of several GPCRs has been recently accomplished, the high-resolution of receptor 3D structures harboring an intact IL3 and CT regions remains challenging for several reasons. Among them is the structural instability of solubilized membrane-bound GPCR proteins, mainly due to the presence of the IL3 and CT regions. The IL3 and CT are very flexible regions of GPCRs, which has interfered with the determination of the folding conformation of solubilized receptors. Therefore, the use of site-directed mutagenesis to study the structure-activity relationships underlying the IL3 central region remains an alternative and compelling experimental strategy.

Several approaches have been used recently to facilitate the crystallographic process of different membrane proteins like the human β2-adrenergic receptor, adenosine 2A receptor (A_{2A}), chemokine receptors type 4 (CXCR4), D3R, turkey β1-adrenoceptor, and more recently opioid and neurotensin receptors (Cherezov et al., 2007, Rasmussen et al., 2007, Warne et al., 2008, Baker et al., 2011, Lebon et al., 2011, Granier et al., 2012, Manglik et al., 2012, White et al., 2012, Wu et al., 2012, Scott and Pluckthun, 2013, Egloff et al., 2014). These approaches include: Alanine-scanning mutations to help GPCR thermostabilization, replacement of the IL3 loop with T4 lysozyme fusion proteins to stabilize the GPCR structure or the use of antibody fragment against the IL3 (Kobilka and Schertler, 2008, Warne et al., 2008, Kobilka, 2011, Lebon et al., 2011, Rasmussen et

al., 2011a, Steyaert and Kobilka, 2011, Scott and Pluckthun, 2013). Although these approaches have unraveled the high resolution of the three-dimensional structure of GPCRs, the IL3 structure could not be predicted. This is explained by the IL3 remaining unstructured and disordered in thermostabilized GPCRs, or being deleted (T4 replacement approach), or being shortened to facilitate the crystallization process (Kobilka, 2011 and Baker et al., 2011).

Overall, these aforementioned studies and many others point to a pivotal role of the IL3 in controlling inactive and active GPCR conformations. Notwithstanding the fact that crystal structure studies have enriched our knowledge regarding the different states of GPCR activation, the paucity of information made available by these studies have not fully uncovered the structural determinants of the IL3 required for receptor activation and G protein coupling yet. Importantly, even if the IL3 is successfully resolved through crystallographic studies of few GPCRs, findings may not be generalizable to the whole GPCR family due to the high degree of diversity seen in the IL3 length and amino acid content of different GPCRs.

In light of this, further research is needed to pinpoint the exact point of interaction between the receptor and the G protein and how these two are oriented relative to each other. More focused research is also needed to further explain how different structural determinants within the IL3 region produced different receptor phenotypes. Additionally, it will be important to study how these different phenotypes would impact the receptor's ability to couple to a specific G protein and to exert different pharmacological effects depending on GPCR binding to different ligands.

4. Rationale, hypothesis, and objectives

As discussed above, dysfunction of DAR signaling has been implicated in many disease states affecting motor and cognitive functions such as Parkinson's, schizophrenia, Huntington's disease, and drug addiction (Papakostas, 2006, Iversen and Iversen, 2007, Tang et al., 2007, Seeman, 2009, Beaulieu and Gainetdinov, 2011, Porcelli et al., 2011). For Parkinson's disease, the only efficient pharmacological treatment is the use of DA precursor, L-dopa. However, the one long-term side effect associated with the use of L-dopa is LID (abnormal involuntary movements), which has been proven to be linked to a hypersensitized human D1R (hD1R) in the striatum (Feyder et al., 2011, Herve, 2011, Murer and Moratalla, 2011).

The broad brain distribution of hD1R and its involvement in many pathological states make them an attractive drug target for LID and other brain disorders. Therefore, high demand is urged for a more focused research to deepen our knowledge regarding the role of the IL3 and potentially shed the light on a critical motif (residue/residues) involved in DAR activation and signaling. Consequently, this will assist in the process of designing subtype-specific drugs and improving drug target selectivity. Perhaps, this can be done by an allosteric modulation of receptor intracellular domains or through the development of subtype selective drug, which are currently viewed as promising therapeutic approaches that may help to reduce the side effects associated with classical treatments.

The development of subtype-selective drugs to differentiate between the DARs has been hampered, most importantly, by the high level of identity between the TM domains of DARs where the key ligand binding sites are found and fully conserved

(Sunahara et al., 1990, Sunahara et al., 1991, Tiberi et al., 1991, Tiberi and Caron, 1994, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). Notably, the functional phenotype differences between hD1-class receptors have been mostly conferred by the IL3 and CT, which are the most divergent between hD1R and hD5R (Iwasiow et al., 1999, Demchyshyn et al., 2000, Jackson et al., 2000, Tumova et al., 2003). For instance, residues located within the IL3, adjacent to the cytoplasmic extension of TM6, have been shown to be critical for the D1-class receptors basal level of activation; however, these residues were not sufficient to explain the different D1R and D5R phenotypes. This suggests the involvement of other structural determinants within the receptor (Charpentier et al., 1996).

It is intriguing to note that a differential regulation of D1-class subtypes can be influenced by the presence of specific residues located within the IL3 central region (Thompson and Whistler, 2011, Plouffe et al., 2012). However, the structural determinant highlighting the function of the IL3 central region, and its involvement in G protein coupling of D1-class receptors is unknown yet.

Preliminary studies in our lab were done to understand the role of the IL3 central region for D1-class receptor activation and signaling. Using a truncation approach of the entire IL3 of human D1-class receptors rendered a non-functional receptor, as no receptor expression was detected in transfected HEK293 cells. This unsuccessful approach was followed by another approach of engineering two truncations dividing the IL3 central region of hD1R and hD5R into two segments relying principally on the primary structure of the receptor. Specifically, the IL3 central region of D1-class receptors consists of the N-terminal (hD1/Q224-T245; hD5/Q255-S275) and the C-terminal (hD1/G246-K265;

hD5/A276-K289), which are the most conserved and divergent IL3 portions between D1R and D5R, respectively **(Fig. 13)**. This preliminary study has pointed to the importance of the IL3 central region. The IL3/N-terminal portion seems to be critical for promoting DAR conformation toward agonist-independent and dependent activation. In contrast, the IL3/C-terminal region appears to play a role in restraining the receptor in the inactive state (Charrette and Tiberi, unpublished).

The results of our recent study suggest an important role of the IL3 central region for the regulation of ligand binding, agonist-independent and agonist-dependent activity of hD1-class receptors. An important issue raised here is whether changes in agonist affinity and G-protein coupling properties are explained by a size reduction of the IL3 central region created by truncation, or points to the presence of key structural motif/motifs controlling agonist binding and receptor activation.

I hypothesize:

1. The functional properties of hD1-class receptors are regulated via a molecular micro-switch present within the IL3 central region modulating the functional properties of the receptor distinctly.

2. The functional differences between D1R and D5R require structural elements from both N- and C-terminal halves of the IL3 central region.

3. The molecular interplay between the N and C-terminal halves of the IL3 central region is dependent on the amino acid chain length and content.

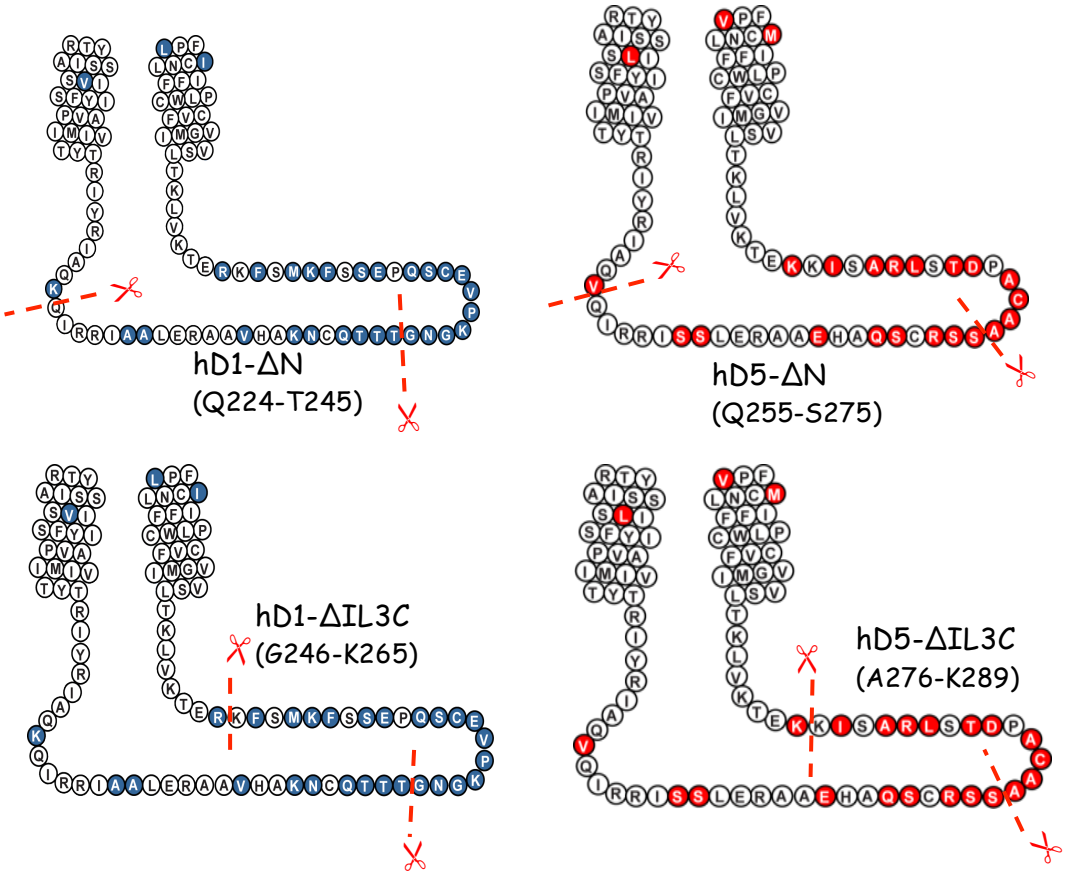


Figure 13. Schematic representation of deletion mutant forms of hD1-class receptors made at the IL3-central region.
Four deletion mutants were generated by PCR and referred to as hD1-ΔN, hD5-ΔN, hD1-ΔIL3C, hD5-ΔIL3C, respectively

Objectives

1. To explore the impact of amino acid chain length and content of the IL3/N-terminal moiety on the human D1R-class functionality (receptor expression, ligand binding, and G-protein coupling). This will be investigated by reducing the number of amino acid residues using a deletion approach and by an alanine replacement approach.

2. To delineate the structural components within the IL3/C-terminal region involved in hD1R functionality using incremental deletions and alanine mutations of stretches of amino acid residues in this region.

3. To identify the presence of a structural motif within the distal region of the IL3/C-terminal moiety using single-point deletion and single-point alanine-scanning mutagenesis approaches.

Chapter 2

MATERIALS AND METHODS

Materials

Ascorbic acid, dopamine, *cis*-flupenthixol, thioridazine, (+)-butaclamol, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Dihydrexidine hydrochloride was from Tocris Bioscience (Ellisville, MO, USA). Fetal Bovine Serum (FBS), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, gentamycin, and trypsin- were from Invitrogen (Burlington, ON, Canada). Minimal essential media (MEM) and phosphate buffer saline (PBS) were obtained from Wisent Bioproducts (Saint-Bruno, QC, Canada). [^{3}H]-adenine and [^{3}H]-SCH23390 were purchased from Perkin Elmer (Boston, MA, USA). [^{14}C]-cAMP was bought from Moravek Biochemicals (Brea, CA, USA). Biosafe II scintillation cocktail was from Research Products International Corp (Mt. Prospect, IL, USA). Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories Inc. (Mississauga, ON, Canada). Paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Expand High Fidelity Taq DNA polymerase was from Roche Diagnostics (Laval, QC, Canada). Restriction enzymes used in generating DNA constructs were acquired from Fermentas Life Sciences (Burlington, ON, Canada) and New England Biolabs (Pickering, ON, Canada). Primers used to generate the mutant receptors were custom made at Sigma-Aldrich (Oakville, ON, Canada). Monoclonal anti-flag M2 antibody was purchased from Invitrogen (Burlington, ON, Canada). Sheep anti-mouse antibody conjugated to horseradish peroxidase was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA).

Methods

1. Construction of mutant receptors

1.1 Construction of the IL3/N-terminal deletion and alanine mutant forms of hD1R-class receptors for Section 1 of the results

The IL3-central region of hD1R and hD5R was divided into two halves. The N-terminal halves (hD1/Q224-T245, hD5/Q255-S275) were given the following names; hD1-ΔN and hD5-ΔN **(Fig. 12)**. The C-terminal halves (hD1/G246-K265, hD5/A276-K289) were given the following names; hD1-ΔIL3C and hD5-ΔIL3C **(Fig. 12)**. Each N-terminal half was further divided into another two moieties for each receptor. The two N-terminal halves of hD1R are Q224-A235 (hD1-N1) and V236-T245 (hD1-N2), which were either deleted or mutated to alanine, were given the following names; hD1-ΔN1, hD1-N1A, hD1-ΔN2, and hD1-N2A **(Fig. 13A)**. The two N-terminal halves of hD5R are Q255-A266 (hD5-N1) and E267-S275 (hD5-N2), which were either deleted or mutated to alanine, were given the following names: hD5-ΔN1, hD5-N1A, hD5-ΔN2, and hD5-N2A **(Fig. 13B)**. hD1-ΔN1, hD1-N1A, hD1-ΔN2, hD1-N2A, hD5-ΔN1, and hD5-ΔN2 constructs were generated using the polymerase chain reaction (PCR)-based overlap extension strategy as previously described (Plouffe et al., 2010). Briefly, two PCR products (megaprimers), A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively. An overlapping region between A and B allowed the amplification of the final PCR product using primers P5 and P6 **(Table 1A, 1B)**. PCR reactions were carried out using Expand High Fidelity Taq DNA as follows: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 50°C for 1

min, 72°C for 1 min), completed by an anneal extension step at 72°C for 8 min. Overlap PCR reactions were done with an equimolar amount of purified P1-P2 and P3-P4 product using the following conditions: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 10 min). At the end of the cycle, P5 and P6 primers were added to the mixture and the final product was generated as follows: 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min), completed by an anneal extension step at 72°C for 8 min. PCR products were separated on 1% agarose gel, excised, and purified on QIAEX resin (Qiagen). Then, the purified PCR products were digested with *Hind*III/*Xba*I for hD1-mutants and *BsmI/EagI* for hD5 mutants, and the purified DNA bands products were subcloned into the hD1R-pCMV5 and hD5R-pCMV5 expression construct linearized with *Hind*III/*Xba*I and *BsmI/EagI* for hD1R and hD5R mutants, respectively. The integrity of mutants in pCMV5 was confirmed by automated fluorescent DNA sequencing done at the StemCore Laboratories of the Ottawa Hospital Research Institute (Ottawa, ON, Canada, www.stemcore.ca). hD5-N1A and hD5-N2A were custom made by gene synthesis into pUC57 plasmid vector (GenScript USA Inc., NJ, USA). Custom-made hD5-IL3N1A cassette subcloned into *EcoRI/XbaI* of pUC57 was used to generate the D5-N1A cassette. To produce the D5-N2A, custom-made D5-IL3N2A cassette subcloned into *EcoR I/Hind III* of pUC57 was used to obtain the DNA cassette. DNA cassettes were then subcloned into hD5R-pCMV5 expression plasmid linearized with *BsmI/EagI* to give hD5-N1A and hD5-N2A in PCMV5. The sequence of the engineered receptors in pCMV5 was verified by automated fluorescent DNA sequencing done at the Centre de Recherche du CHUL (CHUQ) of Laval University.

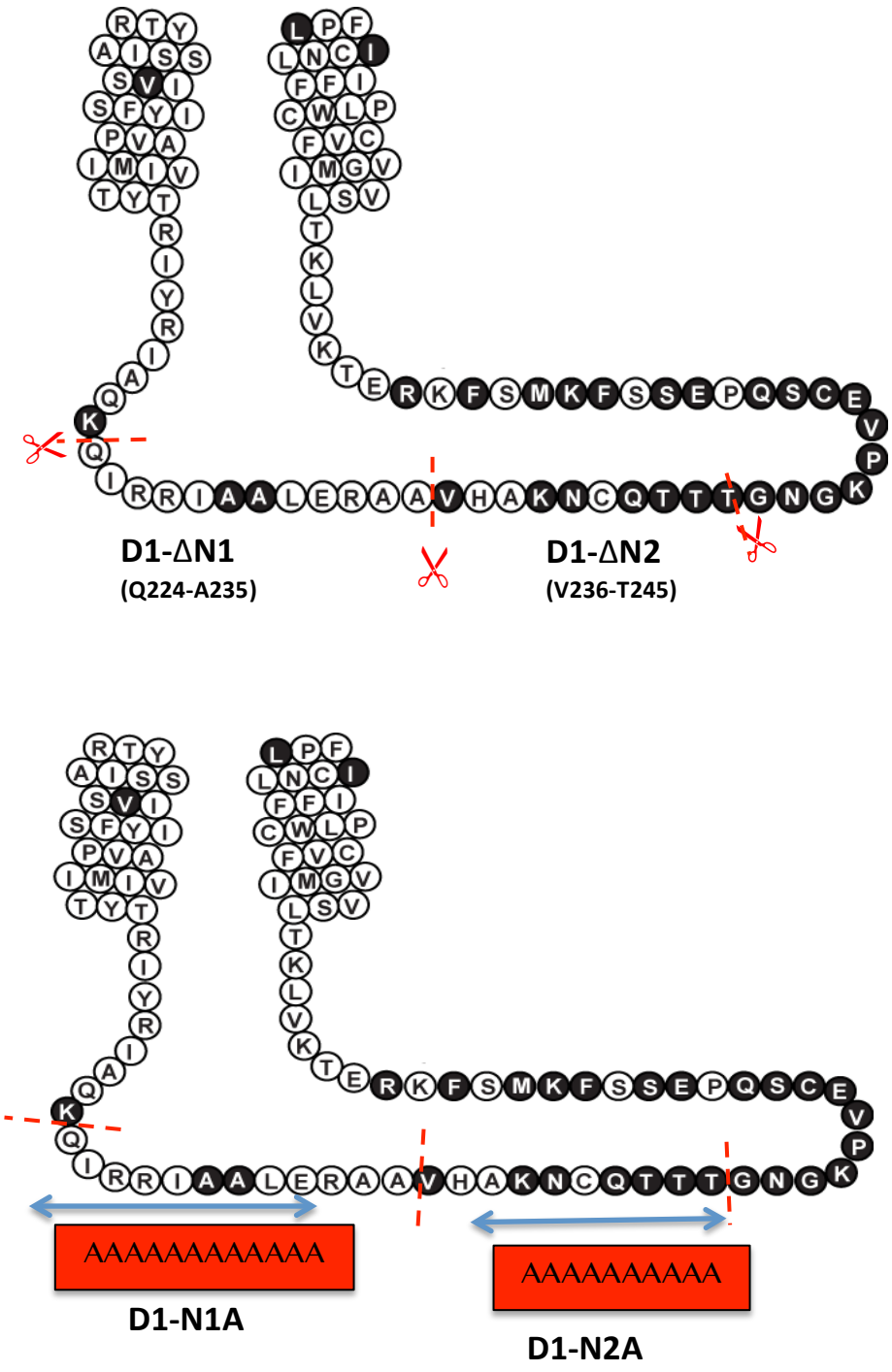


Figure 13A. Schematic representation of the IL3/N-terminal deletion and IL3/N-terminal alanine mutant forms of hD1 receptor.
Deletion (D1-ΔN1, D1-ΔN2) and alanine (D1-N1A, D1-N2A) mutant forms of hD1R are delimited by scissors or by dashed lines, respectively.

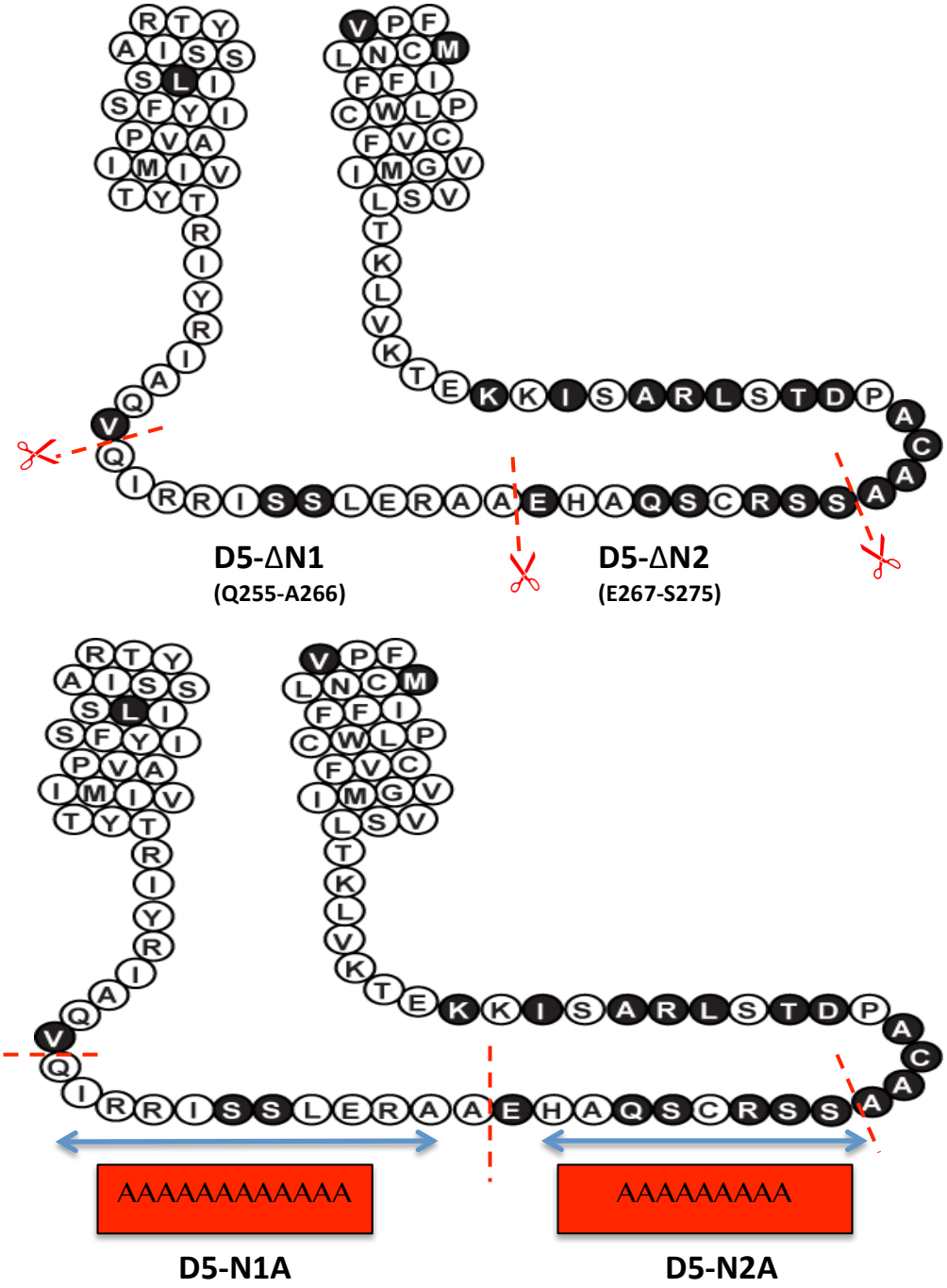


Figure 13B. Schematic representation of the IL3/N-terminal deletion and IL3/N-terminal alanine mutant forms of hD5 receptor.
Deletion (D5-ΔN1, D5-ΔN2) and alanine (D5-N1A, D5-N2A) mutant forms of hD5R are delimited by scissors or by dashed lines, respectively.

Table 1A. Sequences of oligonucleotide primers for construction of the IL3/N-terminal deletion and IL3/N-terminal alanine mutant forms of hD1 receptor using a polymerase chain reaction-based overlapping approach

Constructs	Primers Sequences (5'➔3')
hD1-ΔN1	P1: TTCATCCCAGTGCAGCTC
	P2: ATTCTTGGCGTGGACTTTCTGCGCAATCCTGTAGATCCTGGTGTA
	P3: ATTGCGCAGAAAGTCCACGCCAAGAATTGCCAGACCACCACAGGT
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
hD1-ΔN2	P1: TTCATCCCAGTGCAGCTC
	P2: TTTGAAGGACATTTTAAAAGAACTTTCCGGTTGAGAACATTCGACAGGC TTTCCATTACCTGCTGCCCTCTCCAAGGCCGC
	P3: GAGAGGGCAGCAGGTAATGGAAAGCCTGTCGAATGTTCTCAACCGGAA AGTTCTTTTAAAATGTCCTTCAAAAGAGAAACT
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
hD1-N1A	P1: TTCATCCCAGTGCAGCTC
	P2: ATTCTTGGCGTGGACTGCTGCGGCTGCAGCGGCCGCAGCTGCCGCGGC TGCTTTCTGAGCAATCCTGTAGATCCTGGTGTA
	P3: ATTGCTCAGAAAGCAGCCGCGGCAGCTGCGGCCGCTGCAGCCGCAGC AGTCCACGCCAAGAATTGCCAGACCACCACAGGT
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
hD1-N2A	P1: TTCATCCCAGTGCAGCTC
	P2: TTCCGGTTGAGAACATTCGACAGGCTTTCCATTACCTGCCGCGGCAG CTGCAGCCGCGGCTGCAGCTGCTGCCCTCTCCAAGGC
	P3: CAAATACGGCGCATTGCGGCCTTGGAGAGGGCAGCAGCTGCAGCCG CGGCTGCAGCTGCCGCGGCAGGTAATGGAAAGCCTGTC
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA

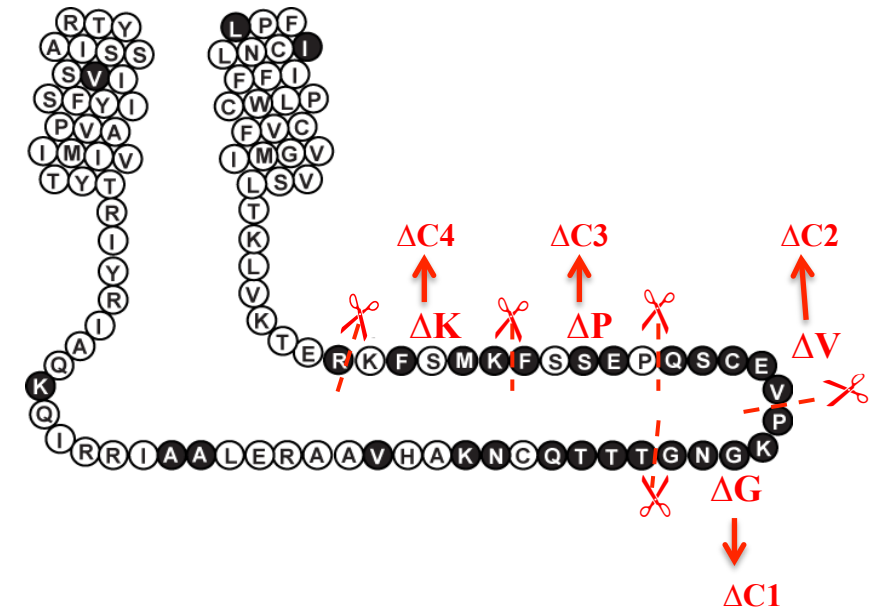
Table 1B. Sequences of oligonucleotide primers for construction of the IL3/N-terminal deletion mutant forms of hD5 receptor using a polymerase chain reaction-based overlapping approach

Constructs	Primers Sequences (5'➔3')
hD5-ΔN1	P1: TACGGTGGGAGG
	P2: CGCGCAGGCAGCGCTGCTCCGGCAGCTCTGCGCGTGCTCCACCTGGG CGATGCGGTAGATGCG
	P3: ATCGCCCAGGTGGAGCACGCGCAGAGCTGCCGGAGCAGCGCTGCCT GCGCGCCCGACACC
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
hD5-ΔN2	P1: TACGGTGGGAGG
	P2: GCTGGTGTCGGGTGCGCAGGCTGCTGCGGCCCTCTCCAGGGAGGA
	P3: GAGAGGGCCGCAGCAGCCTGCGCACCCGACACCAGCCTGCGCGCT
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA

1.2 Construction of IL3/C-terminal deletion and alanine mutant forms of hD1R for Section 2 of the results

Mutant hD1R constructs were designed using a PCR overlap extension strategy. The hD1R subcloned in the mammalian expression vector pCMV5 was used as PCR template. To produce the hD1R-IL3 mutants, the distal region of the IL3/C-terminal, G246-K265, was divided into four moieties (5 amino acids each). These four moieties were either deleted or mutated to alanine and were referred to as ΔC1, ΔC2, ΔC3, ΔC4, C1A, C2A, C3A, and C4A **(Fig. 14A, 14B)**. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively. An overlapping region between A and B allowed the amplification of the final PCR product using primers P5 and P6 **(Table 2, Table 3)**. PCR reactions were carried out as mentioned in 1.1. Flag epitope peptide (DYKAAAAK) was used to tag D1R at the N-terminus, taking advantage of the already-generated mutants and using a Flag-tagged hD1R expression plasmid DNA as a template.

A.



B.

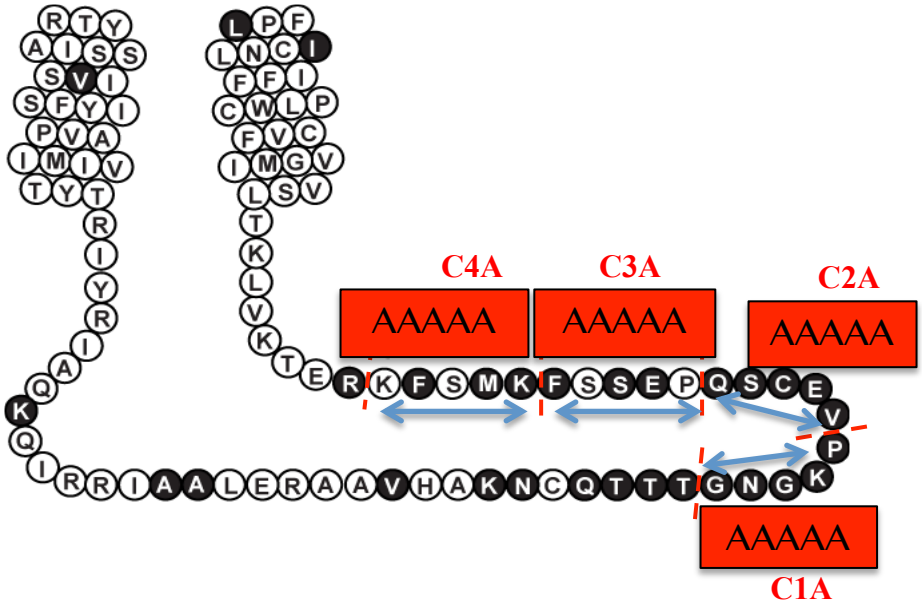


Figure 14. Schematic representation of the IL3/C-terminal deletion and alanine mutant forms of hD1R.

A. Deletion mutants within the hD1-IL3C distal region are shown. Deleted regions of the ICL3 are defined by scissors. **B.** Alanine mutants within the hD1-IL3C distal region are shown. Alanine-mutated regions of IL3 are defined by dashed lines. C1: G246-P250, C2: V251-Q255, C3: P256-F260, C4: K261-K265.

Table 2. Sequence of oligonucleotide primers for the construction of the IL3/C-terminal deletion mutant forms of hD1R using a polymerase chain reaction-based overlapping approach.

Constructs	Primers Sequences (5'➔ 3')
ΔC1	P1: TTCATCCCAGTGCAGCTC
	P2: TCTTTTGAAGGACATTTTAAAAGAACTTTCCGGTTGAGAACATTCG ACTGTGGTGGTCTGGCAATTCTT
	P3: CAGACCACCACAGTCGAATGTTCTCAACCGGAAAGTTCTTTTAAA ATGTCCTTCAAAAGA
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔC2	P1: TACGGTGGGAGG
	P2: TCTTTTGAAGGACATTTTAAAAGAACTTTCCGGAGGCTTTCCATTA CCTGTGGT
	P3: AATGGAAAGCCTCCGGAAAGTTCTTTTAAAATGTCCTTCAAAAGA
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
ΔC3	P1: TTCATCCCAGTGCAGCTC
	P2: GACTTTAGTTTCTCTTTTAAAGGACATCTTTTGAGAACATTCGACAG GCTT
	P3: GAATGTTCTCAAAAGATGTCCTTTAAAAGAGAAACTAAAGTC
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔC4	P1: TACGGTGGGAGG
	P2: GATCACCGACAGAGTCTTGAGGACTTTAGTTTCTCTAAAAGAACTTTC CGGTTGAGA
	P3: GAAAGTTCTTTTAGAGAAACTAAAGTCCTCAAGACTCTGTCGGTGATC
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA

Table 3. Sequence of oligonucleotide primers for the construction of the IL3/C-terminal alanine mutant forms of hD1R using a polymerase chain reaction-based overlapping approach.

Constructs	Primers Sequences (5'➔3')
C1A	P1: TACGGTGGGAGG
	P2: GATCACCGACAGAGTCTTGAGGACTTTAGTTTCTCTAAAAGA ACTTTCCGGTTGAGA
	P3: GAAAGTTCTTTTAGAGAAACTAAAGTCCTCAAGACTCTGTCG GTGATC
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
C2A	P1: TTCATCCCAGTGCAGCTC
	P2: AGAACATTCGACAGCTGCTGCAGCAGCTGTGGTGGTCTGGCA ATTCTT
	P3: CAGACCACCACAGCTGCTGCAGCAGCTGTCGAATGTTCTCAA CCGGAA
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
C3A	P1: TACGGTGGGAGG
	P2: AGAACTTTCCGGTGCAGCTGCTGCAGCAGGCTTTCCATTACCTGTGGT
	P3: AATGGAAAGCCTGCTGCAGCAGCTGCACCGGAAAGTTCTTTTAAGATG
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
C4A	P1: TACGGTGGGAGG
	P2: TTTAGTTTCTCTAGCTGCTGCAGCAGCAAAAGAACTTTCCGGTTGAGA
	P3: GAAAGTTCTTTTGCTGCTGCAGCAGCTAGAGAAACTAAAGTCCTGAAG
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA

1.3 Construction of single point deletion and single point alanine mutant forms of hD1R for Section 3 of the results

Single point deletion or single point alanine substitution mutants of the far distal region of the IL3 of hD1R, K261-K265, were generated using a PCR overlap extension strategy. The hD1R subcloned in the mammalian expression vector pCMV5 was used as PCR template. The deletion mutants were referred to as ΔK261, ΔM262, ΔS263, ΔF264, and ΔK265. The alanine mutants were named K261A, M262A, S263A, F264A, and K265A **(Fig. 15)**. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively. An overlapping region between A and B allowed the amplification of the final PCR product using primers P5 and P6 **(Table 4, Table 5)**. PCR reactions were carried out as mentioned in 1.1. Flag epitope peptide (DYKAAAAK) was used to tag hD1R at the N-terminus taking advantage of the already generated mutants and using a Flag-tagged hD1R expression plasmid DNA as a template.

2. Cell culture and transfection

HEK293 cells were cultured in a 5% CO_2 incubator at 37°C in MEM with Earle's salts supplemented with 10% (v/v) heat-inactivated FBS and gentamycin sulfate (20 μg/ml). Cells were seeded in 100-mm dishes (~2.5×10^6 cells/dish) and transiently transfected using a modified calcium-phosphate precipitation procedure described previously (Plouffe et al., 2010).

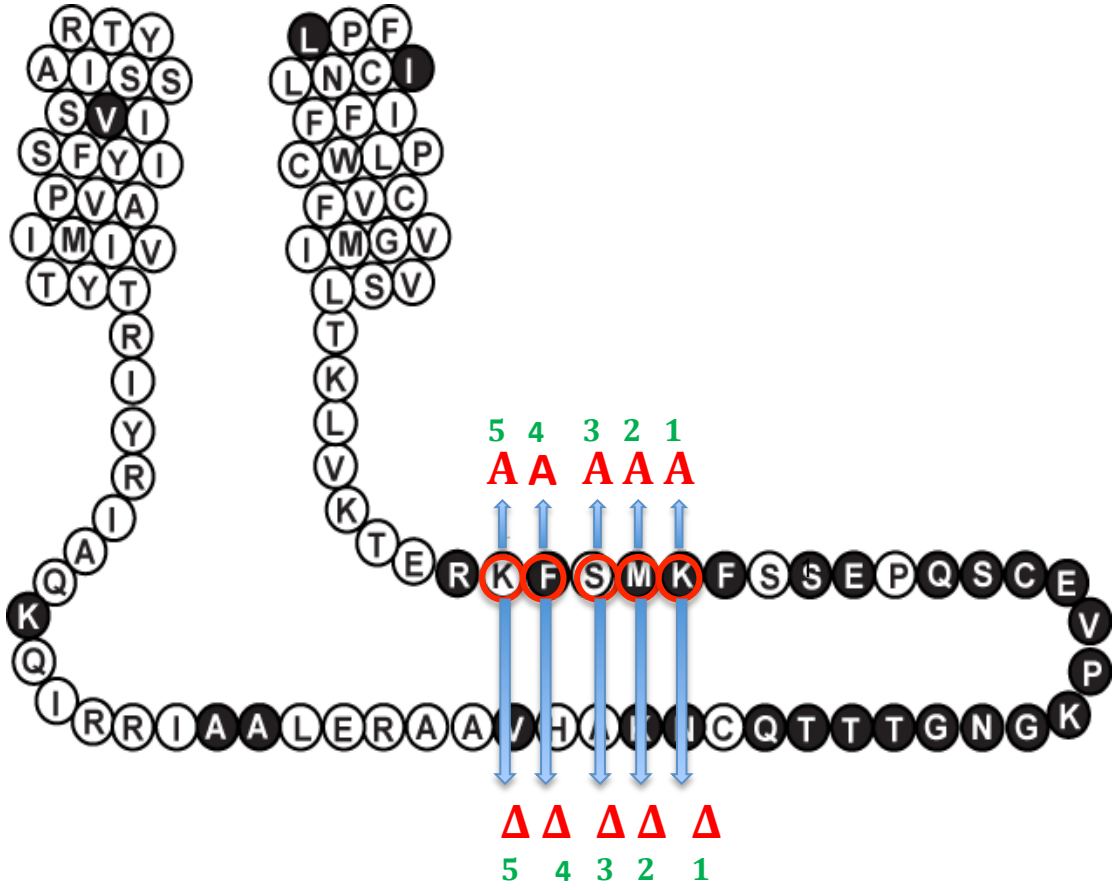


Figure 15. Schematic representation of single point deletion and single point alanine mutant forms of hD1R.
The mutants created by PCR are: **1.** ΔK261, **2.** ΔM262, **3.** ΔS263, **4.** ΔF264, **5.** ΔK265, **1A.** K261A, **2A.** M262A, **3A.** S263A, **4A.** F264A, **5A.** K265A.

Table 4. Sequence of oligonucleotide primers for construction of single point deletion mutant forms of hD1 receptor using a polymerase chain reaction-based overlapping approach.

Constructs	Primers Sequences (5'➔3')
ΔK261	P1: TTCATCCCAGTGCAGCTC
	P2: TTTAGTTTCTCTTTTAAAGGACATAAAAGAACTTTCCGGTTG
	P3: GAAAGTTCTTTTATGTCCTTTAAAAGAGAAACTAAAGTCCTG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔM262	P1: TTCATCCCAGTGCAGCTC
	P2: TTTAGTTTCTCTTTTAAAGGACTTAAAAGAACTTTCCGG
	P3: AGTTCTTTTAAGTCCTTTAAAAGAGAAACTAAAGTCCTG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔS263	P1: TTCATCCCAGTGCAGCTC
	P2: TTCTCTTTTGAACATTTTAAAAGAACTTTCCGGTTGAGA
	P3: GAAAGTTCTTTTAAAATGTTCAAAAGAGAAACTAAA
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔF264	P1: TTCATCCCAGTGCAGCTC
	P2: TTTAGTTTCTCTTTTGGACATTTTAAAAGAACTTTCCGGTTG
	P3: GAAAGTTCTTTTAAAATGTCCAAAAGAGAAACTAAAGTC
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
ΔK265	P1: TTCATCCCAGTGCAGCTC
	P2: GACTTTAGTTTCTCTGAAGGACATTTTAAAAGAACTTTCCGGTTG
	P3: GAAAGTTCTTTTAAAATGTCCTTCAGAGAAACTAAAGTCCTG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA

Table 5. Sequence of oligonucleotide primers for the construction of single point alanine mutant forms of hD1R using a polymerase chain reaction-based overlapping approach.

Constructs	Primers Sequences (5'➔3')
K261A	P1: TTCATCCCAGTGCAGCTC
	P2: TTTAGTTTCTCTTTTAAAGGACATCGCAAAAGAACTTTCCGGTTG
	P3: GAAAGTTCTTTTGCGATGTCCTTTAAAAGAGAAACTAAAGTCCTG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
M262A	P1: TACGGTGGGAGG
	P2: TTTAGTTTCTCTTTTAAAGGACGCCTTAAAAGAACTTTCCGG
	P3: AGTTCTTTTAAGGCGTCCTTTAAAAGAGAAACTAAAGTCCTG
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
S263A	P1: TTCATCCCAGTGCAGCTC
	P2: TTCTCTTTTGAAGGCCATTTTAAAAGAACTTTCCGGTTGAGA
	P3: GAAAGTTCTTTTAAAATGGCCTTCAAAAGAGAAACTAAA
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
F264A	P1: TACGGTGGGAGG
	P2: TTTAGTTTCTCTTTTGGCGGACATTTTAAAAGAACTTTCCGGTTG
	P3: GAAAGTTCTTTTAAAATGTCCGCCAAAAGAGAAACTAAAGTC
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
K265A	P1: TACGGTGGGAGG
	P2: GACTTTAGTTTCTCTTGCGAAGGACATTTTAAAAGAACTTTCCGG
	P3: CCGGAAAGTTCTTTTAAAATGTCCTTCGCAAGAGAAACTAAAGTCCTG
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA

For radioligand binding studies (saturation and competition), each dish was transfected using 5 μg of plasmid DNA for maximal expression. For constitutive activity and dose-response curve experiments, cells were transfected with a lower amount, 0.01-0.3 μg, of plasmid DNA per dish to obtain an equal amount of the receptor expression level for the WT and mutant receptor (1-3 pmol/mg membrane proteins) to obtain an; empty pCMV5 vector was used to normalize the total amount of DNA to 5 μg per 100-mm dish. Cells were incubated overnight following the transfection, then washed with PBS, detached with trypsin, and reseeded in assay dishes. The 100-mm dishes were used for radioligand binding studies, the 150-mm dishes were used for saturation and competition studies, the 6-well plates were used for basal experiments, and the 12-well plates were utilized for dose response curve and ELISA experiments as described in the following sections. The cells were grown for an additional 48 hours before performing the experiments. HEK293 cells used for these experiments were between 40-52 passages.

3. Cell membrane preparation and radioligand binding assays

Crude membrane preparation was done from cells reseeded in 150-mm dishes. Cells were washed with cold PBS, scraped in cold lysis buffer (10 mM Tris–HCl, pH 7.4; 5 mM EDTA, pH 8.0) and centrifuged for 20 min at 40,000×*g* and 4°C. Pellets were washed with lysis buffer and homogenized using a Brinkman Polytron (velocity of 17,000 rpm; 15 s), then centrifuged again for 20 min (40,000×*g*; 4°C). These pellets were homogenized again in lysis buffer. Part of the membrane was used immediately for saturation studies or frozen in liquid nitrogen and stored at -80˚C to be used for competition studies. Membrane samples, either fresh or frozen (thawed on ice) were diluted in resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA, pH 8.0).

Saturation studies were performed using increasing concentrations of D1-class radioligand [^{3}H]-SCH23390 (0.01-10 nM) and 100 µL of fresh membrane preparation in binding buffer (final in assays: 50 mM Tris–HCl, pH 7.4; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$; 1 mM EDTA, pH 8.0) for a total volume of 500 µL. Non-specific binding was assessed in the presence of 10 µM *cis*-flupenthixol. Competition studies were done using thawed frozen membrane preparation. A constant concentration of [^{3}H]-SCH23390 (based on the K_D value measured from saturation studies), and increasing concentrations of cold ligands were used. DA was dissolved in 0.1 mM ascorbic acid while dihydrexidine (DHX), thioridazine, and (+) butaclamol, were dissolved in water. The binding reaction was incubated for 90 min and then was terminated by rapid filtration through glass fiber filters (GF/C, Whatman). The filters were then washed three times with 5 ml cold washing buffer solution (50 mM Tris-HCl, pH 7.4; 100 mM NaCl) and the radioactivity bound to the filter was quantified using the liquid scintillation beta counter (Beckman LS6500). Protein concentration was evaluated with a Bio-Rad protein assay kit with bovine serum albumin (BSA) was used as a standard.

4. Whole cell cAMP assays

For whole cell cAMP assays, transfected cells were reseeded in 6-well (constitutive activity) or 12-well (dose-response curves) plates. One 100-mm dish/condition was plated for the assessment of receptor expression. The day following reseeding, the cells were metabolically labeled with [^{3}H]-adenine (1-2 µCi/ml) in MEM containing 5% (v/v) FBS and gentamicin (20 µg/ml) and incubated overnight. DA-independent (constitutive activity) and DA-dependent effect on the level of AC activity

were assessed in the presence of 1mM IBMX (cAMP phosphodiesterase inhibitor) and 20 mM HEPES as described previously (Plouffe et al., 2010). Briefly, cells were either treated with 0.1 mM ascorbic acid or DA for basal activity assessment or treated with different concentrations of DA for dose-response curve studies and incubated in a 5% CO_2 incubator at 37˚C for 30 min. At the end of the incubation time, the reaction was stopped on ice and the media in wells aspirated. Then the cells were lysed with 1 ml of lysis solution (2 % v/v perchloric acid; 0.1 mM cAMP; [^{14}C]-cAMP) and were incubated for 30 min at 4°C. The amount of intracellular [^{3}H]-cAMP was assessed from KOH-neutralized perchloric acid-lysed samples. Samples then were purified by sequential chromatography over Dowex and alumina columns as detailed previously (Plouffe et al., 2010). The relative amount of AC activity was calculated as the amount of intracellular [^{3}H]-cAMP formed (CA) over the total amount of intracellular [^{3}H]-adenine [total uptake (TU)], and was expressed as CA/TU × 1000. The functional receptor expression (B_{max}) values were determined by applying a saturating concentration of [^{3}H]-SCH23390 (~7-10 nM) in the absence or presence of 10 μM cis-flupenthixol as aforementioned.

5. Enzyme-linked immunosorbent assay (ELISA)

HEK23 cells transfected with different plasmid DNA were reseeded in 12-well dishes, previously coated with 0.01% (w/v) poly-D-lysine. The cells were incubated for 24 hours after reseeding, and then the media was aspirated, and cells were fixed with 3.7% (v/v) paraformaldehyde in PBS for 5 minutes. Each well was washed three times (10 minutes each) with PBS containing 0.2% (w/v) BSA and then incubated with PBS containing 1% (w/v) BSA for 45 minutes. This step was followed by aspiration of the media and incubation of the cells with monoclonal anti-flag M2 antibody diluted 1:10000

in PBS containing 1% (w/v) BSA supplemented with NaN3 for 45 minutes. PBS with 0.2% (w/v) BSA was used to wash the cells three times (10 minutes each), followed by a second blocking step with 1% (w/v) BSA in PBS for 15 minutes; then the media was aspirated and the cells were incubated for 45 minutes with sheep anti-mouse antibody conjugated to horseradish peroxidase diluted 1:2500 in PBS containing 1% (w/v) BSA. This was followed by another three cycles of washing/10 minutes each with 1% (w/v) BSA in PBS. Each well was treated with 400 µl o-phenylenediamine dihydrochloride (OPD) solution (0.4 mg/ml OPD, 0.4 mg/ml urea hydrogen peroxide and 0.05 M phosphate-citrate pH 5.0; SIGMAFAST OPD, P9187). The dishes were then covered with foil and rocked for 10 min before stopping the reaction with 100 µl 3M HCL/well. 250 µl solution from each well was transferred to 96-well plates, and the absorbance was read at 490 nm using a SpectraMaxM5.

6. Statistical Analysis

GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) was used for all statistical analysis and curve fitting. Binding isotherm analysis using a non-linear regression curve fitting was used to determine the equilibrium dissociation constant (K_D, nM), the maximal binding capacity (B_{max}, pmol/mg protein) of [^{3}H]SCH23390 (saturation studies), and inhibitory dissociation constant of cold drugs (K_I, nM) (competition studies). Geometric means of K_D and K_I are reported. Arithmetic means of B_{max} are expressed ± S.E.M. Relative expression, affinity, and constitutive activation values were calculated as the arithmetic mean ± S.E.M Using a four-parameter logistic equation from GraphPad Prism, the raw data for individual dose-response curves of DA were analyzed to obtain the DA half maximal effective

concentration (EC_{50} or potency) and DA-induced maximal activation of AC (E_{max}). Raw data were then normalized as a percent of the best-fitted E_{max} obtained from the wild type receptor. Normalized data were averaged, and the curves were analyzed again using a simultaneous fitting with a shared slope parameter. Best-fitted E_{max} ± S.E.M and half maximal effective concentration of DA (EC_{50}) generated from GraphPad were reported. Significant differences between best-fitted values of wild-type and mutant receptors were determined using curve fitted with unconstrained and constrained parameters. One sample *t*-test and one-way ANOVA with Newman-Keuls and Dunnett's post hoc tests were performed using GraphPad Prism with a critical level of probability of $\alpha < 0.05$.

Chapter 3

RESULTS

Results: Section 1

1. Truncations in N-terminal moiety of the IL3 of hD1-class receptors differentially impact receptor expression (B_{max}) levels

To verify if the mutant receptors are expressed, HEK293 cells were transiently transfected with 5 μg DNA construct per 100-mm dish (a DNA amount leading to the highest achievable B_{max} for DARs) to assess impact of the IL3 truncations on the functional expression of hD1R and hD5R in four different set of experiments as follows: hD1-ΔN, hD1-ΔN1, hD1-ΔN2; hD1-N1A, hD1-N2A; hD5-ΔN, hD5-ΔN1, hD5-ΔN2; or hD5-N1A, hD5-N2A. Saturation studies were done using the classical D1-class receptor specific ligand [^{3}H]-SCH23390 to determine K_D and B_{max}. The K_D values did not change significantly for any of hD1-class mutants, suggesting that mutant receptors retain their ability to bind with high affinity [^{3}H]-SCH23390 **(Fig. 16A-16D, Table 6)**. Truncation of the proximal half of the N-terminal side of the IL3-central area (hD1-ΔN1) resulted in approximately a two-fold reduction in B_{max} while alanine replacement in the same region (hD1-N1A) resulted in further decrease in the level of receptor expression, about five-fold, in comparison to hD1-WT receptor. On the other hand, deletion (hD1-ΔN2) or alanine-substitution (hD1-N2A) of the distal half of the N-terminal side of the IL3-central region resulted in either no change or an increase of about two-fold in B_{max} values, respectively **(Fig. 17A, 17B)**. Reduction in the level of B_{max} of hD1-ΔN1 and hD1-N1A could be explained by an increase in the retention of mutant receptors in the ER thereby lowering the total number of functionally expressed receptors. Another possibility is that the mutant receptors are normally processed to the cell surface, but they are unstable and rapidly internalized and sorted to the degradation pathway, which may have contributed to the low level of receptor expression.

In contrast, neither ΔN1 nor ΔN2 deletions made at N-terminal side of the IL3-central region of hD5R had any effect on B_{max} **(Fig. 17C, Table 6)**. Keeping in mind that the proximal half of the N-terminal moiety of the IL3 of hD1-class receptors differ by just two amino acid residues, AA in hD1R and SS in hD5R, it was unexpected to see these different phenotypes. To further investigate this discrepancy between hD1R and hD5R mutants, and the role of the IL3-N terminal/hD5R on B_{max}, alanine mutants were generated for the proximal and the distal halves of the IL3-N terminal/hD5R. Surprisingly, alanine replacement of the proximal half of the IL3-N terminal/hD5R (hD5-N1A) has resulted in about 85 % reduction in B_{max} in comparison to the hD5-WT while no change was noticed with hD5-N2A **(Fig. 17D, Table 6)**. This led me to speculate that in fact the receptor can adopt different conformations affecting its maturation process upon deletions or alanine substitutions.

Table 6. Equilibrium dissociation constant (K_D) and maximal binding capacity (B_{max}) of [^{3}H]-SCH23390 for WT and N-terminal segment mutant forms of hD1-class receptors.

Receptor	[^{3}H]-SCH23390 pK_D (K_D, nM)	B_{max} (pmol/mg protein) ± SEM
hD1-WT	9.25 ± 0.05 (0.56)	9.80 ± 1.67
hD1-ΔN	9.01 ± 0.05 (0.82)	1.23 ± 0.21
hD1-ΔN1	9.18 ± 0.04 (0.66)	5.19 ± 0.60
hD1-ΔN2	9.19 ± 0.05 (0.65)	11.47 ± 1.05
hD1-WT	9.38 ± 0.02 (0.42)	14.75 ± 1.35
hD1-N1A	9.36 ± 0.08 (0.43)	2.59 ± 0.28
hD1-N2A	9.25 ± 0.05 (0.56)	27.14 ± 3.61
hD5-WT	9.04 ± 0.08 (0.91)	8.65 ± 1.27
hD5-ΔN	8.96 ± 0.02 (1.10)	7.20 ± 1.15
hD5-ΔN1	9.03 ± 0.05 (0.93)	5.28 ± 0.99
hD5-ΔN2	9.14 ± 0.06 (0.73)	7.22 ± 1.43
hD5-WT	9.04 ± 0.04 (0.91)	9.78 ± 0.54
hD5-N1A	9.03 ± 0.09 (0.82)	1.55 ± 0.09
hD5-N2A	9.07 ± 0.04 (0.66)	8.79 ± 0.44

pK_D (negative logarithm of K_D in molar) and B_{max} (pmol/mg of protein) values obtained from saturation curves done in duplicate are expressed as arithmetic means ± S.E.M from 6-7 experiments. The corresponding K_D value in nM is shown in brackets. Experiments for hD1R deletion (hD1-ΔN, hD1-ΔN1, hD1-ΔN2), hD1R alanine substitution (hD1-N1A, hD1-N2A), hD5R deletion (hD5-ΔN, hD5-ΔN1, hD5-ΔN2) and hD5R alanine substitution (hD5-N1A, hD5-N2A) mutants were performed independently.

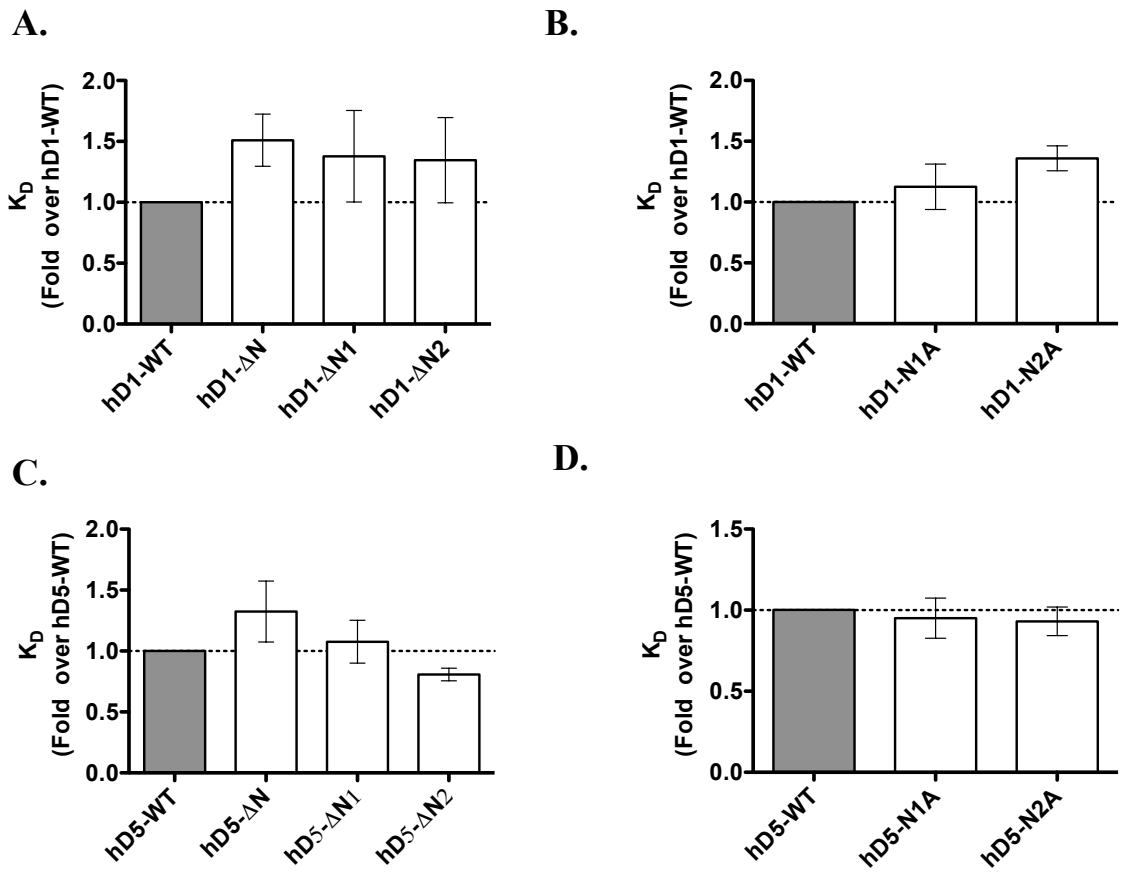


Figure 16: Fold change values of equilibrium dissociation constant (K_D) of mutant hD1-class receptors for [^{3}H]-SCH23390 relative to WT.

Raw K_D values obtained from saturation studies were expressed as fold change relative to their respective WT receptor (value set to 1). Relative K_D values were compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for deletion (hD1-ΔN, hD1-ΔN1, hD1-ΔN2) and alanine (hD1-N1A, hD1-N2A) mutant hD1R were performed independently **(A, B)**. Experiments for deletion (hD5-ΔN, hD5-ΔN1, hD5-ΔN2) and alanine (hD5-N1A, hD5-N2A) mutant hD5R were performed independently **(C, D)**.

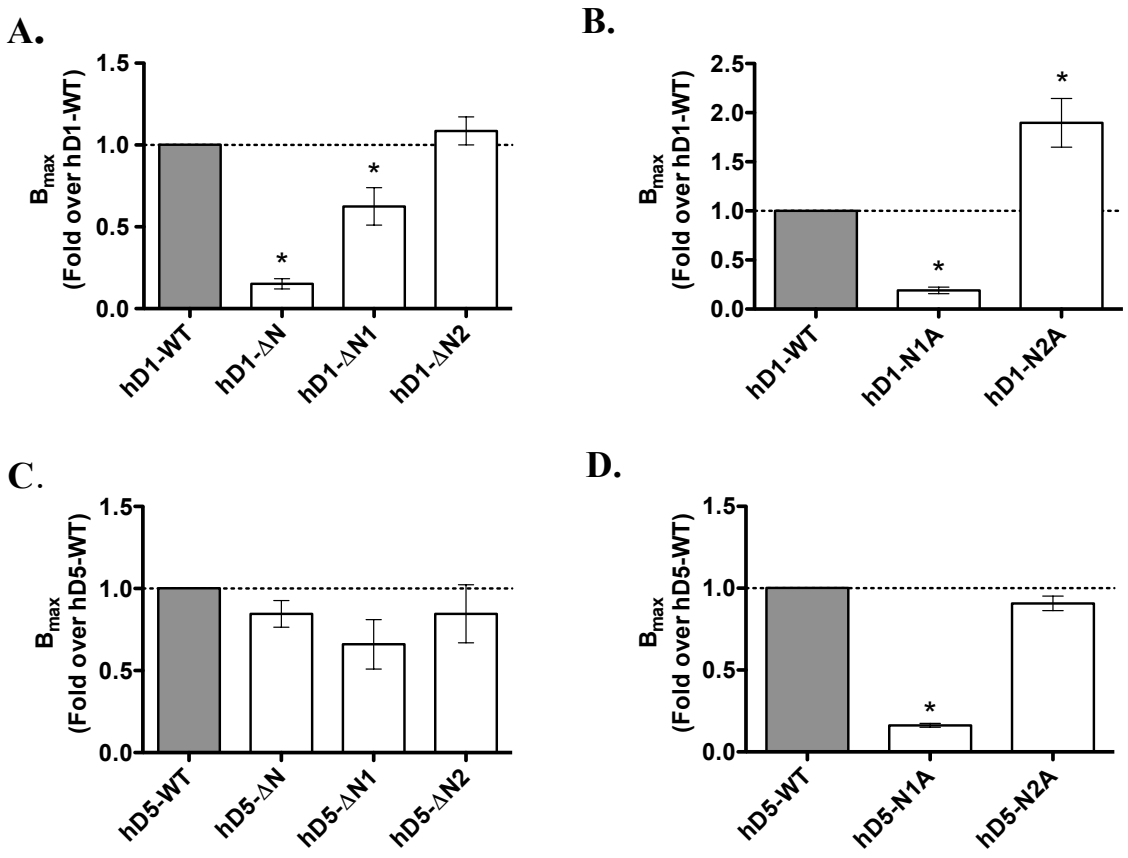


Figure 17: Fold change values of maximal binding capacity (B_{max}) of mutant hD1-class receptors for [^{3}H]-SCH23390 relative to WT.

Raw B_{max} values obtained from saturation studies using duplicate determinations were expressed as fold change relative to their respective WT receptor (value set to 1). Relative B_{max} values compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for deletion (hD1-ΔN, hD1-ΔN1, hD1-ΔN2) and alanine (hD1-N1A, hD1-N2A) mutant hD1R were performed independently **(A, B)**. Experiments for deletion (hD5-ΔN, hD5-ΔN1, hD5-ΔN2) and alanine (hD5-N1A, hD5-N2A) mutant hD5R were performed independently **(C, D)**.

2. Opposite subtype-specific ligand affinities are mediated upon deletion of the proximal and the distal halves of the N-terminal moiety of the IL3 of hD1-class receptors

Competition studies were done to test the affinity of the mutant receptors for different agonists and inverse agonists **(Table 7)**. The affinities of hD1-ΔN1 and hD1-N1A for the agonists used, DA and DHX, were increased by approximately two-fold and four-fold, respectively. On the other hand, no change in the affinity for any of the agonists used was observed with either deletion or alanine mutants of the distal half of the IL3-N terminal of hD1R (hD1-ΔN2 and hD1-N2A**) (Fig. 18A, 18B)**.

Intriguingly, a three-fold reduction in the affinity for dopaminergic agonists of mutant lacking the proximal side of the IL3-N terminal/hD5R (hD5-ΔN1) while alanine replacement in the same region (hD5-N1A) resulted in approximately a two-fold increase in the affinity in comparison to its respective WT. Although the affinity of hD5-ΔN2 for agonists is unchanged, an approximate two-fold increase in the affinity of the alanine mutant receptor (hD5-N2A) was observed in comparison to WT receptor **(Fig. 18C, 18D)**. Meanwhile, the affinity of inverse agonists (thioridazine and butaclamol) remain unchanged for both hD1 and hD5 mutants **(Fig. 18E-18H)**.

Differences in the agonist affinity change pattern (increase for hD1-deletions and decrease for hD5-deletions) with respect to WT receptors suggests that the proximal side of the N-terminal of the IL3 of hD1-class receptors plays a significant role in shaping in a subtype-specific fashion the binding conformation pocket of agonists. The observed differential behaviors between deletion and alanine replacements of the proximal region

Table 7. pK_I values of agonists and inverse agonists for WT and mutant hD1-class receptors.

Receptor	Dopamine		Dihydrexidine		Thioridazine		Butaclamol	
	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)
hD1-WT	5.10 ± 0.09	6944	6.22 ± 0.11	572	7.10 ± 0.04	79	8.30 ± 0.08	5.0
hD1-ΔN	5.80 ± 0.07	1350	6.55 ± 0.11	205	6.85 ± 0.04	140	8.26 ± 0.06	5.5
hD1-ΔN1	5.52 ± 0.04	2822	5.90 ± 0.50	364	7.01 ± 0.04	80	8.25 ± 0.07	5.7
hD1-ΔN2	5.08 ± 0.04	8070	6.00 ± 0.07	750	7.06 ± 0.05	87	8.40 ± 0.12	4.0
hD1-WT	5.23 ± 0.02	5927	6.44 ± 0.05	366	7.17 ± 0.11	53	8.23 ± 0.09	6.0
hD1-N1A	6.0± 0.08	982	7.06 ± 0.05	87	7.05 ± 0.09	74	8.22 ± 0.05	6.0
hD1-N2A	5.09 ± 0.04	8229	6.30 ± 0.03	506	7.04 ± 0.12	70	8.20 ± 0.08	6.3
hD5-WT	6.16 ± 0.05	697	7.15 ± 0.06	71	6.59 ± 0.06	260	7.52 ± 0.07	30
hD5-ΔN	5.33 ± 0.04	4689	6.43 ± 0.04	375	6.64 ± 0.04	231	7.55 ± 0.05	28
hD5-ΔN1	5.74 ± 0.07	1835	6.74 ± 0.05	182	6.73 ± 0.05	188	7.54 ± 0.04	29
hD5-ΔN2	6.26 ± 0.07	553	7.25 ± 0.08	56	6.67 ± 0.06	212	7.57 ± 0.06	27
hD5-WT	5.93 ± 0.03	1178	7.03 ± 0.05	93	6.56 ± 0.08	274	7.53 ± 0.03	30
hD5-N1A	6.28 ± 0.03	528	7.19 ± 0.11	55	6.43 ± 0.18	245	7.62 ± 0.07	24
hD5-N2A	6.06 ± 0.06	870	7.20 ± 0.05	62	6.61 ± 0.07	246	7.65 ± 0.07	22

pK_I values (negative logarithm of K_I in molar) determined from competition studies done in duplicate with [^{3}H]-SCH23390 and increasing concentrations of dopaminergic ligands are reported as arithmetic means ± S.E.M from 5-7 experiments. The corresponding K_I values (nM) are also shown. Experiments for deletion (hD1-ΔN, hD1-ΔN1, hD1-ΔN2) and alanine (hD1-N1A, hD1-N2A) mutants of hD1R were performed independently. Likewise, deletion (hD5-ΔN, hD5-ΔN1, hD5-ΔN2) and alanine (hD5-N1A, hD5-N2A) mutants of hD5R were performed separately.

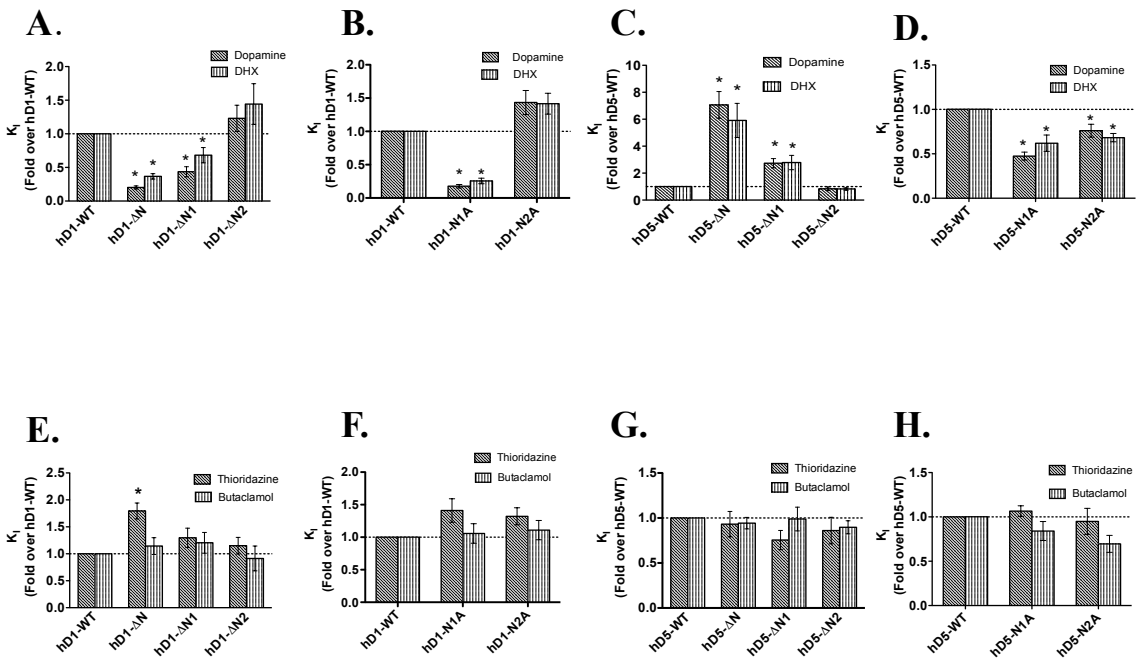


Figure 18. Fold change values for dopaminergic agonists (DA, DHX) and inverse agonists (thioridazine, butaclamol) affinities of mutant hD1-class receptors relative to WT.

Raw K_I values obtained from competition studies were expressed as fold change relative to their respective WT receptor (value set to 1). Relative K_I values were compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for deletion (hD1-ΔN, hD1-ΔN1, hD1-ΔN2) and alanine (hD1-N1A, hD1-N2A) mutant hD1R were performed independently **(A, B, E, F)**. Experiments for deletion (hD5-ΔN, hD5-ΔN1, hD5-ΔN2) and alanine (hD5-N1A, hD5-N2A) mutant hD5R were performed independently **(C, D, G, H)**.

of hD1-class receptors suggest that both the amino acids content and preserving the receptor length are important for modulating the receptor affinity for agonists binding.

3. The proximal side of the N-terminal moiety of the IL3 of hD1-class receptors is necessary for agonist-independent intracellular cAMP production

D5R has higher constitutive activity in comparison to D1R (Tiberi and Caron, 1994). To test the effect of these mutants on agonist-independent activity, HEK293 cells were transiently transfected with the WT receptors or with the truncated hD1R and hD5R forms, and then whole cAMP studies were performed. Agonist-independent activity for hD1-ΔN1 and hD1-N1A was reduced by approximately two-fold and was comparable to the reduction in the level of basal activity observed when the whole IL3-N terminal moiety of hD1R was deleted. On the other hand, no change in the basal activity was observed when the distal half of the IL3-N terminal/hD1R was deleted or mutated to alanine **(Fig. 19A, 19B)**. Likewise for hD5-ΔN1 and hD5-N1A, agonist-independent activity was sharply reduced to the level of mock-transfected cells. However, basal activity remained virtually unchanged when the distal side of the IL3-N terminal/hD5R was modified, albeit with a tendency to increase **(Fig. 19C, 19D)**.

My data suggest that deletion or alanine substitution made at the proximal half of the IL3/N-terminal of hD1-class receptors promotes a reduction or a full loss of agonist-independent G-protein. In contrast, when the distal half of the IL3/N-terminal was altered in either hD1R or hD5R, the mutated receptors were able to retain a conformation resembling their respective WT receptor as indexed by intracellular cAMP levels in the absence of agonists.

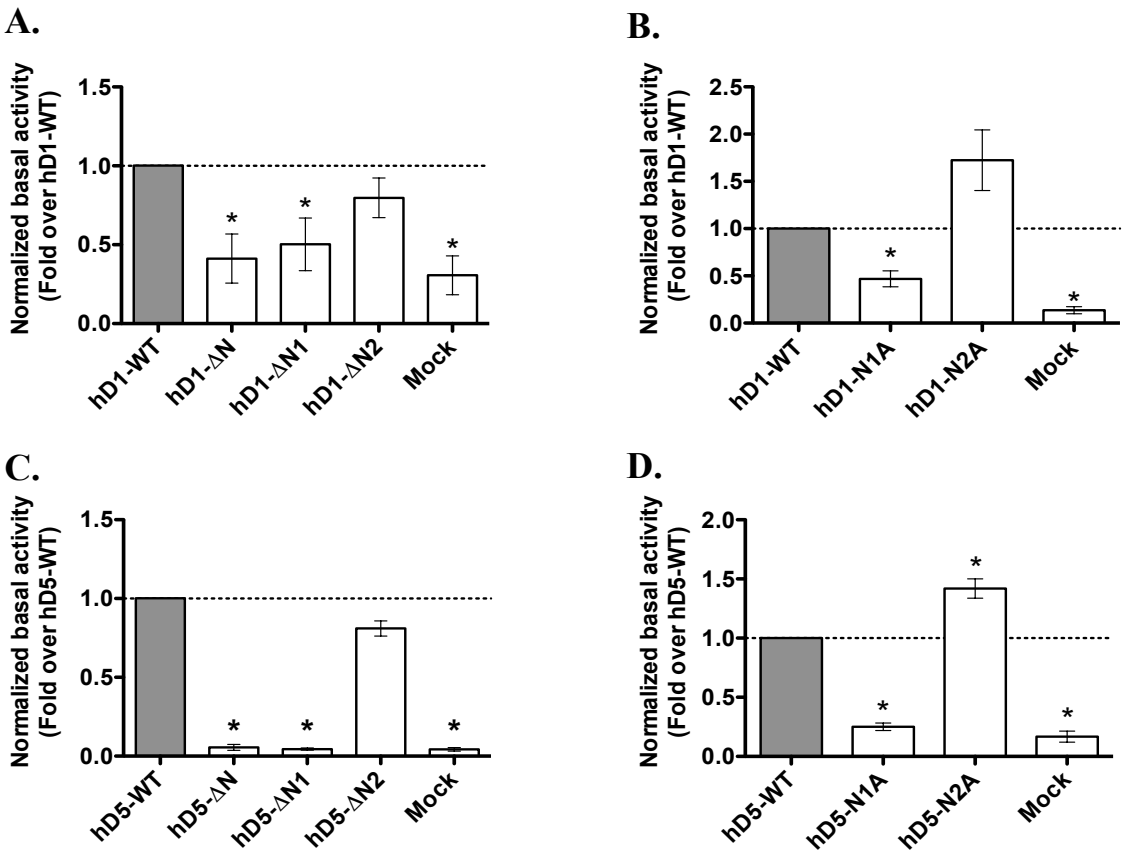


Figure 19. Constitutive activity of WT and mutant hD1-class receptors expressed in HEK293 cells.

Cells transfected with low DNA concentration (0.01 μg-0.2 μg/dish) of WT, and mutant forms of hD1-class receptor mutants were seeded in 6-well plates and labeled with [^{3}H] adenine**.** Mock represents the condition in which the cells were transfected with empty DNA vector. Cells were treated with vehicle (ascorbic acid, 100 μM) or DA (10 μM) for 30 min at 37°C and intracellular cAMP levels were determined. DA-independent and dependent activation of AC were performed in triplicate determinations and are expressed as the arithmetic mean ± S.E.M of six experiments and normalized as the fold change relative to respective WT receptors (value set to 1). Relative values were compared to 1 (WT) using a one-sample t-test (* $p < 0.05$). Agonist-dependent maximal activation of AC assessed with 10 μM DA was observed for each condition and used as positive control for receptor responsiveness (not shown). B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 2.40, hD1-ΔN: 1.32, hD1-ΔN1: 2.09, hD1-ΔN2: 2.60. **(B)** hD1-WT: 2.90, hD1-N1A: 1.97, hD1-N2A: 3.28. **(C)** hD5-WT: 1.56, hD5-ΔN: 1.44, hD5-ΔN1: 1.48, hD5-ΔN2: 1.61. **(D)** hD5-WT: 1.75, hD5-N1A: 1.22, hD5-N2A: 1.95. Each group of experiments for deletion and alanine mutant forms of hD1R and hD5R was performed independently.

These results pointed to the importance of the proximal side of the IL3/N-terminal of hD1-class receptors for agonist-independent activity. Overall, changes in the constitutive activity of receptors imply a significant role of the length and amino acids content of the proximal region of the IL3/N-terminal moiety.

4. Deletion of N1 and N2 segments of the IL3 central region of hD1-class receptors differentially modulates E_{max} and EC_{50} values

Dose-response curves for DA were performed in intact cells to assess E_{max} and EC_{50} of N1 and N2 mutant forms of hD1R and hD5R. A robust decrease in E_{max} was observed for hD1-ΔN1 mutant (40%), reminiscent of the 50% reduction in E_{max} when the whole IL3-N terminal moiety was deleted **(Fig. 20A, 20B, Table 8)**. Meanwhile, no significant effect was noticed for hD1-N1A in comparison to hD1-WT. A rightward shift in EC_{50} (potency loss) was observed with hD1-ΔN1 and hD1-N1A mutant receptors, although not statistically significant. The lower E_{max} and potency suggest a reduced ability of the mutant receptors to couple to $G\alpha_s$ proteins. In contrast, no significant effect on E_{max} and EC_{50} values was observed with hD1-ΔN2 and hD1-N2A in comparison to hD1-WT **(Fig. 20A, 20B, Table 8)**.

In contrast to the results obtained with deletion of the proximal region of the IL3/N-terminal of hD1R, deletion of the corresponding segment in hD5R (hD5-ΔN1) resulted in a dramatic loss of $G\alpha_s$ coupling as shown by the ~90% reduction in E_{max} and the large EC_{50} rightward shift (3780 nM in comparison to 1.6 nM for hD5-WT) **(Fig. 20C, Table 8)**. These results suggest that hD5-ΔN1 adopts a stable inactive state which cannot couple efficiently to $G\alpha_s$-protein following DA stimulation. Interestingly, replacement of N1 with alanine (hD5-N1A) resulted in a better phenotype in comparison

to hD5-ΔN1, but yet displayed a robust (60 %) reduction in E_{max} and a 35-fold rightward shift in EC_{50} (55 nM compared to 1.6 nM for hD5R-WT) **(Fig. 20D, Table 8)**. On the other hand, deletion or alanine mutations of the distal region (N2) of the IL3/N-terminal of hD5R resulted in approximately a 25 % reduction in E_{max} of hD5-ΔN2 and no significant change for hD5-N2A. Also, no significant change in the EC_{50} rightward shift was observed for either mutant **(Fig. 20C, 20D, Table 8)**. These results suggest that the length of N2 plays a significant role in maintaining optimal function of the IL3 for DA-mediated maximal activation but is dispensable for agonist potency.

Conclusion

Together, the results of this section suggest a critical role of the proximal side of the N-terminal of the IL3 of hD1-class receptors for the receptor expression and agonist affinity, while has no impact on the inverse agonist affinity. In addition, the proximal side of the N-terminal moiety of hD1-class receptors is important for the constitutive activity of the receptor and its agonist-dependent G-protein coupling properties. Overall, these results suggest that the hD1/N-terminal side of the IL3 plays an important structural role in controlling the ligand binding conformation, regulating agonist binding, and G-protein coupling efficiency. As this section of the result dealt with investigating the functional role of the IL3/N-terminal region of hD1-class receptors, the focus of the following two sections of the result will be on studying the importance of the IL3/C-terminal moiety, solely focusing on hD1R **(Table S1)**.

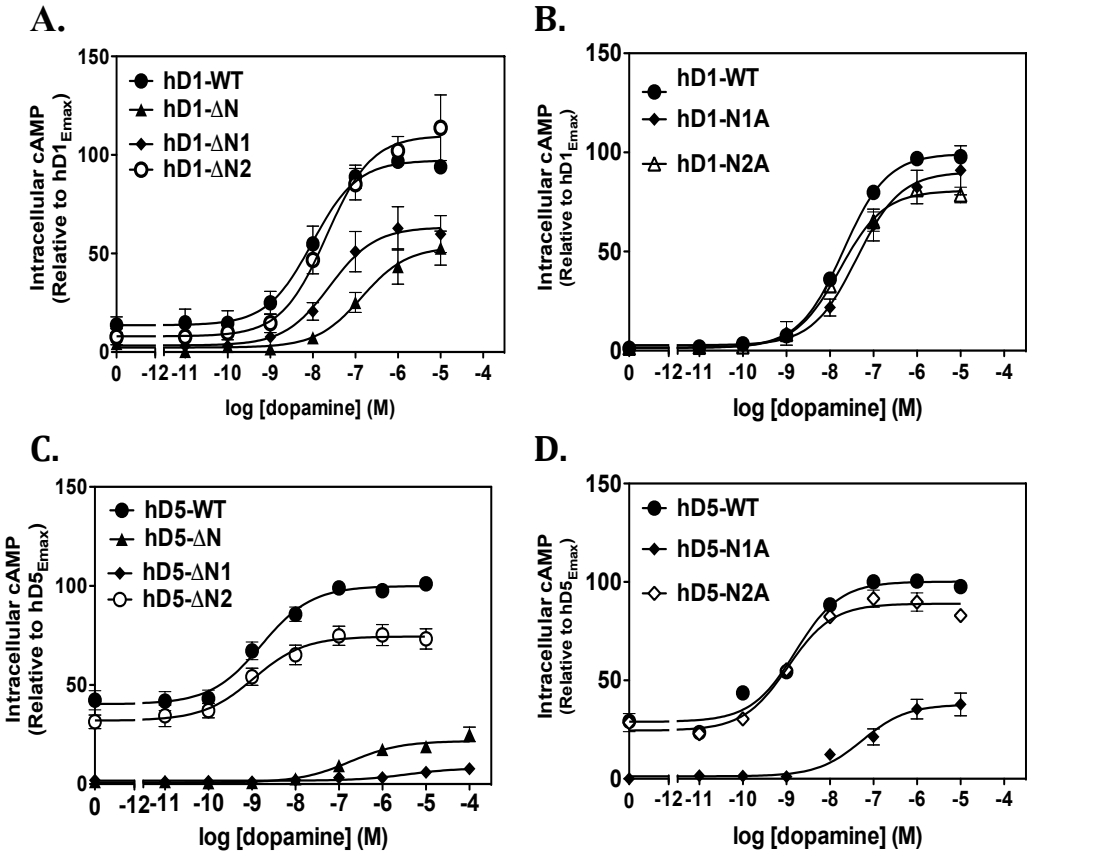


Figure 20. Dose-response curves for dopamine-induced stimulation of adenylyl cyclase activity in HEK293 cells expressing WT and mutant forms of hD1-class.

Cells transfected with a low amount of different DNA receptor constructs (0.01 μg-0.2 μg/dish) were seeded in 12-well plates and labeled with [^{3}H] adenine. Dose-response curves were determined in the presence of increasing DA concentrations (0 to 10^{-4} M) done in triplicate. Each point represents the arithmetic mean of six experiments. Data were plotted as a function of log [DA] in molar. Simultaneous nonlinear curve fitting using raw data was performed using GraphPad Prism. Curves were then normalized relative to 100% of the maximal activation (E_{max}) obtained with WT and then refitted to determine the EC_{50} and relative E_{max} values for mutant receptors. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 2.33, hD1-ΔN: 1.70, hD1-ΔN1: 2.11, hD1-ΔN2: 2.44. **(B)** hD1-WT: 2.29, hD1-N1A: 2.46, hD1-N2A: 2.67. **(C)** hD5-WT: 2.66, hD5-ΔN: 2.49, hD5-ΔN1: 1.94, hD5-ΔN2: 2.90. **(D)** hD5-WT: 1.94, hD5-N1A: 1.73, hD5-N2A: 2.30. Each group of experiments (A-D) for deletion and alanine mutant forms of hD1R and hD5R was performed independently.

Table 8. pEC_{50} and E_{max} values for DA-induced stimulation of adenylyl cyclase activity in HEK293 cells expressing WT and mutant hD1-class receptors.

Receptor	pEC_{50} ± SEM	EC_{50} (nM)	E_{max} ± SEM
hD1-WT	7.95 ± 0.20	9.65	100
hD1-ΔN	6.72 ± 0.10	141.3	49 ± 9
hD1-ΔN1	7.55 ± 0.12	24.68	55 ± 9
hD1-ΔN2	7.48 ± 0.32	20.92	104 ± 17
hD1-WT	7.62 ± 0.08	20.21	100
hD1-N1A	7.38 ± 0.05	41.04	91 ± 12
hD1-N2A	7.65 ± 0.11	16.93	79 ± 4
hD5-WT	8.78 ± 0.11	1.648	100
hD5-ΔN	6.02 ± 0.35	178.3	19 ± 2
hD5-ΔN1	8.90 ± 0.15	3770	6 ± 1
hD5-ΔN2	8.90 ± 0.15	1.085	73 ± 5
hD5-WT	8.85 ± 0.17	1.614	100
hD5-N1A	7.42 ± 0.31	55.12	43 ± 7
hD5-N2A	9.33 ± 0.32	1.079	96 ± 13

Best-fitted values for pEC_{50} (negative logarithm of EC_{50} in molar) and the corresponding EC_{50} in nM and E_{max} obtained from curve-fitting of dose-response curves shown in Fig. 18 are reported.

Results: Section 2

1. Truncations in the C-terminal moiety of the IL3 of hD1-class receptors differentially impact receptor expression (B_{max}) and the cell surface receptor expression

To investigate the functional role of the IL3/C-terminal, a series of four mutants with sequential deletion or alanine replacements of 20 amino acids located at the C-terminal portion of the IL3 were generated (5 amino acids each) as shown in **Figure 14**. Studies were performed in two independent series of experiments; one series was done with the deletion mutants while the other series was done with alanine mutants.

To assess the functional expression of the mutant receptors, saturation studies were done using the classical D1-class receptor specific ligand [^{3}H]-SCH23390. HEK293 cells were transiently transfected at high expression level (using 5 μg DNA/dish) with WT or deletion mutant (ΔC1, ΔC2, ΔC3, ΔC4, ΔIL3C) forms of hD1R. The K_D and the B_{max} values were determined **(Table 9)**. The K_D did not change significantly for any of hD1R-mutants, which indicated that the affinity of the mutant receptors for [^{3}H]-SCH23390 is not altered relative to WT **(Fig. 21A, 21B)**. The B_{max} for ΔC1 is slightly reduced, while being significantly decreased for ΔC2, ΔC3, and ΔIL3C. Notably, the most robust decrease in B_{max} was observed with ΔC4 (95%) relative to hD1-WT **(Fig. 21C)**.

In contrast, there was a significant increase in B_{max} level when the C1, C2 or C3 segment was replaced with alanine (C1A, C2A, C3A). Nevertheless, the B_{max} level for C4A remains lower with a 3.5 fold reduction in comparison to hD1-WT **(Fig. 21D)**, but it was improved relative to ΔC4. B_{max} values represent the total number of functional receptors including those inside the cells and those on the cell surface. As some of these

mutant receptors caused a reduction in B_{max} level, we tested the cell surface expression level using ELISA assay. Notably, the level of cell surface expression observed from the ELISA experiments is, in fact, relevant to the B_{max} values obtained for the same mutant receptor **(Fig. 22)**.

Table 9. Equilibrium dissociation constant (K_D) and maximal binding capacity (B_{max}) of [^{3}H]-SCH23390 for WT and C-terminal segment mutant forms of hD1-class receptors.

Receptor	[^{3}H]-SCH23390 pK_D (K_D nM)	B_{max} (pmol/mg protein) ± SEM
hD1-WT	9.29 ± 0.04 (0.52)	14.74 ± 0.73
ΔC1	9.27 ± 0.03 (0.54)	10.69 ± 0.68
ΔC2	9.36 ± 0.04 (0.43)	4.89 ± 0.55
ΔC3	9.35 ± 0.05 (0.45)	2.628 ± 0.33
ΔC4	9.31 ± 0.06 (0.49)	0.617 ± 0.09
ΔIL3C	9.16 ± 0.06 (0.69)	4.352 ± 0.23
hD1-WT	9.25 ± 0.09 (0.56)	13.86 ± 1.77
C1A	9.16 ± 0.04 (0.69)	27.45 ± 3.08
C2A	9.28 ± 0.04 (0.52)	18.35 ± 1.27
C3A	9.10 ± 0.06 (0.80)	27.63±2.16
C4A	9.22 ± 0.13 (0.60)	3.97 ± 0.66

pK_D (negative logarithm of K_D) and B_{max} values (pmol/mg of protein) obtained from saturation curves done in duplicate are expressed as arithmetic means ± S.E.M from six experiments. The corresponding K_D values in nM are shown in brackets. Experiments for hD1-IL3C/deletion (ΔC1, ΔC2, ΔC3, ΔC4, ΔIL3C) and hD1-IL3C/alanine substitution (C1A, C2A, C3A, and C4A) mutants were performed independently.

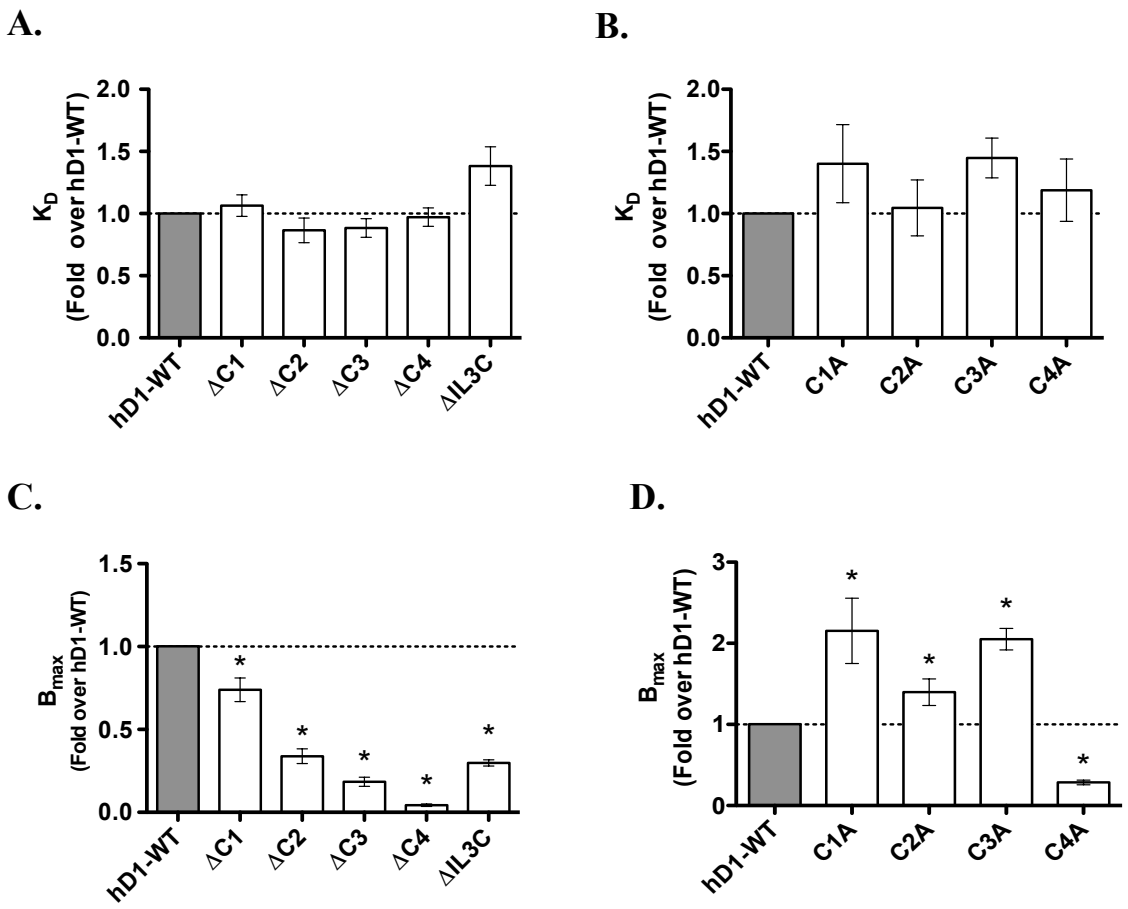
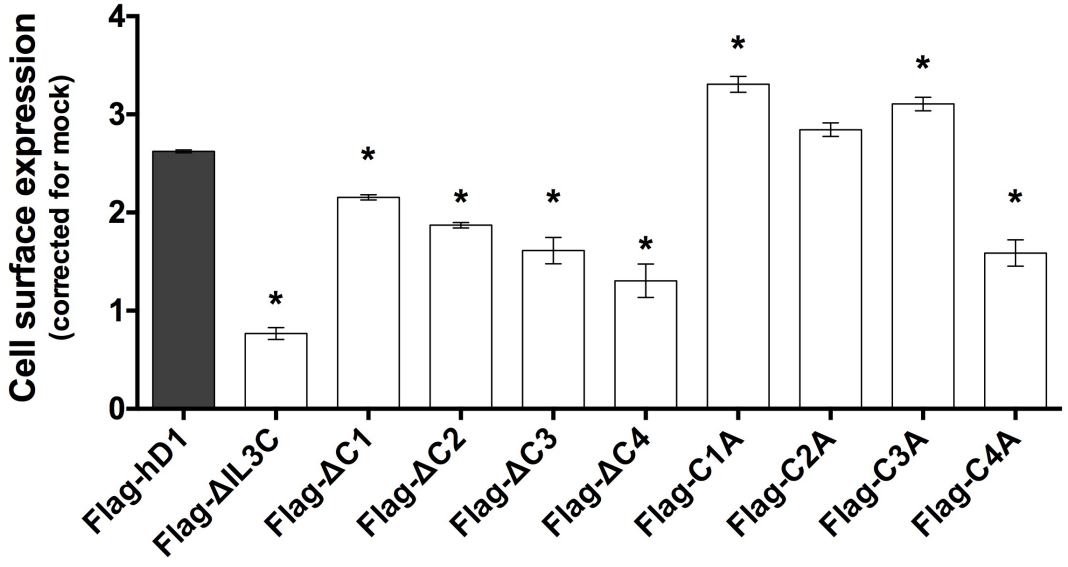


Figure 21: Fold change values of equilibrium dissociation constant (K_D) and maximal binding capacity (B_{max}) of hD1R-IL3C mutants for [3H]-SCH23390 relative to hD1R-WT

Raw K_D and B_{max} values obtained from saturation studies were expressed as fold-change relative to their respective WT receptor (Value set to 1). Relative K_D and B_{max} values compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for hD1-IL3C/deletion (ΔC1, ΔC2, ΔC3, ΔC4, ΔIL3C) and hD1-IL3C/alanine (C1A, C2A, C3A, and C4A) mutant forms were performed independently.

A.



A.

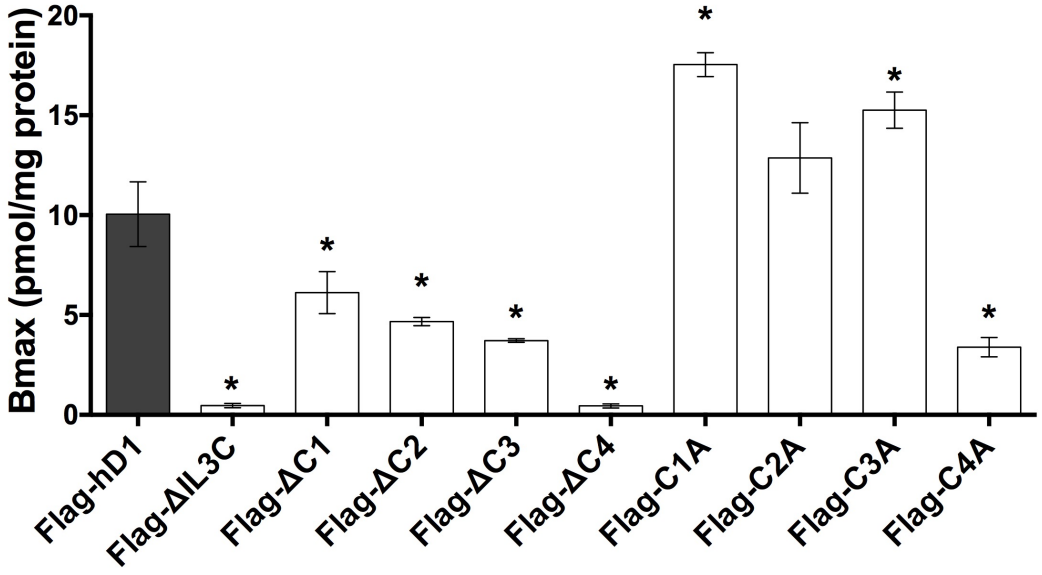


Figure 22: Cell surface expression of Flag-tagged WT and mutant forms of hD1R expressed in HEK293 cells

(A) Raw cell surface expression values obtained from ELISA were subtracted from control (mock-transfected cells) and expressed as the mean ± S.E.M of four experiments done in triplicate determination. **(B)** B_{max} in pmol/mg membrane proteins for [^{3}H]-SCH23390 (expressed as arithmetic means ± S.E.M.) is shown. Values are as follows: Flag-hD1-WT: 10.05, Flag-ΔIL3C: 0.47, Flag-ΔC1: 6.12, Flag-ΔC2: 4.67, Flag-ΔC3: 3.72, Flag-ΔC4: 0.45, Flag-C1A: 17.53, Flag-C2A: 12.86, Flag-C3A: 15.26, Flag-C4A: 3.39. * $p < 0.05$ when compared with Flag/hD1-WT using a one-way ANOVA followed by Dunnett's multiple comparisons test. All experiments were performed in HEK293 cells transfected with 5 μg per 100-mm dish of WT and mutant receptor constructs in pCMV5 or empty pCMV5 (mock).

Deletion of these different segments of the receptor (ΔC1, ΔC2, ΔC3) may have caused the loss of key structural determinants (e.g. motif for maturation) for optimal receptor expression. Alternatively, it is possible that the remained IL3 residues may have led to refolding/repacking of GPCR structure to compensate for the lost sequences and thus potentially masking some of the structural determinants for receptor expression. In agreement with this, restoring the receptor length with alanine substitution produced a better phenotype than the parent receptor. However, the noticeable reduction in B_{max} of C4 mutants (ΔC4, C4A) could suggest the importance of this moiety for the correct folding conformation of a mature and functional receptor, which requires the presence of a particular residue or (residues) located within this segment. Overall, the results here suggest that the far distal region of the IL3/C-terminal, K261-K265, plays a critical role in controlling the expression level of hD1R.

2. Regulation of agonist and inverse agonist affinities by mutations in the IL3/C-terminal moiety of hD1R

Competition studies using [^{3}H]-SCH23390 were done to test the affinity of the mutant receptors using two agonists, DA and DHX. The affinity of the mutant receptors for different inverse agonists [thioridazine and (+) butaclamol] was also tested **(Table 10)**. The affinity of ΔC1 and ΔC2 for the agonists did not change significantly from hD1R-WT as observed too for alanine substitution mutants of the same regions (C1A, C2A). On the other hand, about a four-fold increase in the affinity of ΔC3 was observed while no change in the agonist affinity was seen for alanine substitution made in the same segment (C3A) **(Fig. 23A, 23B)**. Noticeably, the agonist affinity of both ΔC4 and ΔIL3C was drastically increased by about 30-fold **(Fig. 23A)**. The same striking increase was

also observed with C4A mutant **(Fig. 23B)**. Meanwhile, the affinity of inverse agonists was unchanged for neither deletion nor alanine mutations done in the IL3/C-terminal moiety **(Table 10, Fig. 23C, 23D)**.

Overall, the results obtained here emphasize the importance of the C-terminal segment encompassing residues K261-K265 of hD1R for the agonist affinity. Indeed, deletion or alanine mutations of the K261-K265 segment (ΔC4, C4A) result in an improved agonist binding phenotype (30-fold increase) in comparison to WT. Constitutively active mutants (CAMs) GPCRs are known to exhibit a higher agonist affinity (Samama et al., 1993, Lattion et al., 1999, Costa and Cotecchia, 2005, Cotecchia, 2007). Whether this is also the case for K261-K265 mutants or not, was next investigated.

3. Modulation of agonist-independent intracellular cAMP production by mutations generated in the IL3/C-terminal moiety of hD1R

The effect of mutations in the IL3/C-terminal moiety on the agonist-independent activation of hD1R was probed in HEK293 cells expressing similar levels of WT and mutant forms using whole cell cAMP studies. As observed previously, ΔIL3C resulted in a significant increase in the agonist-independent activity (6-fold) while sequential deletion or alanine mutations of smaller segments of the IL3/C-terminal moiety did not alter the receptor basal activity except for one segment, C4. Indeed, either deletion (ΔC4) or alanine replacement (C4A) of this segment has resulted in a robust increase of basal activity by 7-fold and 5-fold, respectively. The agonist-independent activity detected was comparable to the increase in the basal level of activity observed with ΔIL3C **(Fig. 24A, 24B)**. The results obtained here suggest that the amino acid contents in this segment of

the IL3, K261-K265, has a negative impact on the receptor transition from R (inactive) to R* (active) conformation.

4. The effect of inverse agonists on the basal activity level of mutant forms of the IL3/C-terminal moiety of hD1R

To determine the inverse agonist efficacy on the constitutive activity of mutant and WT forms, the basal level of AC activity was evaluated in the absence or presence of different inverse agonists (thioridazine and (+) butaclamol). HEK293 cells were transiently transfected to achieve similar B_{max} levels for WT and mutant forms (ΔC1, C1A, ΔC2, C2A, ΔC3, C3A, ΔC4, C4A, ΔIL3C). Interestingly, the drugs used exhibited inverse agonist activity at all mutant receptors with the strongest efficacy relative to WT seen with mutant receptors having a higher level of basal activity **(Fig. 25)**.

Table 10. pK_I values of agonists and inverse agonists for hD1-WT and hD1-IL3/C-terminal mutant receptors

Receptor	Dopamine		Dihydrexidine		Thioridazine		Butaclamol	
	pK_I ± SEM	K_I (nM)	pK_i ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)
hD1-WT	5.08 ± 0.04	8280	6.28 ± 0.06	524	7.39 ± 0.05	41	9.02 ± 0.16	1.0
ΔC1	5.05 ± 0.04	8970	6.25 ± 0.03	569	7.38 ± 0.04	38	9.06 ± 0.11	0.9
ΔC2	5.28 ± 0.04	5292	6.37 ± 0.05	430	7.04 ± 0.03	40	9.14 ± 0.10	0.7
ΔC3	5.71 ± 0.06	1956	6.75 ± 0.03	180	7.43 ± 0.09	41	9.15 ± 0.14	0.7
ΔC4	6.56 ± 0.03	277	7.47 ± 0.06	34	7.41 ± 0.08	47	8.75 ± 0.16	1.6
ΔIL3C	6.51 ± 0.02	311	7.44 ± 0.04	36	7.30 ± 0.04	44	8.87 ± 0.12	1.3
hD1-WT	5.22 ± 0.11	5992	6.49 ± 0.07	322	7.42 ± 0.08	39	8.43 ± 0.07	3.7
C1A	5.08 ± 0.04	8271	6.27 ± 0.08	494	7.22 ± 0.09	60	8.37 ± 0.02	4.3
C2A	5.18 ± 0.07	6414	6.42 ± 0.04	373	7.21 ± 0.12	62	8.37 ± 0.05	4.3
C3A	5.06 ± 0.09	8778	6.29 ± 0.05	509	7.22 ± 0.10	69	8.37 ± 0.10	4.2
C4A	6.21 ± 0.09	616	7.28 ± 0.06	52	6.98 ± 0.31	59	8.34 ± 0.08	4.6

pK_I values (negative logarithm of K_I in molar) obtained from competition curves done in duplicate with [^{3}H]-SCH23390 and increasing concentrations of dopaminergic ligands are expressed as arithmetic means ± S.E.M from five experiments. The corresponding K_I values (nM) are also shown. Experiments for hD1-IL3C/deletion (ΔC1, ΔC2, ΔC3, ΔC4, ΔIL3C) and hD1-IL3C/alanine substitution (C1A, C2A, C3A, and C4A) mutants were performed independently.

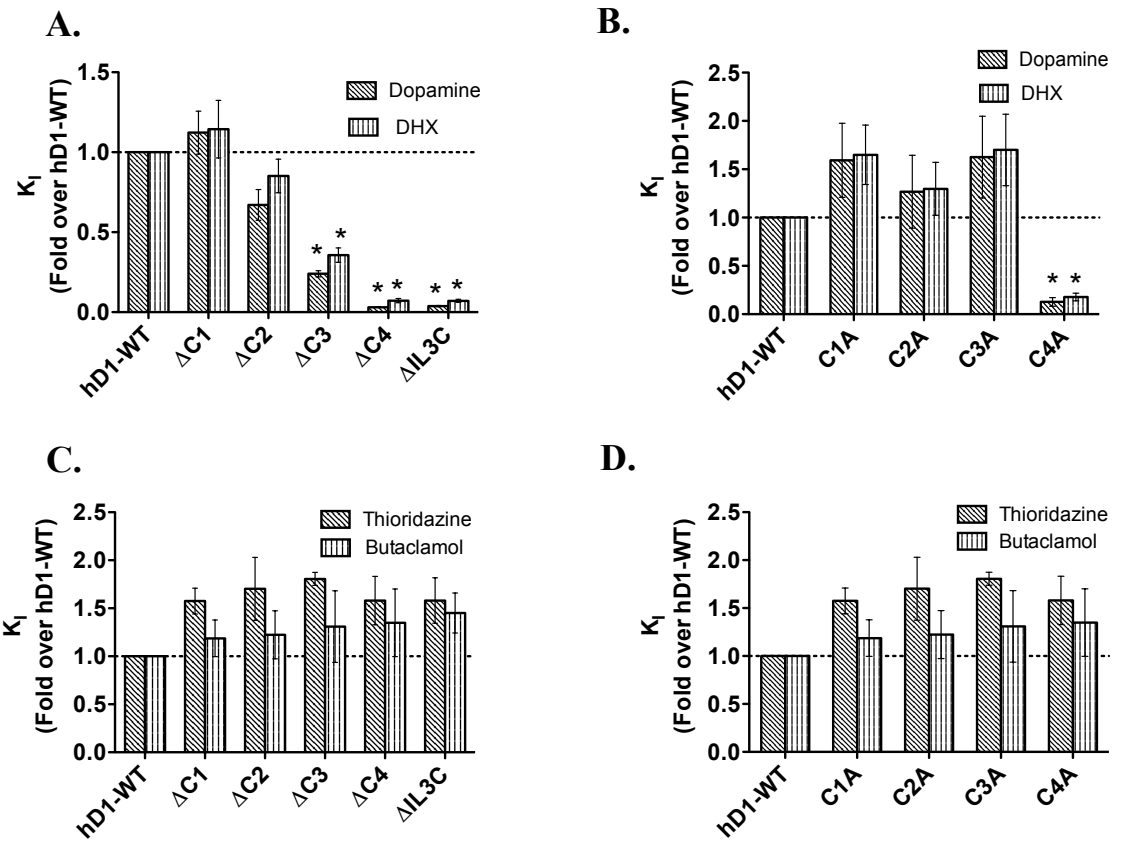


Figure 23. Fold change values for dopaminergic agonists (DA, DHX) and dopaminergic inverse agonists (thioridazine, butaclamol) affinities of hD1R mutants relative to hD1R WT

Raw K_I values obtained from competition studies were expressed as a fold-change relative to their respective WT receptor (Value set to 1). Relative K_I values were compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for hD1-IL3C/deletion (ΔC1, ΔC2, ΔC3, ΔC4, ΔIL3C) **(A, C)** and hD1-IL3C/alanine substitution (C1A, C2A, C3A, and C4A) **(B, D)** mutants were performed independently.

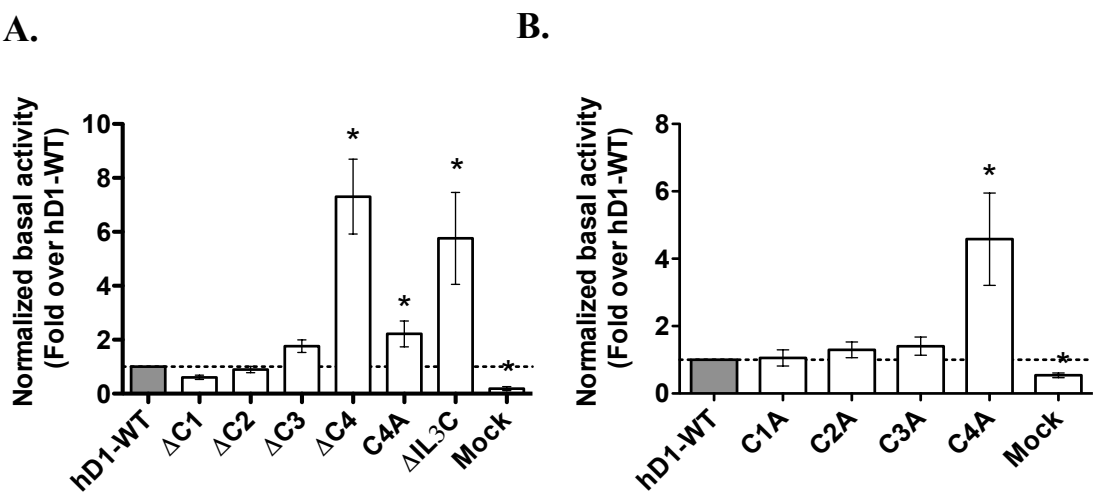


Figure 24. Constitutive activity of WT and hD1 mutant receptors expressed in HEK293 cells.

Cells transfected with a low DNA concentration (0.01 µg-0.15 µg/dish) of WT or hD1-mutants were seeded in 6-well plates and labeled with [^{3}H] adenine**.** Mock represents the condition in which the cells were transfected with empty DNA vector. Cells were treated with vehicle (ascorbic acid, 100 µM) or DA (10 µM) for 30 min at 37°C and intracellular cAMP levels were determined. DA-independent and dependent activation of AC were performed in triplicate determinations and were expressed as the arithmetic mean ± S.E.M of five experiments and normalized as the fold change relative to their respective WT receptor (Value set to 1). Relative values compared to 1 using a one-sample t test (* $p < 0.05$). Agonist-dependent maximal activation of AC assessed with 10 µM DA was observed for each condition and used as positive control for receptor responsiveness (not shown). B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 1.20, ΔC1: 1.11, ΔC2: 0.93, ΔC3: 1.03, ΔC4: 1.08, ΔIL3C: 1.08. **(B)** hD1-WT: 2.39, C1A: 2.17, C2A: 2.19, C3A: 2.20, C4A: 2.10. Each group of experiments for deletion and alanine mutant forms of hD1R was performed independently.

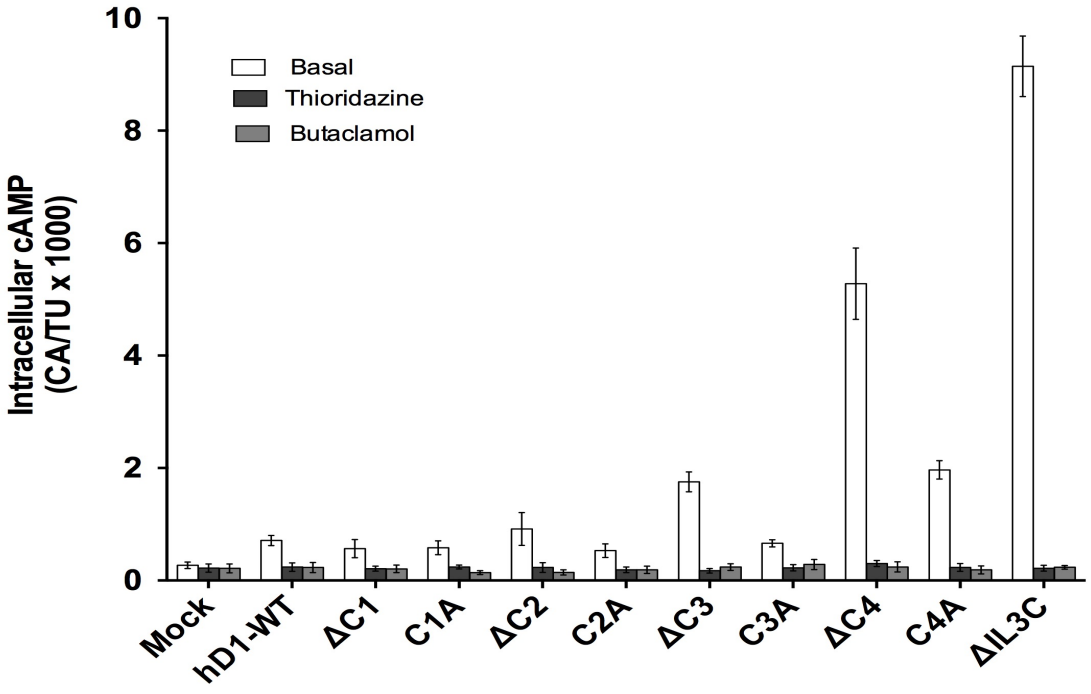


Figure 25. The effect of inverse agonists on the constitutive activity of hD1R-WT and mutant forms of hD1R in HEK293 cells.

Average raw data obtained from HEK293 cells transfected with low DNA concentration (0.01 µg-0.15 µg/dish) of WT or hD1-mutant forms. Mock represents the condition in which the cells were transfected with empty DNA vector. Transfected cells were seeded in 6-well plates in triplicate and labeled with [^{3}H] adenine, and then they were treated with vehicle (H_2O + 0.01 % v/v ethanol), thioridazine, or (+) butaclamol for 30 min at 37°C. Intracellular cAMP levels were determined in the absence or presence of inverse agonists and were expressed as arithmetic means ± S.E.M of three experiments. The inverse agonists used, thioridazine and butaclamol produced a significant reduction in the receptor basal activity of hD1-WT and all hD1-mutant forms. * $p < 0.05$ when compared with basal activity of each receptor using a one-way ANOVA followed by Sidak's multiple comparisons test. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are hD1-WT: 1.86, ΔC1: 1.78, C1A: 2.23, ΔC2: 2.29, C2A: 1.92, ΔC3: 1.65, C3A: 1.78, ΔC4: 0.70, C4A: 1.72, ΔIL3C: 1.67.

5. Deletion of IL3/C-terminal segments of hD1R differentially modulates E_{max} and EC_{50} values

Amplification of agonist potency is one of the hallmark characteristics of constitutively active GPCRs (Tiberi and Caron, 1994). Since, C4 (hD1/K261-K265) resembles constitutively active mutant receptors, we wanted to assess the G-protein coupling efficiency of these mutants versus WT. HEK293 cells were transiently transfected with hD1-WT or hD1-receptor mutants, and the level of dopamine-mediated AC activation was determined. In agreement with our previous study (Charrette and Tiberi, unpublished), ΔIL3C resulted in a robust augmentation of E_{max} and a significant leftward shift in EC_{50} (0.81 nM compared to 34 nM for hD1-WT), indicative of increased potency for DA. Small deletions generated on the IL3/C-terminal resulted in small changes in E_{max} and EC_{50} except for ΔC4 mutant. Substantial increase in the E_{max} associated with a significant increase in DA potency (~18-fold) in comparison to hD1-WT (1.8 nM for ΔC4 in comparison to 34 nM for hD1-WT) **(Fig. 26, Table 11)**. In agreement with my deletion results, the most significant change obtained with alanine mutations was observed with C4A mutant, which highlight how crucial this region is for the agonist-dependent activity of hD1R **(Fig. 26, Table 11)**. Small changes noted in the receptor potency and maximal activation of other IL3/C-terminal deletions/alanine mutant receptors (ΔC1, ΔC2, ΔC3, C1A, C2A, C3A) suggest that these segments play a modulatory role in the C4 section. With respect to deletion mutants, I cannot rule out that changes in IL3 length potentially promote the formation of different hD1R conformations with altered G-protein coupling sites leading to a reduced or an increased E_{max} and agonist potency.

Conclusion

K_D values from saturation studies indicate that neither deletions nor alanine-substitutions of the hD1-IL3/C-terminal have an aberrant effect on the overall ligand binding conformation of mutant receptors. The C-terminal moiety of the IL3 mediates a significant modulatory effect on the affinity of agonists while having no impact on the inverse agonist affinity. The far distal region of the IL3 of hD1R (Adjacent to TM6), K261-K265, plays a significant role in restraining the receptor in the inactive state as deletion or alanine replacement in this region (ΔC4, C4A) resulted in a high receptor constitutive activity, and a better DA efficacy and potency in comparison to hD1-WT **(Table S2)**. To complement this study and to further explore the impact of the hD1/K261-K265 on the receptor functionality, a single point mutation approach (deletion/alanine) was employed to map the major structural determinant-motif interaction site within the IL3-central region; the focus of the results reported in the following section 3.

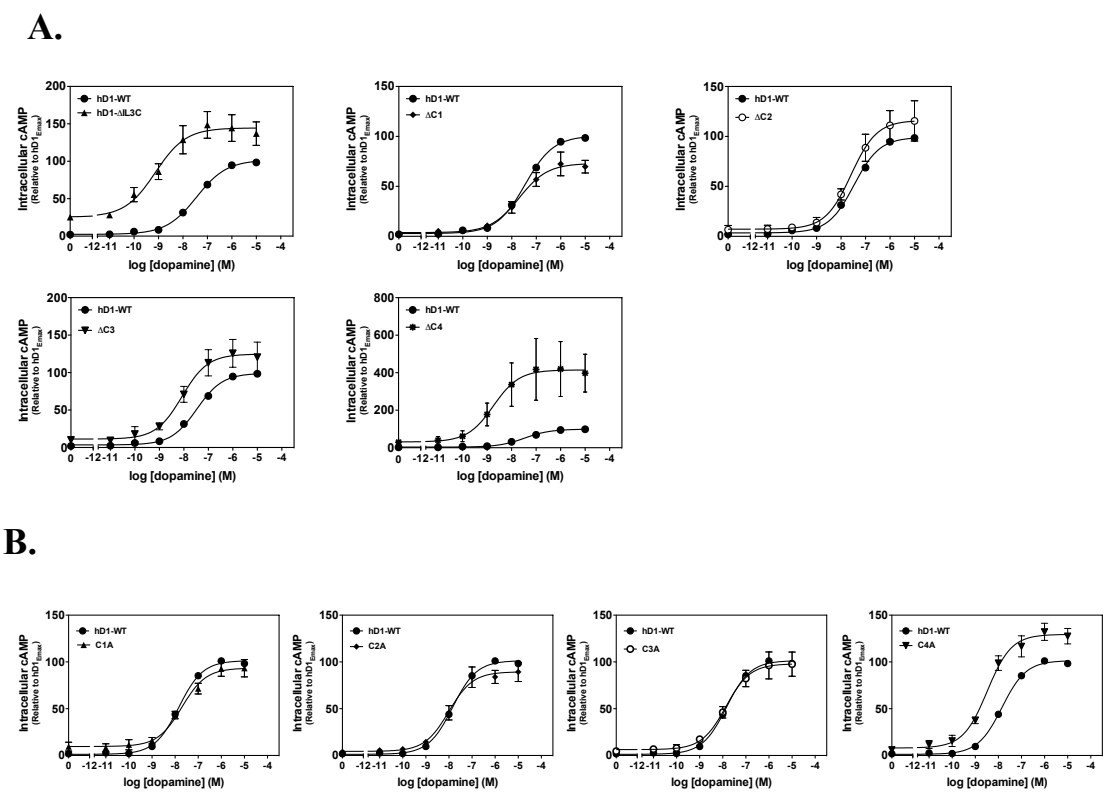


Figure 26. Dopamine-induced adenylyl cyclase activity of hD1-WT and hD1-IL3/C-terminal mutant receptors expressed in HEK293 cells.

Cells transfected with a low amount of different DNA receptor constructs (0.01 μg-0.15 μg/dish) were seeded in 12-well plates and labeled with [^{3}H] adenine. Dose-response curves were determined in the presence of increasing DA concentrations (0 to 10^{-4} M) done in triplicate. Each point represents the arithmetic mean of 4-6 experiments. The data were plotted as a function of log [DA] in molar. Simultaneous nonlinear curve fitting using raw data was performed using GraphPad Prism. Curves were normalized relative to 100% of the maximal activation (E_{max}) obtained with WT and then refitted to determine the EC_{50} and relative E_{max} of the mutant receptors. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 1.10, ΔIL3C: 1.28, ΔC1: 1.36, ΔC2: 1.21, ΔC3: 0.69, ΔC4: 0.97. **(B)** hD1-WT: 2.20, C1A: 2.42, C2A: 2.18, C3A: 2.09, C4A:1.89. Each group of experiments for hD1-IL3C/deletion **(A)** and hD1-IL3C/alanine mutant receptors **(B)** was performed independently.

Table 11. pEC_{50} and E_{max} values for DA-induced stimulation of adenylyl cyclase activity in HEK293 cells expressing WT and hD1-IL3/C-terminal mutant forms of hD1R

Receptor	pEC_{50} ± SEM	EC_{50} (nM)	E_{max}± SEM
hD1-WT	7.69 ± 0.18	33.91	100
ΔC1	7.92 ± 0.19	19.68	70 ± 6
ΔC2	7.52 ± 0.17	25.73	116 ± 20
ΔC3	8.15 ± 0.08	8.39	121 ± 20
ΔC4	8.65 ± 0.08	1.81	398 ± 50
ΔIL3C	9.08 ± 0.05	0.810	137 ± 16
hD1-WT	7.83 ± 0.05	14.33	100
C1A	7.94 ± 0.07	19.52	93 ± 9
C2A	7.99 ± 0.07	9.840	89 ± 10
C3A	7.90 ± 0.09	13.62	98 ± 13
C4A	8.42 ± 0.03	3.299	128 ± 8

Best-fitted values for pEC_{50} (negative logarithm of EC_{50} in molar) and the corresponding EC_{50} in nM and E_{max} obtained from curve-fitting of dose-response curves shown in Fig. 23 are reported.

Results: Section 3

1. Effect of single point deletions and single point alanine mutations made in the C4 segment of the IL3/C-terminal moiety on the functional expression (B_{max}) and the cell surface expression of hD1R

Saturation studies were done using the classical D1-class receptor specific ligand [^{3}H]-SCH23390 **(Table 12)**. The experiments were done in two separate sets. For the first set, HEK293 cells were transiently transfected at high expression level (5µg DNA/dish) with hD1-WT or hD1-single point deletions made at the far distal region of the hD1/IL3C-terminal, adjacent to TM6, (ΔK261, ΔM262, ΔS263, ΔF264, or ΔK265). For the second set, HEK293 cells were transiently transfected with 5µg DNA/dish with hD1-WT or hD1-single point alanine mutants (K261A, M262A, S263A, F264A, or K265A). The saturation studies showed that mutations had no effect on K_D of hD1R-deletion or alanine mutant receptors suggesting that the affinity of the mutant receptors for [^{3}H]-SCH23390 is not altered **(Table 12, Fig. 27A, 27B).**

Saturation studies also showed that the B_{max} values for the single point mutant receptors were reduced. The most significant B_{max} reductions relative to the hD1-WT were observed with deletions of F264 and K265. Additionally, among single point alanine substitutions performed only S263A and F264A mutants exhibited changes in B_{max} values when compared to WT. Notably, a significant increase was observed in B_{max} of S263A while the B_{max} value obtained for F264A mutant was strikingly reduced, by about five-fold and similar to the value measured with ΔF264 mutant **(Table 12, Fig. 27C, 27D)**. The reduction in B_{max} of the single point deletion mutants (ΔK261, ΔM262, ΔS263, ΔK265) was restored to a phenotype resembling the hD1-WT or even to a better phenotype in the case of ΔS263 upon alanine replacement. On the other hand, as K265

and F264 are adjacent to each other, the noticeable reduction in B_{max} of ΔK265 may be explained by the negative impact of its deletion on intramolecular interactions imposed by F264. Interestingly, the receptor expression of F264 deletion or alanine substitution was almost abolished, suggesting an important functional role of this residue in the maturation process and/or structural stability of hD1R. As some of these mutant receptors caused a reduction in B_{max} level, we tested the cell surface expression level using ELISA assay. Notably, the level of cell surface expression observed from the ELISA experiments is, in fact, relevant to the B_{max} values obtained for the same mutant receptor **(Fig. 28)**.

2. Effect of single point mutations within the far distal segment of the IL3-C terminal moiety on the ligand affinities of hD1R

Competition studies were done to test the affinity of the mutant receptors for different agonists (DA and DHX), and inverse agonists [thioridazine and (+) butaclamol] **(Table 13)**. The affinity of the deletion mutants (ΔK261, ΔM262, ΔS263) for the agonists used was virtually unchanged from hD1R-WT except for ΔF264 and ΔK264. Their affinity was markedly increased by about 20- and 30-fold respectively **(Fig. 29A)**. Interestingly, single point alanine substitutions gave different results for K261 and S263. A ~2-fold reduction in the affinity of K261 and S263 for DA was observed but not for DHX, which was also reduced but to a lesser extent. Again, the same prominent increase in the affinity of F264A was noticed and was comparable to that seen with deletion of this same amino acid **(Fig. 29B)**. Interestingly, the affinity for the inverse agonists (thioridazine and butaclamol) remained unchanged with either single point deletion or single point alanine mutant receptors except for F264A that displayed a ~2-fold reduction **(Fig. 29C, 29D)**.

The results obtained here reflect the importance of F264 for regulating the agonist affinity as highlighted by the deletion or alanine mutation of this amino acid. These alterations have led to an improved version of the receptor with respect to agonist binding. My results also point to the role played by F264 on modulating the inverse agonist binding affinity. Notably, CAM receptors usually exhibit a higher agonist binding affinity and a lower inverse agonist affinity. In the following sections, I have assessed whether F264 deletion and alanine mutant receptors behave as CAM receptors.

Table 12. Equilibrium dissociation constant (K_D) and maximum binding capacity (B_{max}) values of [^{3}H]-SCH23390 for WT and single point mutant forms of hD1R.

Receptor	[^{3}H]-SCH23390 pK_D (K_D nM)	B_{max} (pmol/mg protein) ± SEM
hD1-WT	9.35 ± 0.04 (0.45)	16.55 ± 1.29
ΔK261	9.41 ± 0.04 (0.39)	7.91 ± 0.61
ΔM262	9.40 ± 0.07 (0.40)	9.74 ± 0.89
ΔS263	9.20 ± 0.07 (0.63)	12.57 ± 0.76
ΔF264	9.28 ± 0.03 (0.52)	3.04 ± 0.34
ΔK265	9.21 ± 0.05 (0.62)	1.62 ± 0.15
ΔC4	9.19 ± 0.05 (0.65)	1.82 ± 0.11
hD1-WT	9.31 ± 0.02 (0.49)	20.78 ± 1.59
K261A	9.25 ± 0.04 (0.56)	16.96 ± 1.63
M262A	9.25 ± 0.01 (0.56)	20.37 ± 1.58
S263A	9.20 ± 0.01 (0.62)	29.78 ± 1.96
F264A	9.10 ± 0.07 (0.79)	4.25 ± 0.54
K265A	9.17 ± 0.04 (0.67)	20.81 ± 1.31
C4A	9.18 ± 0.04 (0.66)	6.66 ± 0.54

pK_D (negative logarithm of K_D) and B_{max} values (pmol/mg of protein) obtained from saturation curves done in duplicate and are expressed as means ± S.E.M from six experiments. The corresponding K_D and B_{max} values in nM are shown between brackets Experiments for hD1-IL3C/deletion (ΔK261, ΔM262, ΔS263, ΔF264, ΔK265, ΔC4) and hD1-IL3C/alanine mutant receptors (K261A, M262A, S263A, F264A, K265A, C4A) were performed independently.

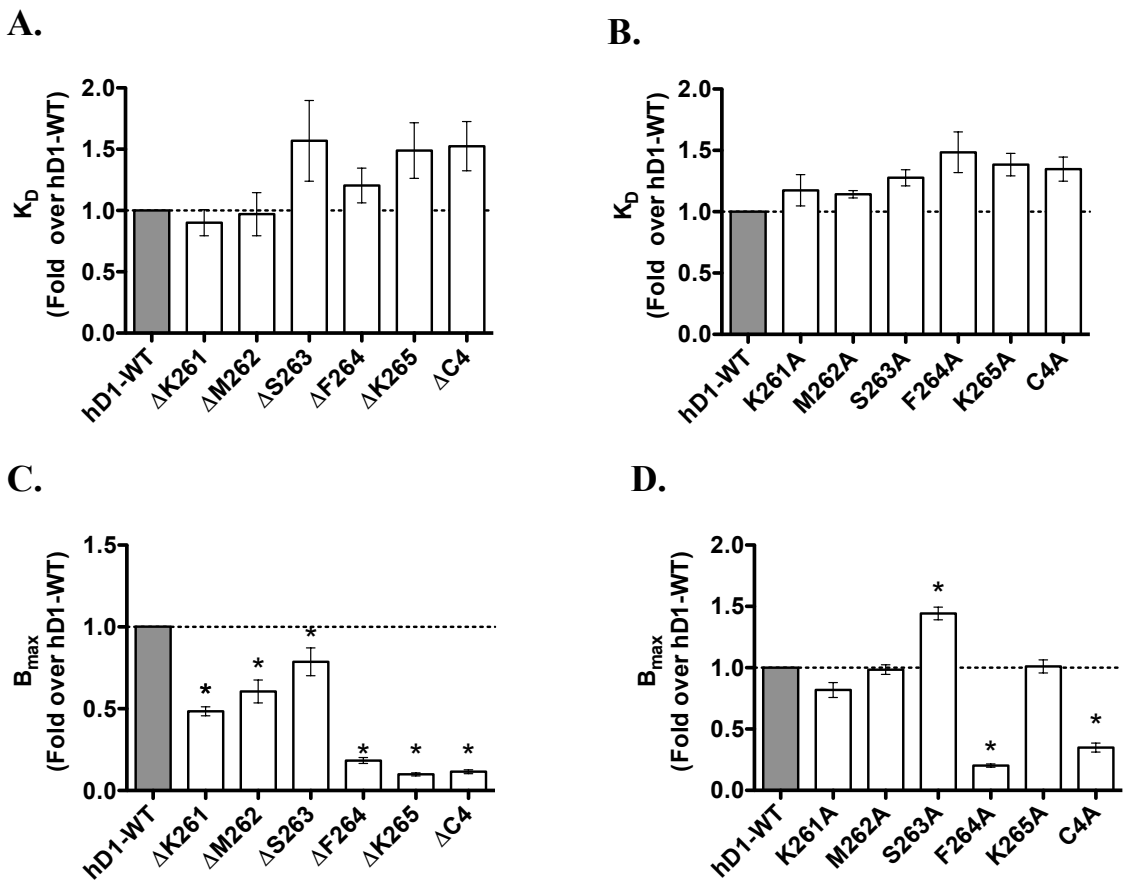


Figure 27: Fold change values of equilibrium dissociation constant (K_D) and maximal binding capacity (B_{max}) of single point mutant receptors for [^{3}H]-SCH23390 relative to WT.

Raw K_D and B_{max} values obtained from saturation studies were expressed as fold-change relative to their respective WT-receptor (value set to 1). Relative K_D and B_{max} values were compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for hD1-IL3C/deletion (ΔK261, ΔM262, ΔS263, ΔF264, ΔK265, ΔC4) and hD1-IL3C/alanine substitution (K261A, M262A, S263A, F264A, K265A, C4A) mutant hD1R were performed independently.

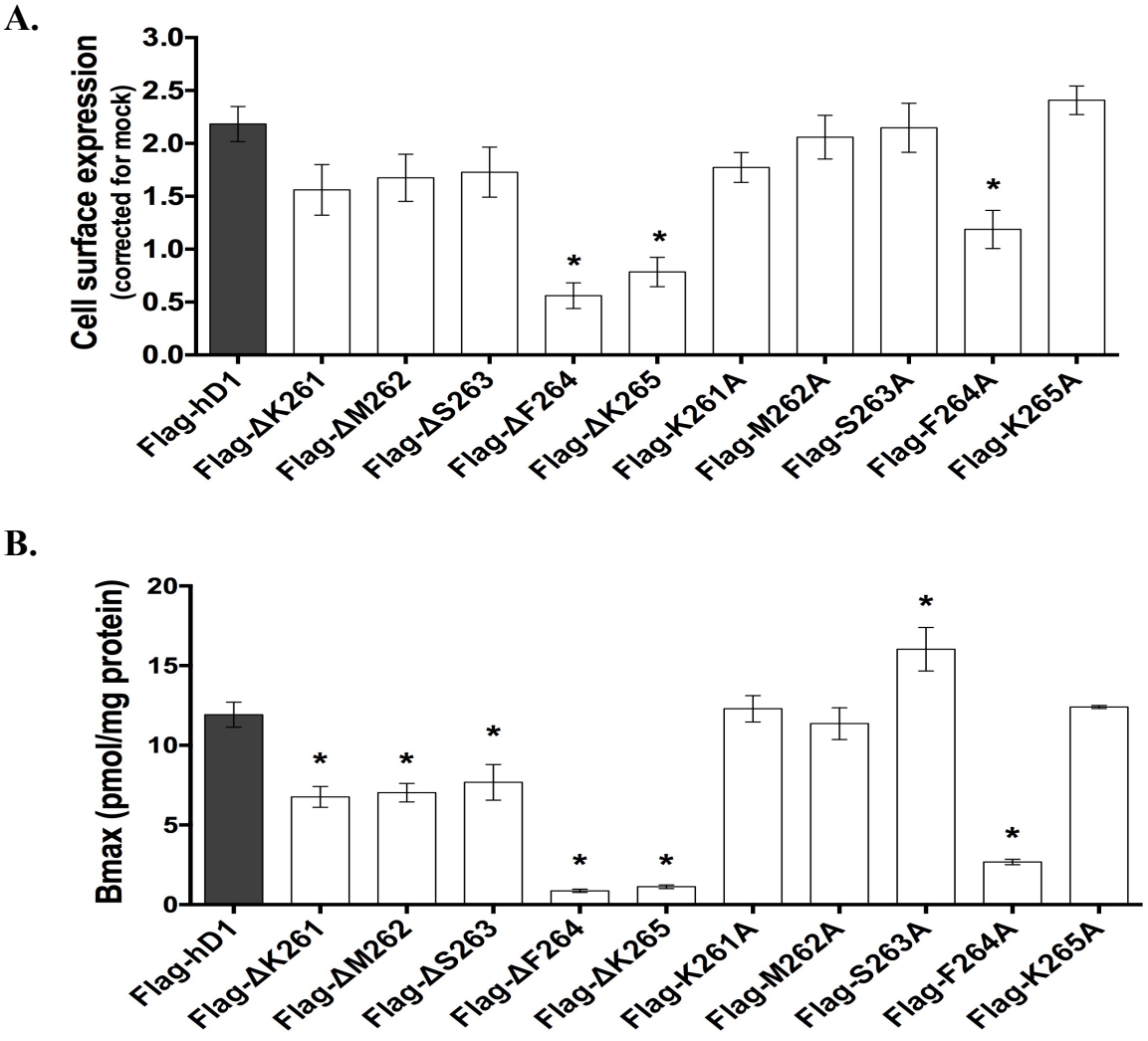


Figure 28: Cell surface expression of Flag-tagged WT and single point mutant forms of hD1R expressed in HEK293 cells

(A) Raw cell surface expression values obtained from ELISA were subtracted from control (mock-transfected cells) and expressed as the mean ± S.E.M of four experiments done in triplicate determination. **(B)** B_{max} in pmol/mg membrane proteins for [^{3}H]-SCH23390 (expressed as arithmetic means ± S.E.M.) is shown. Values are as follows: Flag/hD1-WT: 11.92, Flag-ΔK261: 6.76, Flag-ΔM262: 7.03, Flag-ΔS263: 7.68, Flag-ΔF264: 0.87, Flag-ΔK265: 1.13, Flag-K261A: 12.29, Flag-M262A: 11.36, Flag-S263A: 16.03, Flag-F264A: 2.68, Flag-K265A: 12.41. * $p < 0.05$ when compared with Flag/hD1-WT using a one-way ANOVA followed by Dunnett's multiple comparisons test. All experiments were performed in HEK293 cells transfected with 5 μg per 100-mm dish of WT and mutant receptor constructs in pCMV5 or empty pCMV5 (mock).

Table 13. pK_I values of agonists and inverse agonists for WT and single point mutant forms of hD1R

Receptor	Dopamine		Dihydrexidine		Thioridazine		Butaclamol	
	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)	pK_I ± SEM	K_I (nM)
hD1-WT	5.21 ± 0.06	6167	6.36 ± 0.05	439	7.36 ± 0.04	44	8.84 ± 0.18	1.5
ΔK261	5.44 ± 0.11	3658	6.64 ± 0.04	227	7.36 ± 0.04	43	8.87 ± 0.21	1.4
ΔM262	5.38 ± 0.12	4169	6.46 ± 0.04	345	7.38 ± 0.07	41	8.89 ± 0.25	1.3
ΔS263	5.20 ± 0.08	6257	6.29 ± 0.06	410	7.38 ± 0.03	42	8.89 ± 0.15	1.3
ΔF264	6.56 ± 0.13	274	7.42 ± 0.10	38	7.30 ± 0.04	50	8.78 ± 0.12	1.7
ΔK265	6.78 ± 0.12	168	7.44 ± 0.05	37	7.28 ± 0.10	53	8.80 ± 0.24	1.6
ΔC4	6.84 ± 0.25	146	7.46 ± 0.12	35	7.13 ± 0.04	74	8.81 ± 0.26	1.6
hD1-WT	5.14 ± 0.03	7269	6.17 ± 0.03	684	7.32 ± 0.04	48	8.72 ± 0.04	1.9
K261A	4.90 ± 0.04	12489	6.06 ± 0.03	874	7.35 ± 0.05	44	8.78 ± 0.06	1.7
M262A	4.99 ± 0.04	10190	6.09 ± 0.03	811	7.31 ± 0.04	49	8.67 ± 0.05	2.1
S263A	4.87 ± 0.04	13645	6.02 ± 0.05	948	7.31 ± 0.03	49	8.72 ± 0.07	1.9
F264A	6.35 ± 0.11	451	7.35 ± 0.04	45	7.13 ± 0.02	74	8.58 ± 0.04	2.7
K265A	5.22± 0.23	10075	6.22 ± 0.19	935	7.28 ± 0.06	53	8.68 ± 0.05	2.1
C4A	6.13 ± 0.03	746	7.01 ± 0.08	97	7.12 ± 0.02	75	8.61 ± 0.03	2.5

pK_I values (negative logarithm of K_I in molar) determined from competition studies done in duplicate with [^{3}H]-SCH23390 using increasing concentrations of dopaminergic ligands are reported as means ± S.E.M from 3-6 experiments. The corresponding K_I values (nM) are also shown. Experiments for hD1-IL3C/deletion (ΔK261, ΔM262, ΔS263, ΔF264, ΔK265, ΔC4) and hD1-IL3C/alanine substitution (K261A, M262A, S263A, F264A, K265A, C4A) mutants were performed independently.

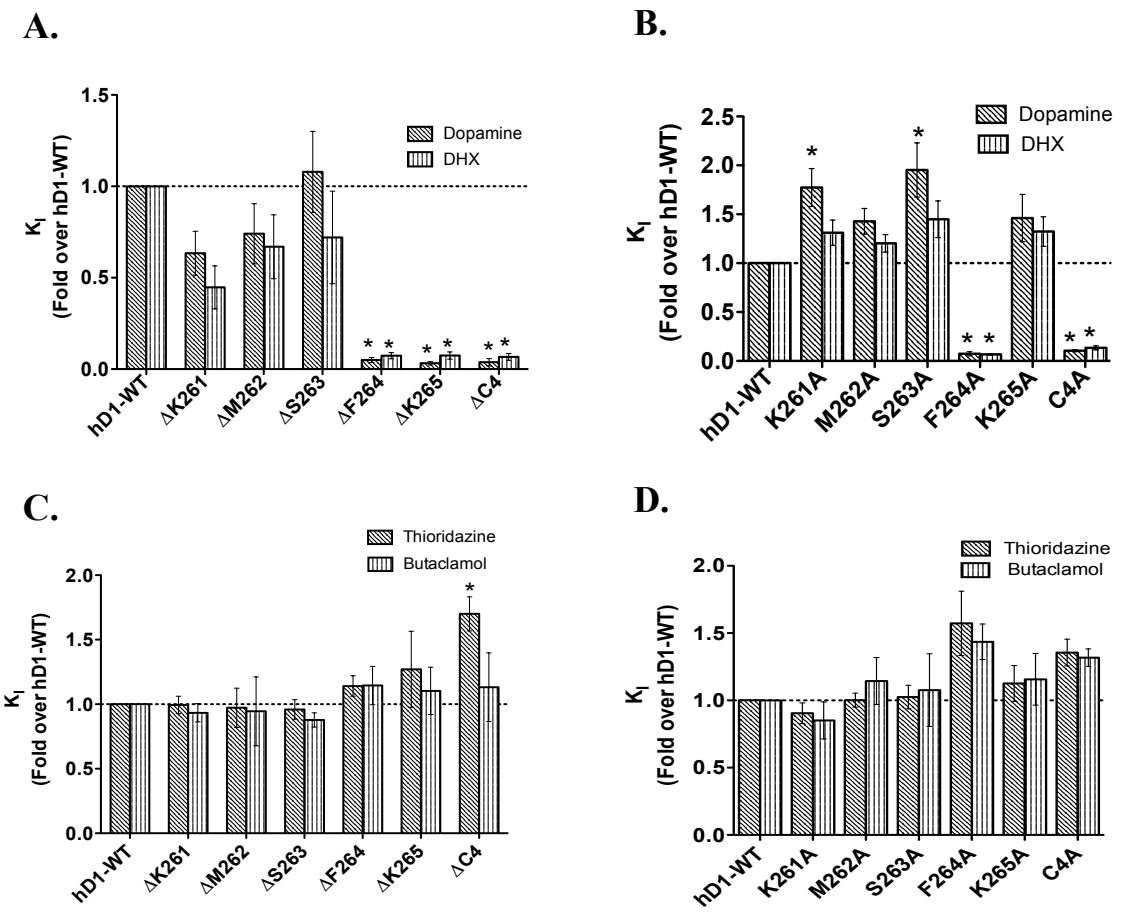


Figure 29. Fold changes values for dopaminergic agonists (DA and DHX) and inverse agonists (thioridazine, butaclamol) affinities of single point mutant receptors relative to WT.

Raw K_I values obtained from competition studies were expressed as fold change relative to the hD1-WT receptor (value set to 1). Relative values were compared to 1 using a one-sample t-test (* $p < 0.05$). Experiments for hD1-IL3C/deletion (ΔK261, ΔM262, ΔS263, ΔF264, ΔK265, ΔC4) **(A,C)** and hD1-IL3C/alanine substitution (K261A, M262A, S263A, F264A, K265A, C4A) **(B, D)** mutants were performed independently.

3. Agonist-independent activity of WT and single point mutant forms of hD1R

Whole cell cAMP studies show a drastic augmentation in the agonist-independent activity in cells transfected with F264 mutant receptors (ΔF264, F264A). These findings along with the increase in the agonist affinity observed with these two mutants are reminiscent of the properties reported for CAM receptors. An increase in the basal activity of ΔK265 mutant but not with K265A mutant was also observed. A similar pattern was noticed with binding studies, which demonstrated increased agonist affinity with ΔK265 but not with K265A **(Fig. 30A, 30B)**. As discussed above, the effect K265 deletion on agonist-independent activity is potentially explained by an alteration of the intramolecular interactions dependent on F264 caused by the spatial rearrangement of F264. This effect is likely explained by a change in the net charge brought by the removal of the basic residue lysine as its substitution with alanine (A) led to basal activity that was unchanged relative to WT. Overall, these results suggest that F264 imparts a negative constraint on the receptor ability to adopt an active conformation (R*) in the absence of agonists.

4. The effect of inverse agonists on the constitutive activity of single point mutant forms of hD1R.

Since some of these mutants have a very high level of basal activity and appear to resemble the CAM receptors, we tested whether inverse agonists retain their efficacy for blocking the constitutive activity of mutants. AC activity was evaluated in the presence or absence of two inverse agonists, thioridazine and (+) butaclamol. Interestingly, these two

drugs exhibited inverse agonism at all mutant receptors but not to the same extent relative to WT receptor. The strongest effect was seen with the mutants displaying a very high level of basal activity such as ΔF264 and F264A **(Fig. 31)**.

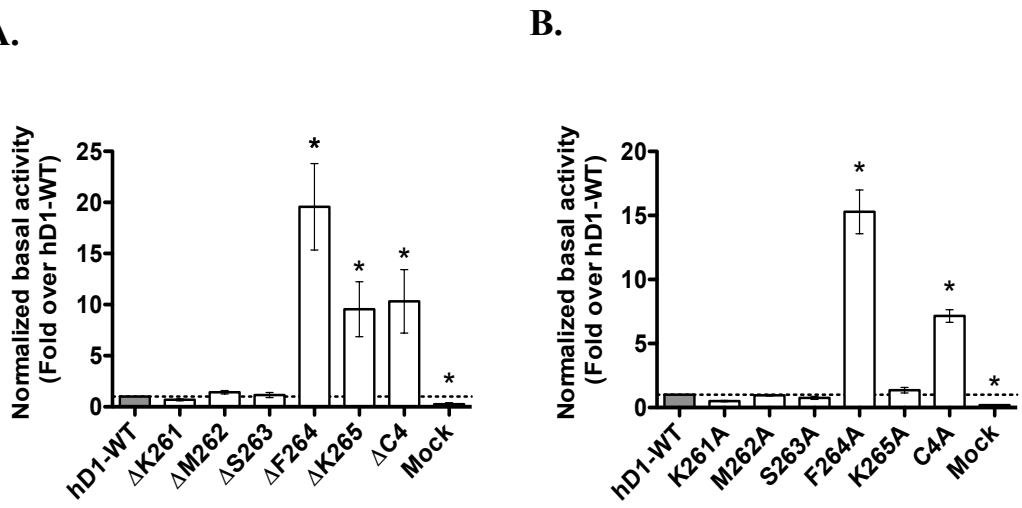


Figure 30. Constitutive activity of hD1-WT and hD1-single point mutant receptors expressed in HEK293 cells.

Cells transfected with a low DNA concentration (0.01 μg-0.2 μg/dish) of WT or hD1-mutants were seeded in 6-well plates and labeled with [^{3}H] adenine**.** Mock represents the condition in which the cells were transfected with empty DNA vector. Cells were treated with vehicle (ascorbic acid, 100 μM) or DA (10 μM) for 30 min at 37°C and intracellular cAMP levels were determined. DA-independent and dependent activation of AC were performed in triplicate determinations and were expressed as the arithmetic mean ± S.E.M of five experiments and normalized as the fold change relative to their respective WT receptor (Value set to 1). Relative values compared to 1 using a one-sample t-test (* $p < 0.05$). Agonist-dependent maximal activation of AC assessed with 10 μM DA was observed for each condition and used as positive control for receptor responsiveness (not shown). B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 1.20, ΔK261: 1.62, ΔM262: 2.20, ΔS263: 1.84, ΔF264: 2.29, ΔK265: 1.38, ΔC4: 1.50 **(B)** hD1-WT: 1.20, K261A: 1.11, M262A: 0.93, S263A: 1.03, F426A: 1.08, K265A: 1.28, C4A: 1.08. Each group of experiments for single point deletion and single point alanine mutant forms of hD1R was performed independently.

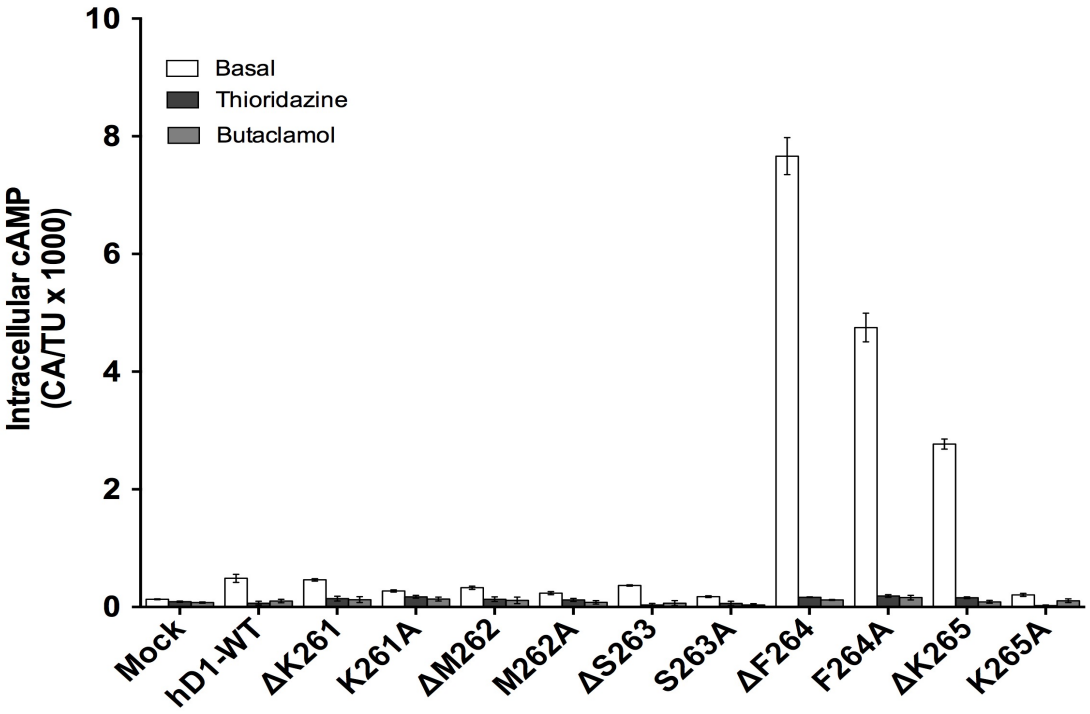


Figure 31. The effect of inverse agonists on the constitutive activity of hD1R-WT and hD1-single point mutant receptors expressed in HEK293 cells.

Average raw data obtained from HEK293 cells transfected with a low DNA concentration (0.01 µg-0.2 µg/dish) of WT or hD1-mutant forms. Mock represents the condition in which the cells were transfected with empty DNA vector. Transfected cells were seeded in 6-well plates in triplicate and labeled with [^{3}H] adenine, and then they were treated with vehicle (H_2O+ 0.01 % v/v ethanol), thioridazine, or (+) butaclamol for 30 min at 37°C. Intracellular cAMP levels were determined in the presence or absence of inverse agonists and were expressed as arithmetic means +S.E.M of three experiments. The inverse agonists used, thioridazine and butaclamol produced a significant reduction in the receptor basal activity of hD1-WT and all hD1-mutant forms. * $p < 0.05$ when compared with basal activity of each receptor using a one-way ANOVA followed by Sidak's multiple comparisons test. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are hD1-WT: 1.98, ΔK261: 2.41, K261A: 2.69, ΔM262: 1.89, M262A: 2.08, ΔS263: 2.02, S263A: 2.24, ΔF264: 2.12, F426A: 2.00, ΔK265: 0.74, K265A: 2.09.

5. The effect of single point deletion and alanine substitution in the C4 segment of hD1R on EC_{50} and E_{max} of dopamine.

Dose-response curves for DA were performed to measure EC_{50} and E_{max} values in cells expressing the mutant receptors relative to that transfected with hD1-WT **(Table 14)**. Consistent with my previous results, single point mutation of the amino acid residues of the C4 segment of IL3/C-terminal moiety resulted in either no change or increase of E_{max}. Deletion of ΔK261, ΔM262, ΔS263 has produced no or a slight change in DA potency (index with EC_{50}) relative to WT receptor. Interestingly, the single point mutants K261A, M262A and S263A behaved similarly as their deletion counterpart with respect to E_{max} but displayed greater EC_{50} rightward shift ranging from 2 to 5-fold relative to hD1-WT; implying a loss in DA potency **(Fig. 32A, 32B)**. In contrast, deletion of F264 or K265 resulted in a robust increase in DA-mediated maximal AC activation, which indicates an increase in the DA efficacy (~2.5-fold). Additionally, the deletion of these two residues (F264, K265) has resulted in augmentation in DA potency by about 100-fold and 20-fold, respectively **(Fig. 32A)**. In fact, these findings are consistent with the increased constitutive activity of ΔF264 and ΔK265 **(Fig. 30A)**. It is remarkable that alanine substitution made at K265 led to a phenotype resembling E_{max} and EC_{50} of hD1-WT. As discussed above, this rescue phenomenon of ΔK265 could be explained by the role played by this residue in the spatial modulation of intramolecular interactions involving F264. In fact, the exchange of Lys (K) with Ala (A) might have left the molecular constraints imparted by F264 on agonist-induced activation of hD1R. Indeed, the effect seen with a ΔF264, F264A evoked the same level of increase in E_{max} but with a ~13-fold increase in EC_{50} in comparison to 100-fold for ΔF264. Therefore, F264 controls

the receptor ability to be activated in presence or absence of DA, which highlight further the crucial role played by this F264 in constraining the receptor in the inactive state.

Conclusion

The results of this study showed that the lower D1R basal activity could be caused by the presence of single amino acid residue, F264, located in the C-terminal moiety of IL3, just adjacent to TM6. Deletion or alanine replacement of this single amino acid has led to a phenotype resembling CAM receptors, and to some extent D5R, which naturally displayed CAM properties such as increased agonist affinity and potency, and higher basal activity. How mutation of this single amino acid residue would induce these changes in the receptor activity, and whether this effect is solely mediated by F264 via its interaction with other structural elements of the receptor will need to be further addressed in future studies **(Table S3)**.

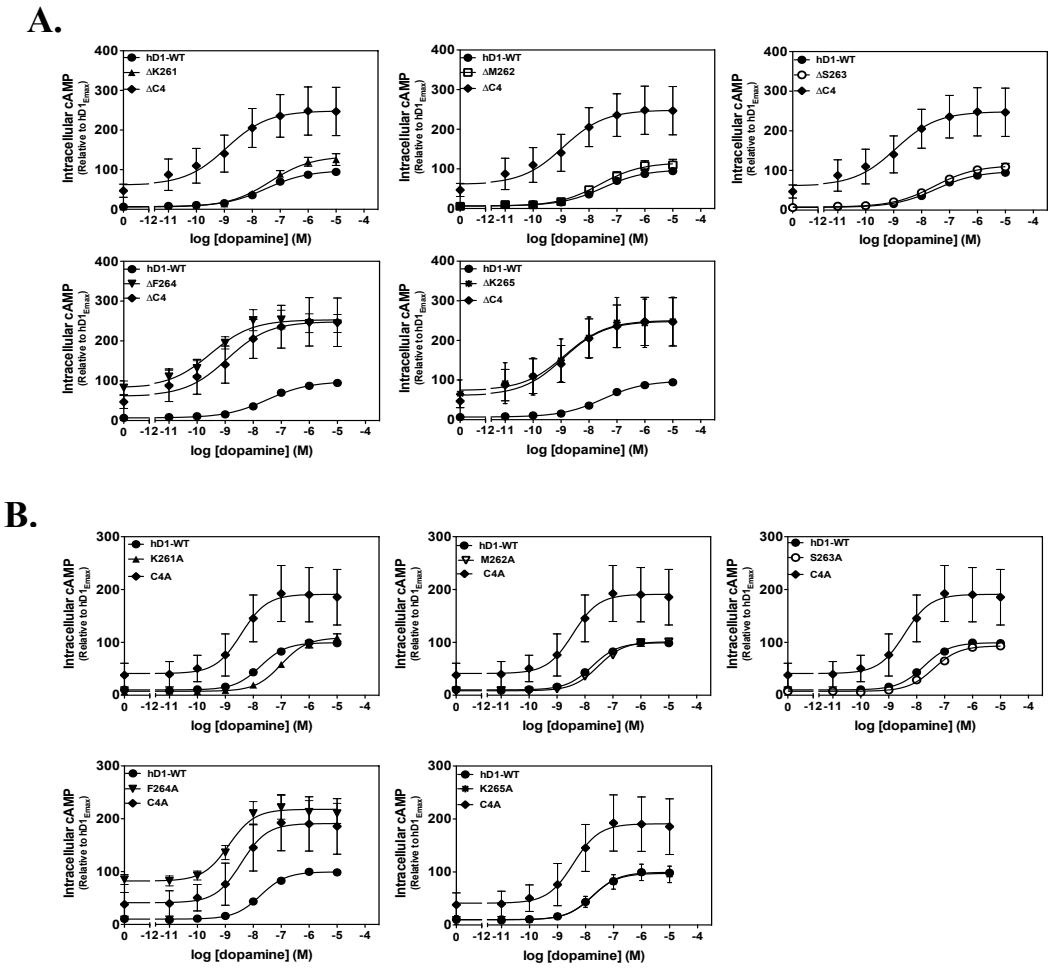


Figure 32. Dose-response curves for dopamine-induced stimulation of adenylyl cyclase activity in HEK293 cells expressing WT and hD1-single point mutants.

Cells transfected with a low amount of different DNA receptor constructs (0.01 μg-0.15 μg/dish) were seeded in 12-well plates and labeled with [^{3}H] adenine. Dose-response curves were determined in the presence of increasing concentrations of dopamine (DA) (0 to 10^{-4} M) done in triplicate. Each point represents the arithmetic mean of 6-7 experiments. Data were plotted as a function of log [DA] in molar. Simultaneous nonlinear curve fitting using raw data was performed using GraphPad Prism. Curves were normalized relative to 100% of the maximal activation (E_{max}) obtained with WT and then refitted to determine the EC_{50} and relative E_{max} values for the mutant response. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are **(A)** hD1-WT: 1.56, ΔK261: 1.39, ΔM262: 1.52, ΔS263: 1.47, ΔF264: 1.51, ΔK265: 1.05, ΔC4: 1.08 **(B)** hD1-WT: 2.13, K261A: 2.11, M262A: 2.06, S263A: 2.17, F264A: 2.93, K265A: 2.30, C4A: 3.55. Each group of experiments for single point deletion and single point alanine mutant forms of hD1R was performed independently.

Table 14. pEC_{50} and E_{max} values for DA-induced stimulation of adenylyl cyclase activity in HEK293 cells expressing hD1 WT and hD1-single point mutant forms of hD1R.

Receptor	pEC_{50} ± SEM	EC_{50} (nM)	E_{max} ± SEM
hD1-WT	7.45 ± 0.14	31.82	100
ΔK261	7.36 ± 0.12	44.26	126 ± 15
ΔM262	7.62 ± 0.14	24.60	110 ± 14
ΔS263	7.77 ± 0.29	28.68	109 ± 9
ΔF264	9.42 ± 0.17	0.306	244 ± 23
ΔK265	9.14 ± 0.20	1.365	248 ± 25
ΔC4	8.90 ± 0.06	1.198	247 ± 25
hD1-WT	7.72 ± 0.07	18.05	100
K261A	6.83 ± 0.09	100.3	109 ± 7
M262A	7.53 ± 0.05	29.66	101 ± 6
S263A	7.37 ± 0.08	40.46	93 ± 5
F264A	8.87 ± 0.05	1.334	210 ± 19
K265A	7.76 ± 0.06	17.00	96 ± 6
C4A	8.40 ± 0.08	3.704	186 ± 20

Best-fitted values for pEC_{50} (negative logarithm of EC_{50} in molar) and the corresponding EC_{50} in nM and E_{max} obtained from curve-fitting of dose-response curves shown in Fig. 23 are reported.

Chapter 4

DISCUSSION

Discussion

Although crystallography has provided valuable information regarding the structural basis and molecular dynamics of GPCRs, the structural role of the IL3 remains unclear as this region was substantially modified or excluded in to resolve these crystal structures. As discussed earlier, this is explained by the high degree of flexibility of the IL3 in solubilized GPCRs, which does not provide the required degree of stability needed for GPCR crystallization (Palczewski et al., 2000, Cherezov et al., 2007, Rasmussen et al., 2007, Kobilka and Schertler, 2008, Warne et al., 2008, Baker et al., 2011, Lebon et al., 2011, Rasmussen et al., 2011a, Rasmussen et al., 2011b, Steyaert and Kobilka, 2011, Granier et al., 2012, Manglik et al., 2012, White et al., 2012, Wu et al., 2012, Scott and Pluckthun, 2013, Egloff et al., 2014).

In spite of intensive research on the structural basis of GPCRs, the underlying functional role of variable IL3 length and amino acid composition among GPCRs remains elusive. In fact, the IL3 structural determinants implicated in G-protein binding and activation, as well as in the formation of the high-affinity ligand binding site and regulation of proper protein folding conformation for the vast majority of GPCRS, notably DARs, have yet to be identified (Cotecchia et al., 1990, Kjelsberg et al., 1992, Ostrowski et al., 1992, Samama et al., 1993, Nielsen et al., 1998, Cotecchia, 2007). Importantly, the IL3 length and primary structure vary from one GPCR to another and from one GPCR class to another, which makes it difficult to draw a general conclusion from the extensive body of studies conducted mostly with the prototypical rhodopsin and β2-adrenergic receptors. For instance, a proline-rich region, consisting of 24 residues, found exclusively within the central area of the IL3 of the β1-adrenergic receptor was

shown to be responsible for the differential activation profile between β1- and β2 adrenergic receptors (Green and Liggett, 1994).

A previous effort in our lab was employed to investigate the IL3 functional significance of hD1-class receptors. The work suggests that the central region of the IL3 is an important element for hD1-class receptor activation and G-protein coupling. The results showed that the conserved N-terminal moiety of the IL3 is important for regulating agonist affinity of the receptor, basal (constitutive) activity, efficacy and potency of DA. On the other hand, the divergent C-terminal moiety of the IL3 plays a role in constraining the receptor in the inactive state. A deletion of the IL3/C-terminal moiety facilitates the transition of the receptor from the inactive to the active state and leads to a more constitutively active hD1R and hD5R mutants relative to their respective WT receptor (Charrette and Tiberi, unpublished).

In this Ph.D. thesis, my aim was to unmask the amino acids, and potentially structural motifs, within the IL3 central region of hD1-class receptors regulating ligand binding and G protein coupling (basal activity and DA-dependent receptor activation). Herein, I have used a deletion and alanine scanning mutational approaches to pinpoint the amino acids within the IL3 central region, which differentially modulate hD1-class receptors. The focus of the first section of this thesis was mainly on studying the role of the IL3/N-terminal moiety of hD1-class receptors. In this section, merely based on the primary structure of the receptor, the IL3/N-terminal region of hD1R and hD5R was divided into two halves where either a deletion or an alanine substitution was used to address the role of the IL3/N-terminal moiety. The scope of the second and the third sections of this thesis were solely on the IL3/C-terminal region of hD1R. In the second

part, a series of four consecutive deletions and alanine substitution for the corresponding deleted regions (5 amino acid stretches) were generated within the carboxyl end of the hD1-IL3/central moiety. The focus of the third section was designed based on the result of the second section where a single amino acid deletion or a single-point alanine mutation was introduced in the far distal region of the hD1/IL3-C-terminal in order to identify the particular structural element modulating hD1R functionality.

1. The effect of deletion/alanine modification of the IL3 central region on folding conformation and cell surface protein expression of hD1-class receptors

Binding assays using the classical D1-class receptor selective ligand [^{3}H]-SCH23390 assisted in quantifying the affinity of the mutant receptors to [^{3}H]-SCH23390 and also to determine the receptor level of expression. Interestingly, all of the mutants generated retained their ability to bind to [^{3}H]-SCH23390 with high affinity, which indicates that none of the mutant receptors displayed any aberrant effect in the ligand binding pocket. Notably, deletion or alanine replacement generated at different regions of the IL3 central region produced different levels of B_{max}. Although there is a structural similarity in the IL3/N-terminal moiety of hD1-class receptors, mutations introduced in this cytoplasmic region have yielded different phenotypes with regard to receptor expression (B_{max}). Remarkably, both deletion and alanine substitution made at the far proximal side of the IL3/N-terminal moiety of hD1, hD1-ΔN1, and hD1-N1A, have resulted in a significant reduction of B_{max} as opposed to hD5 mutants. Only alanine substitution at the first half of the N-terminal moiety of hD5, hD5-N1A, has resulted in a decrease in B_{max} while deletion, hD5-ΔN1, made in the same region of hD5R had no

effect on B_{max} **(Fig. 17C, 17D)**. In agreement with our study, reduction in the B_{max} level was also observed with mutant μ-opioid receptor lacking the first five amino acids of the IL3, located at the far proximal side of the IL3/N-terminal moiety (Chaipatikul et al., 2003).

Alteration of the primary protein structure can have a negative effect on the receptor maturity level and their exportation to the plasma membrane. For instance, mutations in GPCRs could influence the ability of the ER to recognize these modified receptors. It is possible that the ER has identified these mutant receptors as faulty ones and directed them to lysosomes for degradation. Additionally, changing the receptor folding conformation by deletion or alanine scanning could have influenced the receptor binding ability to different ER shuttling partners required for GPCR delivery to plasma membrane leading to ER retention of hD1-class mutants and reduction of B_{max} (index of the binding capacity of functionally mature receptors). Indeed, GPCRs have been shown to interact with GPCR interacting proteins (GIPs). GIPs have several functions; among them is the trafficking of GPCRs to and from the plasma membrane; therefore, aids in the receptor expression at the cell surface. Most of these GIPs are soluble proteins, which have been shown to interact with the CT tail of GPCRs while a very few have been shown to interact with the IL3 (Kim et al., 2002b, Bockaert et al., 2004). It is possible that shortening the receptor length made by deletion or altering the receptor structure by alanine insertion could have resulted in new intermolecular interactions between different receptor domains. As a result, an improvement or a loss in the interaction between the CT or the IL3 and GIPs may have led to the observed changes in B_{max}. Indeed, D1R was shown to interact with different GIPs like DRiP78 and neurofilament M. Notably, it has

been demonstrated that neurofilament-M interacts with the IL3 of D1R in vivo and HEK293 cells leading to a reduction in D1R expression at the cell surface (Kim et al., 2002b).

Concerning mutants engineered at the IL3/C-terminal moiety, there was a general pattern observed concerning the effect of these mutants on the B_{max} level. Notably, deletion of the first three consecutive regions located at the distal side of the IL3/C-terminal moiety of hD1R (ΔC1, ΔC2, ΔC3) produced a reduction in B_{max}. On the other hand, alanine substitution for the same deleted regions (C1A, C2A, C3A) either restored B_{max} to WT receptor level or produced a higher B_{max}. Importantly, both deletion and alanine replacement made at the far distal side of the IL3/C-terminal moiety (K261-K265: ΔC4, C4A) evoked a marked reduction in B_{max} with a phenotype similar to the level detected previously with hD1-ΔIL3 mutant **(Fig. 21C, 21D)** (Charrette and Tiberi, unpublished).

In light of these results, single point deletion/alanine mutations were engineered to ascertain which one of the five amino acids within the C4 region, is responsible for lowering the B_{max} level. Notably, the reduction in B_{max} observed upon deletion or alanine substitution of C4 is likely to be caused by rearrangement of the three-dimensional conformation of the F264 side chain, located at the far distal side of the IL3/C-terminal moiety (C4). In fact, low level of B_{max} was observed upon either deletion or alanine substitution of F264 and was comparable to the reduction observed with ΔC4, C4A **(Fig. 27C, 27D)**.

Notably, D1R contains a hydrophobic motif in the H8 region of the CT, FXXXFXXXF, which is a highly conserved motif among GPCRs. This motif was shown to act as ER-exit motif for D1R via interaction with the dopamine-receptor-interacting protein of 78 kDa (DRiP78). Indeed, functional inhibition or overexpression of DRiP78 leads to ER retention of D1R and reduced receptor biogenesis, which decrease cell surface receptor expression. Furthermore, mutations of single or multiple phenylalanines within this motif (FXXXFXXXF) impairs the transport of D1R from the ER to the cell surface, also leading to a retention of the protein in the ER (Bermak et al., 2001). Intriguingly, a similar motif ^{432}FXXXF^{436}XXXF^{440} motif was shown to be also important for α_{2B}-adrenegic receptor transport to the cell surface. However, in contrast to D1R, deletion of only F^{436} was enough to alter the export of α_{2B}-adrenegic receptor from the ER while deletion of the other two F residues had no effect on the receptor ability to exit the ER (Duvernay et al., 2004). This motif also appears to be necessary for cell surface expression of M2 muscarinic and angiotensin II AT_{1A} receptors (Bermak et al., 2001, Leclerc et al., 2002).

Potentially, F264 in the IL3 of D1R may form intramolecular interactions with residues located outside the IL3, notably within the CT. In fact, deletion or alanine replacement of F264 could interrupt these interactions, which could then hinder DRiP78 ability to bind to the FXXXFXXXF motif located in the CT of D1R **(Fig. 33)**.

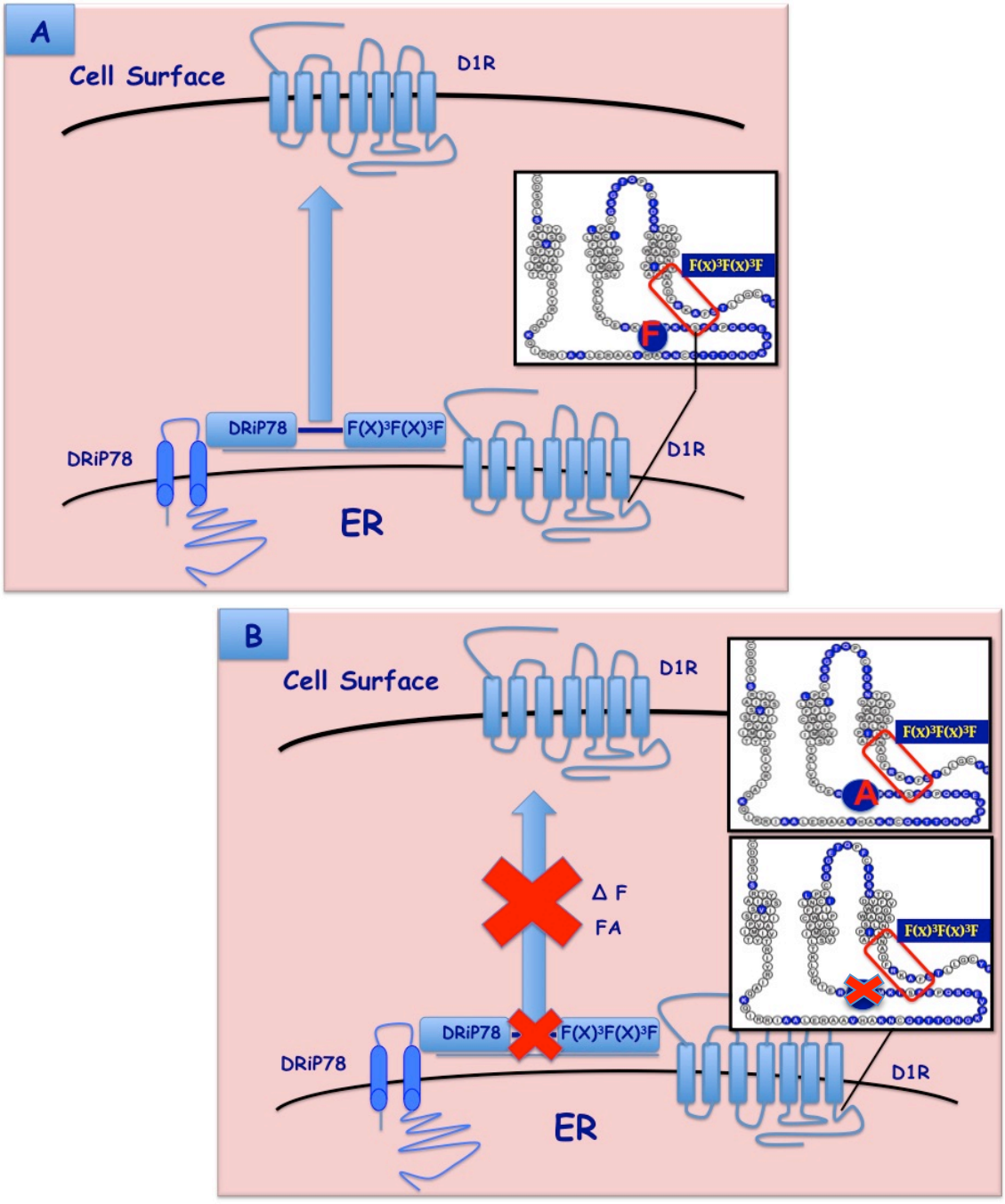


Figure 33. The effect of deletion and alanine substitution of F264$^{6.27}$ on hD1R expression.

A. F(X)^{3}F(X)^{3}F is a highly conserved motif among GPCRs and acts as ER-exit motif for D1R via interaction with DRiP78. **B.** Deletion or alanine substitution of F264$^{6.27}$ (ΔF, FA) may have interrupted the interaction between F(X)^{3}F(X)^{3}F and DRiP78 causing ER-retention of D1R.

2. The proximal part of the IL3/N-terminal moiety modulates agonist binding affinity of hD1-class receptors

While some crystallized GPCRs such as rhodopsin and $A_{2A}R$ harbors their native IL3 (Li et al., 2004, Dore et al., 2011), their resolved 3D structure have not provided any insight into the role of the IL3 in the modulation of the ligand binding pocket. Meanwhile, results obtained through my Ph.D. thesis highlight the importance of the IL3/N-terminal moiety in shaping the agonist binding pocket. Alanine substitution or deletion of the proximal region of the IL3/N-terminal moiety of hD1-class receptors has resulted in different phenotypes in terms of agonist affinity. For instance, removal of the first half of the N-terminal moiety of hD5 (hD5-ΔN1 mutant) leads to a receptor with a reduced affinity for DA and DHX. On the contrary, alanine substitution done in the same region (hΔ5-N1A mutant) promotes an increase in agonist affinity of the mutant receptor. The reduction in the affinity of hD5-ΔN1 for agonists could be explained by its propensity to reside in the inactive state, R (uncouple to G-protein), in comparison to hD5-WT.

Unlike hD5 mutants, deletion or alanine substitution in the corresponding region from hD1R (hD1-ΔN1, hD1-N1A) resulted in a significant increase in agonist affinity, which cannot be explained by a greater propensity of the mutants to adopt an active state. Indeed, these two mutants display lower basal activity. It is possible that deletion or alanine mutation of these residues in hD1R has resulted in optimized repacking of the key residues involved in agonist binding like the serine residues located in TM5 ($S^{5.42}$, $S^{5.43}$, $S^{5.46}$) (Pollock et al., 1992, Tomic et al., 1993). It is worth mentioning that the only difference in this region between hD1-class receptors is the presence of AA in hD1R and

SS in hD5R. The differential changes in the affinity observed between hD1R and hD5R could be explained by the different ability of each mutant receptor to adopt a certain conformation upon deletion or alanine replacement. Perhaps, a differential packing of residues involved in the ligand binding pocket occurs in mutated hD1R and hD5R leading to the observed differences in both DA and DHX affinities between the hD1-class receptors. These findings underscore the notion that D1R and D5R display distinct agonist binding modalities regardless of the fact that both are harboring the canonical amino acids for interacting with catecholamines.

Crystal structure studies have indicated that GPCRs ligands mostly bind in the cavity between TM3, TM5, TM6, and TM7 (Caltabiano et al., 2013). Notably, serine residues within the TM5 ($S^{5.42}$, $S^{5.43}$, $S^{5.46}$) of hD1R were identified to be important for the ligand-receptor interaction (Pollock et al., 1992, Tomic et al., 1993). Modification in the proximal region of the IL3/N-terminal moiety, adjacent to TM5 containing those serine residues, may have undergone conformational changes, possibly altering the orientation of these residues within the vicinity of the receptor-ligand network. Therefore, these potential conformational changes may have led to the observed increase or decrease in the agonist affinity depending on the ability of each mutant receptor to rearrange their TM5 amino acids to compensate for the deleted/modified IL3 sequence. Indeed, the reciprocal serine residues in TM5 of other catecholamine receptors like β_2-adrenergic receptor, α_{2A}-adrenergic receptor, and D2R have been shown to be important for ligand binding and receptor activation; however, the function of each of these serine residues is different among catecholamine receptors (Strader et al., 1989, Wang et al., 1991, Cox et al., 1992, Liapakis et al., 2000).

3. The positive impact of the distal part of the IL3/C-terminal moiety on the binding affinities of hD1R

Preliminary work in our lab shows that deletion of the whole IL3/C-terminal moiety of hD1-class receptors has a positive impact on hD1R and hD5R agonist affinity. To further pinpoint the structural motif within the carboxyl side of the IL3 central region of hD1R involved in modulating agonist affinity, I further divided this area into four parts (C1, C2, C3, C4). Each section contains five amino acids, which were either deleted or replaced by alanine. Alterations in the far distal part, C4, have resulted in a robust increase in agonist affinity. This drastic increase in the agonist affinity of C4 mutants was later found to be attributed to a single amino acid residue, F264, present within this short amino acid sequence, C4 (K261-K265). The magnitude of increase in DA and DHX affinities of ΔF264 and F264A was very much similar to the level of increase seen with ΔC4 and C4A.

It is remarkable that deletion of the basic residue, K265, adjacent to F264 has resulted in the same level of increase in agonist affinity observed upon deleting F264 while the alanine mutant, K265A, recapitulated the parent WT receptor. The increase in agonist affinity observed with K265 deletion could be impacted by the overall effect produced by the removal of this positively charged amino acid, leading to in increased agonist affinity of the receptor. However, replacement of this basic residue, K265, with alanine (A, uncharged and hydrophobic residue) mediates a slightly lower agonist affinity relative to WT rather than robustly increasing agonist affinity as observed with the deletion of K265. Perhaps having two hydrophobic residues [alanine (A)-, phenylalanine, (F)] adjacent to each other has impacted the agonist-binding pocket of the receptor.

Intriguingly, TM6 phenylalanine residues, $F^{6.51}$ and $F^{6.52}$, are highly conserved hydrophobic residues in different GPCR structure including hD1R, hD5R, rat D1R, and β2-adrenergic receptors. These residues have been shown to be important for ligand recognition and binding (Malo et al., 2012, Mente et al., 2015). F264 could be interacting with other residues within or outside the IL3 and remotely control the spatial orientation of the TM6 hydrophobic residues, $F^{6.51}$ and $F^{6.52}$, to promote a more open ligand binding pocket and hence higher affinity for agonists. The effect of these mutants on the ligand-binding pocket appears to be specific for agonists as no change was observed with respect to inverse agonists affinity for any of the mutant receptors.

4. The impact of the proximal part of the IL3/N-terminal moiety on modulating the agonist-independent and dependent signaling of hD1-class receptors

The results from our preliminary study (Charrette and Tiberi, unpublished) emphasize the role of the N-terminal and C-terminal moieties of the IL3 central region in promoting the agonist-free receptor to the inactive or active state, respectively. This was specifically highlighted by changes in the constitutive activity of hD1-class receptors lacking N-terminal (decrease) and C-terminal (increase) moieties. Previous studies using chimeric GPCRs have implied a critical role of both of these IL3 regions in G-protein coupling. However, their exact contribution remains unclear as it may vary from one class of GPCRs to another and also among members of the same family (Bluml et al., 1994b, a, Strader et al., 1994, Burstein et al., 1996, Conchon et al., 1997, Chakraborty et al., 2013).

Despite the fact that hD1R and hD5R share 80 % sequence identity in their TM domains, they display different pharmacological profiles. Relative to hD1R, hD5R exhibit a higher level of basal activity (Tiberi and Caron, 1994). As seen in **Fig. 19C**, the abolition of the basal activity level of hD1-class receptors lacking the full IL3/N-terminal moiety can be mainly ascribed to the proximal part of this region (N1), whereas no change was observed when the distal part of this region (N2) was modified. Structural alterations in this moiety (N1) by deletion or alanine mutagenesis (hD1-ΔN1, hD1-N1A; hD5-ΔN1, hD5-N1A) provoked a significant reduction in the receptor constitutive activity in a similar fashion to removal of the whole IL3/N-terminal moiety (hD1-ΔN, hD5-ΔN).

Interestingly, the extent of the reduction of DA-mediated maximal AC activation and DA potency seen in cells expressing hD1-ΔN were not fully recapitulated in cells expressing hD1-ΔN1 or hD1-ΔN2. In fact, both hD1-ΔN1 and hD1-ΔN2 display a lower DA potency relative to hD1-WT but higher as compared to hD1-ΔN. Interestingly, the lower DA-mediated maximal AC activation evoked by hD1-ΔN can be explained by the deletion of the N1 part while N2 removal had no effect on this parameter. Remarkably, deletion of the N1 in hD5R (hD5-ΔN1 mutant) had a more drastic impact. Indeed, the deletion of N1 almost entirely removed the ability of this mutant receptor to elicit DA-mediated stimulation of AC **(Fig. 20C)**. However, this drastic loss associated with hD5-ΔN1 was rescued in cells expressing hD5-N1A, although still dramatically reduced relative to hD5-WT. The loss of function associated with hD5-ΔN1 suggests that N1 harbors critical $G\alpha_s$ coupling determinants. Although the N1 part is highly conserved between hD1R and hD5R, the loss of coupling of hD5-ΔN1 to G-protein but not hD1-

ΔN1 implies a negative impact imposed by the rest of amino acids sequence left in hD5R and potentially on the optimal packing conformation required for G-protein coupling. In fact, mutagenesis studies reporting loss of function in GPCRs such as β2-adrenergic receptors, parathyroid hormone (PTH) and thyrotropin receptors, have pointed to the importance of the amino acid content of the IL3/N-terminal moiety for G-protein recognition (Cheung et al., 1992, Kosugi et al., 1993, Huang et al., 1996). It is possible that a new network of intramolecular interactions with other receptor domains has formed to partially and fully conceal the consensus sequence required for G-protein coupling of hD1R and hD5R, respectively. Furthermore, change in the protein conformation and charge rather than the primary sequence has been suggested to be of crucial importance for G-protein activation (Punn et al., 2012). Notwithstanding these different possibilities, my data strongly indicate that highly homologous hD1R and hD5R exhibit significant differences in $G\alpha_s$ coupling mechanisms.

Consistent with our results, the loss of efficacy and potency observed with hD5-ΔN1, was also observed upon deleting the far proximal region of the IL3 of α_{2A}-adrenergic receptor, adjacent to the cytoplasmic extension of TM5 (Eason and Liggett, 1995). Evidently, our results concur with other GPCR studies pointing out to the importance of the IL3/N-terminal domain for G-protein coupling. Mutagenesis studies done with β_2-adrenergic receptor denoted the significance of the cytoplasmic extension of TM5 as a key for G protein coupling (Dixon et al., 1987, Liggett et al., 1991). Another study conducted with the rat angiotensin II AT_{1A} receptor also suggested that the proximal part of the IL3, adjacent to TM5, is responsible for $G\alpha_q$ coupling, while the distal region, adjacent to TM6, is important for G-protein selectivity and activation

(Franzoni et al., 1999). Additionally, the effect observed with hD1-N1A and hD5-N1A also underlines the significance of preserving the IL3 length, which was shortened by deleting the proximal half part of the IL3/N-terminal moiety of the hD1-class receptors. To support this idea, a study investigating the role of the IL3 of thyrotropin receptor has shown that a reduction of the IL3 length could affect the conformation of neighbouring TM domains, TM5 and TM6 (Wonerow et al., 1998).

5. The far distal part of the hD1-IL3/C-terminal moiety modulates the agonist-independent and dependent signaling

Mutagenesis studies have highlighted the significance of the distal region of the IL3 of GPCRs for G-protein activation (Liggett et al., 1991, Kosugi et al., 1992, Hogger et al., 1995, Liu et al., 1996, Burstein et al., 1998, Beinborn et al., 2004). As a matter of fact, mutations made at the C-terminal moiety of the IL3 have often produced CAMs (Allen et al., 1991, Kjelsberg et al., 1992, Lefkowitz et al., 1993, Samama et al., 1993, Charpentier et al., 1996, Zhang et al., 2014). However, none of these studies were done with D1-class receptors.

To further explore the impact of the carboxyl terminal moiety of the hD1/IL3-central region, the effect of mutant receptors made at this region on DA-dependent and independent signaling was investigated. The evoked increase in hD1R constitutive activity observed with hD1-ΔIL3 from our previous study is imparted in fact by the far distal part of the IL3/C-terminal moiety of hD1R **(Fig. 24)**. Notably, deletion or alanine replacement of K261-K265 (ΔC4 and C4A mutants) has removed structural constraints on hD1R-WT and thus allowing the mutants to mimic CAM receptors. In a similar

fashion to increased agonist affinity measured with ΔC4 and C4A mutants, F264 plays a major role in controlling the extent of constitutive activity of hD1R. Indeed, deletion or alanine substitution of F264 (ΔF264, F264A) has resulted in ~ 20-fold increase in basal activity in comparison to WT **(Fig. 30)**. Moreover, a drastic increase in DA-mediated maximal activation and DA-potency was also observed with ΔF264 and F264A.

The augmented agonist-independent activation has been observed for several adrenergic receptors following modifications made in the IL3/C-terminal moiety. For instance, single point mutations generated at the IL3/C-terminal of α_{1a} and α_{1B}-adrenergic receptors have resulted in a constitutively active receptor in the absence of agonist stimulation (Kjelsberg et al., 1992, Rossier et al., 1999). Also, the replacement of the IL3/C-terminal of β_2-adrenergic receptor with that of α_{1B}-adrenergic receptor produced a receptor phenotype which exerted a high level of basal activity (Samama et al., 1993). In addition to that, single point mutations at the IL3/C-terminal moiety of β1-adrenergic receptor have resulted in a constitutively active receptor (Lattion et al., 1999). Notably, studies done with adrenergic receptors, α_{1a}, α_{1B}, and β1-adrenergic receptor, to address the importance of the IL3/C-terminal moiety for the tonic receptor activity, mainly focused on residues located closer to the junction of TM6. These residues, in fact, are not located at the same position as the residues mutated for my study. My results, up to my knowledge, are first of kind to address the importance of the IL3/C-terminal moiety for hD1R basal activity, which attributed mainly to $F264^{6.27}$.

Altogether, the results of my studies are in line with the role of the far distal part of the IL3/C-terminal moiety in restraining the receptor in the inactive state through intermolecular interaction within the IL3 or intramolecular with other receptor domains.

Thus, alterations in the K261-K265 sequence, and more specifically the F264 residues, may have facilitated the movement of TM5 and TM6 through a rearrangement of critical amino acids within this sequence needed for G-protein coupling and receptor activation. In fact, intramolecular interactions between different TM domains constraining the receptor preferentially in the inactive state have been observed from previous studies. Indeed, interactions between TM5 and TM6 were shown to stabilize the follicle stimulating hormone (FSH) and muscarinic receptor type 5 (M5R) in the inactive state (Kudo et al., 1996, Spalding et al., 1998). Likewise, studies have also pinpointed the importance of the proper packing of TM domains including TM6 for restraining the GPCR in the inactive state (Robinson et al., 1992, Rao et al., 1994, Hwa et al., 1996, Kosugi et al., 1996, Kudo et al., 1996, Porter et al., 1996, Lin et al., 1997, Dube and Konopka, 1998). Furthermore, it has been shown that rhodopsin is maintained in the inactive state by interaction between the TM6 and the NPXXY motif located in TM7 (Han et al., 1998). Thus, I believe that F264 may be critical in shaping the molecular interactions between TM5, TM6 and TM7.

The cytoplasmic extension of TM5 and TM6 play a coordinate role in GPCR mechanism of activation. TM5 cytoplasmic extension has been proposed to be essential for G-protein coupling while that of TM6 is responsible for G protein specificity and activation (Shirai et al., 1995, Conchon et al., 1997, Franzoni et al., 1999). In fact, the crystal structure of the active β_2AR-G_s complex has supported this notion, by demonstrating an α-helical extension of the TM5 cytoplasmic end and an outward movement of the cytoplasmic side of TM6 (Rasmussen et al., 2011b). Studies have suggested that the IL3/central region moieties indeed have a modulatory role on the

function of TM5 and TM6 (Green and Liggett, 1994, Franzoni et al., 1999). Based on that, elements from both sides of the IL3 of hD1-class receptors seems to be required for fine tuning the G-protein coupling, activation, and selectivity.

Interestingly, the junction between the TM6 and the IL3/C-terminal moiety of hD1R contains a BBXXB motif (KRETK, 265-269; B is a basic amino acid while X is any residue). BBXXB motif has been proposed to act as a conserved GPCR motif, which is important for GPCR activation and G-protein coupling **(Fig. 34A)** (Okamoto and Nishimoto, 1992, Wu et al., 1995, Lee et al., 1996, Pauwels et al., 1999, Timossi et al., 2004, Ulloa-Aguirre et al., 2007). For instance, constitutive activation of muscarinic receptor was noted when the basic amino acids of this motif were mutated by deletion or alanine insertion (Liu et al., 1996, Spalding et al., 1998). Also, deletion of ^{276}RRITR^{280}, the BBXXB motif in μ-opioid receptor leads to a differential effect on the receptor activation in response to different opioid agonists used (Chaipatikul et al., 2003). Intriguingly, it has been suggested that the basic amino acids residues within the BBXXB motif form intramolecular interactions within GPCRs, which stabilize the receptor in the inactive state. Notably, F264 is located upstream and adjacent to BBXXB motif (**F**KRETK) in hD1R. Upon deletion or alanine mutation of F264, it is possible that the orientation of TM6 moves outward. Thus disrupting the F264-dependent intramolecular interactions may likely lead to opening the receptor cavity, and exposing the G-protein contact residues **(Fig. 34B)**.

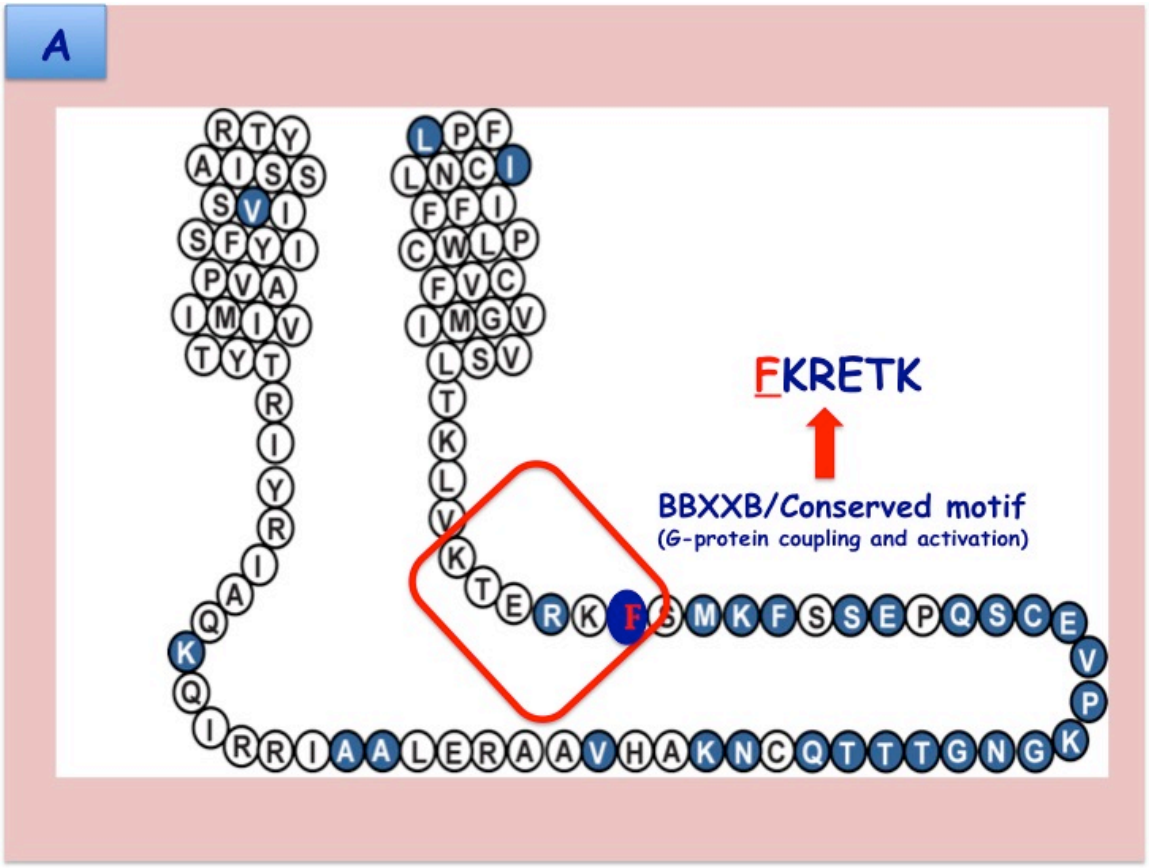


Figure 34A. The effect of deletion and alanine substitution of F264$^{6.27}$ on G-protein coupling and activation of hD1R.
BBXXB motif acts as a conserved GPCR motif (necessary for GPCR coupling and G-protein activation).

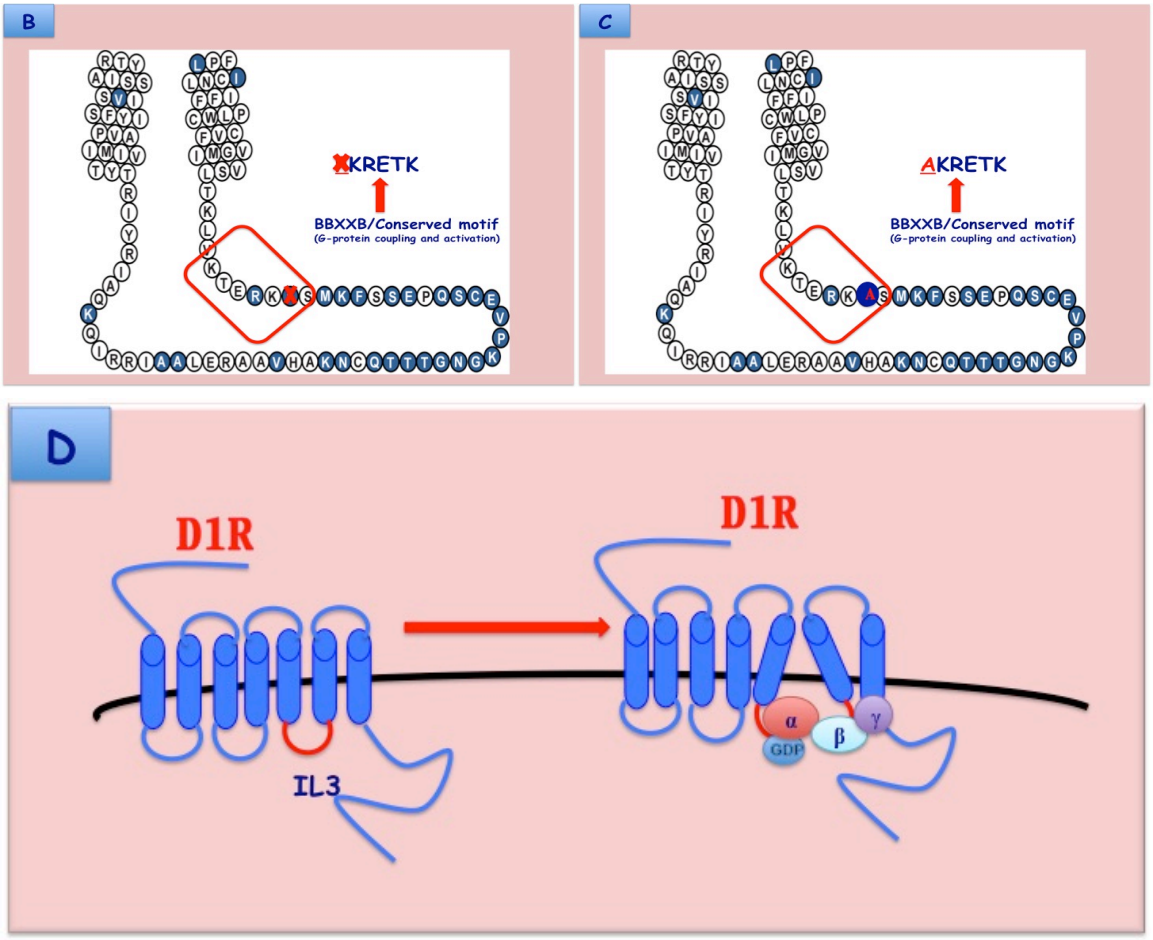


Figure 34B. The effect of deletion and alanine substitution of F264$^{6.27}$ on G-protein coupling and activation of hD1R.

Deletion **(B)** or alanine substitution **(C)** of F264$^{6.27}$ disrupted the intramolecular interactions involving the BBXXB, possibly changing the orientation of TM6 that leads to opening of the receptor cavity for G-protein binding **(D)**.

Moreover, the high constitutive activity observed upon deletion or alanine substitution of F264 might have been caused by the disruption of what is called "the hydrophobic arginine cage". It has been suggested that there is an interaction between the conserved TM3 hydrophobic residue 3.46 and the TM6 hydrophobic residue 6.37, constraining the highly conserved residue $R^{3.50}$ within this arginine cage. Interrupting this interaction between TM3 and TM6 abolishes this constraint imposed on $R^{3.50}$; thus, leading to a constitutively active receptor, which has been shown for many GPCRs (Ringkananont et al., 2006, Smit et al., 2007).

Additionally, deletion or alanine-substitution of F264 may have potentially modified the interaction of the IL3 residues with other intracellular domains to switch the receptor from an inactive to an active state. For instance, DRY is a highly conserved triplet of amino acids in the rhodopsin-like family of GPCRs located at the junction between the IL2 and TM3. DRY is known to be essential for stabilizing the inactive state of the receptor. An intramolecular interaction referred to as the "ionic lock" between arginine ($R^{3.50}$) and glutamate ($E^{6.30}$) residue has been shown to keep the receptor in the inactive state **(Fig. 35A)** (Ramon et al., 2007, Rovati et al., 2007, Rosenbaum et al., 2009, Ambrosio et al., 2011, Huang and Tesmer, 2011). Notably, $E^{6.30}$ is located downstream of F264, separated by only two basic residues (K265, R266). In fact, mutating $E^{6.30}$ to different residues (A, F, K, D, R) in α_{1b}-adrenergic receptor has resulted in CAM receptors with high agonist affinity. It was suggested that these mutations indeed broke the ionic lock between TM3 and TM6 (Greasley et al., 2002). Therefore, I speculate that alteration of F264 has perturbed the interaction between TM6 ($E^{6.30}$) and TM3 ($R^{3.50}$) and lead to a receptor exhibiting a greater ability to adopt an active state in the absence of

agonist such as CAMs **(Fig. 35B, 35C)**. Most importantly, the IL3 of GPCRs has been mostly excluded or modified to allow GPCR crystallization for the reasons discussed above. Crystal structures of inactive GPCRs (inverse agonist/antagonist-bound GPCRs) obtained using this approach has unexpectedly shown a broken ionic-lock (Rasmussen et al., 2007, Warne et al., 2008, Rasmussen et al., 2011b). The basis for such observation remains to be fully appreciated. It has been suggested that replacement of most of the IL3 with T4 lysozyme may have promoted a broken ionic-lock. However, several laboratories were able to crystallize $A_{2A}R$ with an intact IL3 (Dore et al., 2011, Hino et al., 2012). These studies have suggested that the IL3 is essential for the ionic lock formation to maintain the inactive state of the receptor; therefore, maintaining a low level of basal activity.

The hD1R has a low level of basal activity, in comparison to hD5R, which could be attributed to F264 (Charpentier et al., 1996). My results showing that deletion or alanine mutation of F264 transformed the hD1R into a highly constitutively active receptor shed further light on the role of this residue in modulating the basal activity of hD1R and hD5R differentially. Indeed, hD5R harbors an isoleucine (I288) at the corresponding 264 position of hD1R. A F264I mutation in hD1R leads to a mutant hD1R with higher basal activity whereas hD5R with a I288F mutation displays lower constitutive activation (Charpentier et al., 1996).

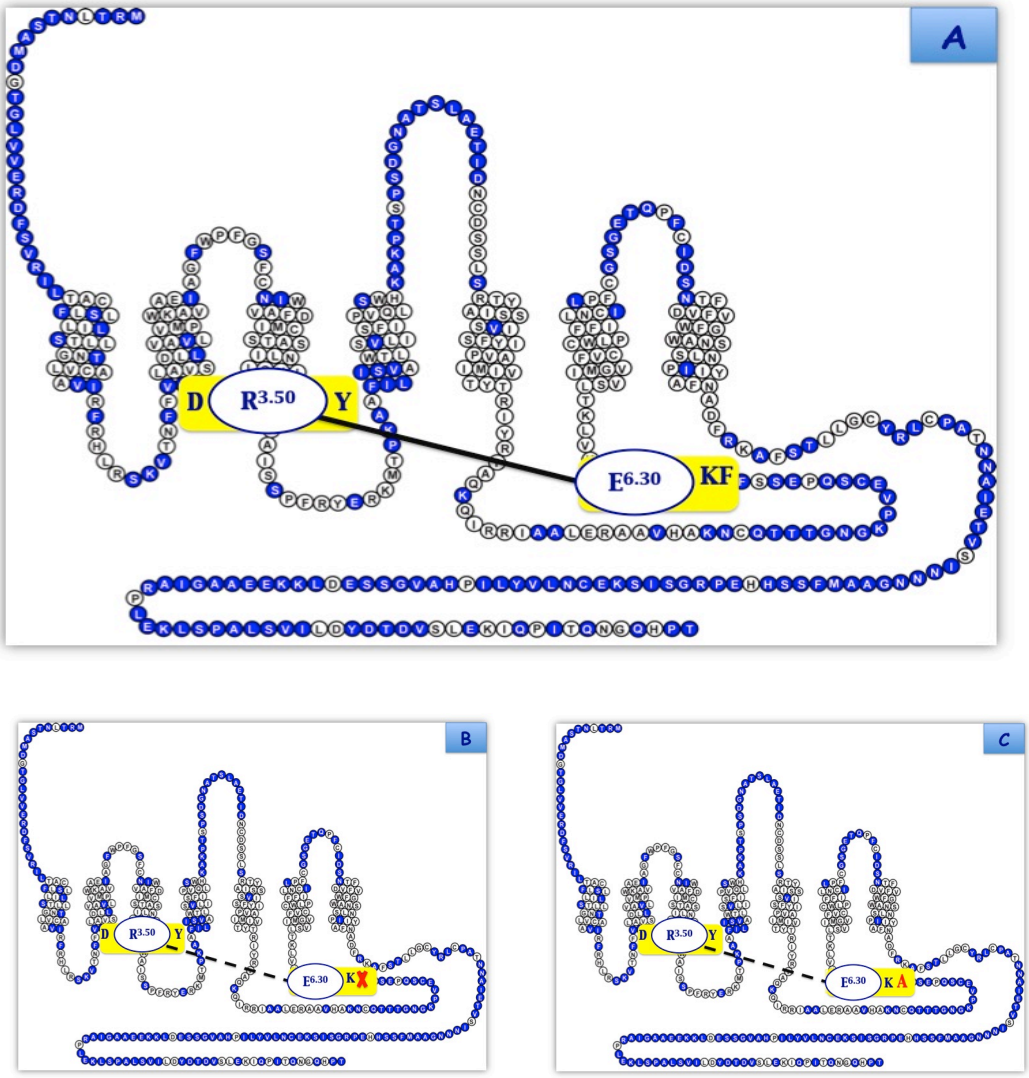


Figure 35. The effect of deletion and alanine substitution of F264$^{6.27}$ on hD1R activation. DRY is a highly conserved triplet of amino acids in the rhodopsin-like family of GPCRs located at the junction between the IL2 and TM3. DRY is essential for stabilizing the inactive state of the receptor through the "ionic lock" [Intramolecular interaction between E$^{6.30}$ (TM6) and R$^{3.50}$ (TM3)] **(A)**. Alteration of F264 by deletion or alanine substitution has perturbed this interaction leading to a receptor exhibiting a greater ability to adopt an active state in the absence of agonist **(B & C)**.

Moreover, the results of my study are also in accordance with the outcome of another study evaluating the role of the IL3 of NPY1 receptor. Chimeric substitution of the IL3 of NPY1 with that of μ-opioid receptor, which is known to have a high level of basal activity, has resulted in a constitutively active receptor indicating that the IL3 of NPY1 plays a crucial role in constraining the receptor in the inactive state (Chee et al., 2008). My studies also are in agreement with a previous study investigating the role of the reciprocal phenylalanine, $F286^{6.27}$, found in α_{1b}-adrenergic receptor. Notably, mutation of $F286^{6.27}$ to alanine in α_{1b}-adrenergic receptor produced the same phenotype we have observed with our F264 mutants with respect to the observed increase in agonist affinity, basal activity, agonist-dependent activation, and potency (Greasley et al., 2002).

6. Efficacy of inverse agonists on the inhibition of basal activity of receptors harboring mutations in the distal region of the hD1-IL3/C-terminal moiety

Most of the GPCRs are known to display low to moderate level of basal activity. Enhanced basal activity of GPCRs has been associated with different disease states such as hereditary thyroid adenomas caused by mutation in thyroid stimulating hormone (TSH) receptors, and male precocious puberty resulted from mutations in the luteinizing hormone (LH) receptor. Thus, inverse agonists can be an important therapeutic approach to silence GPCRs with augmented basal activity (Gether, 2000, Smit et al., 2007).

In an opposite fashion to the effect of agonists promoting the transit from R to R*, inverse agonists stabilize the receptor in the inactive state and shift the GPCR thermodynamic equilibrium toward the R state (Cotecchia, 2007). It has been suggested

that agonists binding could modify the $W^{6.48}$ conformation thus resulting in the movement of $W^{6.48}$ in TM6 toward TM5 and thus receptor activation. The active state of GPCRs appeared to be further stabilized by an aromatic-aromatic interaction between $W^{6.48}$ and $F^{6.52}$. On the other hand, inverse agonists impact the transition of $W^{6.48}$ toward TM5; therefore, reduce the receptor basal activity (Smit et al., 2007). Since some of the mutant receptors we generated, behaved as CAM receptors, the effect of inverse agonists on the mutant receptors basal activity was investigated. Thioridazine and (+) butaclamol exhibited inverse agonist activity at all of the mutant receptors tested relative to their respective WT receptor. Full inverse agonist activity was also observed with those mutant receptors having markedly high level of basal activity like C4 and F264 mutants.

7. Physiological relevance and potential impact of the results for drug design

Since dopamine is involved in many physiological functions, it is not surprising that hypo and hyper-activation of D1-class receptors have been implicated in a plethora of neuropsychiatric and peripheral disorders (Missale et al., 1998, Beaulieu and Gainetdinov, 2011). Although the tonic activity of several GPCRs has been recognized for multiple GPCRs; many of these CAM GPCRs, in fact, are predisposing factors for numerous pathophysiological conditions. For instance, a naturally occurring isoform of the human histamine H3R, which lacks 80 amino acids in the central region of IL3, exhibits a high level of basal activity, increased agonist affinity and potency in comparison to the longer H3R isoform (Bongers et al., 2007). Additionally, mutation in the IL3 of melanocortin 4 receptor (MC4R), P230L, is associated with obesity. Interestingly, characterization of this mutant has revealed a high basal level of activity in

comparison to MC4R WT, which caused the disruption in the maintenance of the normal body energy balance predisposing to obesity (Hinney et al., 2003, Kim et al., 2008). Moreover, mutation of Asp617Tyr located in the IL3 of TSH receptor is associated with hereditary non-autoimmune hyperthyroidism (Nishihara et al., 2007). Other CAM examples include mutations in rhodopsin causing retinitis pigmentosa and congenital night blindness, autosomal dominant mutations in Ca^{2+}-receptor associated with hypo-parathyroidism, mutations in LH-receptor leading to familial male precocious puberty (Seifert and Wenzel-Seifert, 2002). Despite that, the role of hD1-class receptors basal activity in normal and pathological conditions is yet to be appreciated; notably, due to the lack of subtype selective drug to distinguish between D1R and D5R functions in vivo.

Overall, it has been a challenge to develop DAR subtype-selective drugs, most importantly due to the high level of identity between their TM domains, which contain the conserved key ligand binding sites. For instance, the only efficient pharmacological treatment for PD is the use of dopamine precursor; L-dopa. However, the long-term side effect associated with the use of L-dopa is LID, which has been proven to be related to hypersensitized D1R in the striatum (Feyder et al., 2011, Herve, 2011, Murer and Moratalla, 2011).

The main goal of this Ph.D. thesis was to focus comprehensively on identifying the molecular determinants within the IL3 central region of hD1-class receptors, which may provide a drug-targetable receptor region using a diffusible compound to modulate allosterically GPCR function. Importantly, our current study unraveled the importance of a key residue, F264, implicated in restraining hD1R activation in the absence of agonists, shaping lower agonist affinity and potency. By uncovering this residue within the IL3 of

hD1R and by utilizing the knowledge we have gained during this project, one may then envision that once F264-dependent molecular interactions are uncovered, these could be allosterically modulated in hD1R with the use of a diffusible small molecule (<500 daltons). As a proof of concept, it would be worthwhile to test if diffusible lipidated peptides derived from key parts of the IL3 central region can modulate hD1R activation and G protein coupling. Recently, lipidated peptides, referred to as pepducins, have been utilized to target GPCRs and have been shown to be useful in modulating the receptor signaling. Pepducins derived from different intracellular regions of GPCRs such as the IL1, IL2, and IL3 have been successfully designed for various GPCRs like protease-activated receptors (PAR-1, 2, 4); chemokine receptors (CXCR-1, 2, 4) (Kaneider et al., 2005, Tchernychev et al., 2010, Dowal et al., 2011, Tressel et al., 2011, O'Callaghan et al., 2012, Quoyer et al., 2013, Carr et al., 2014). Some of these pepducins are in fact in clinical studies and shown to have a promising therapeutic effect (Gurbel et al., 2016).

Concluding remarks

The findings of this Ph.D. thesis have highlighted the functional importance of the hD1/IL3-central region for hD1-class receptors in terms of receptor expression, agonist affinity, and potency, agonist-independent and dependent activity. Structural elements from the both the proximal and distal regions of the IL3/central region of hD1-class receptors are needed for full receptor activation. The results of this study also underscored the importance of preserving the length of the IL3, regardless of the amino acid content, in shaping ligand binding and G-protein coupling properties. This issue is may also be of functional relevance for the pharmacological differences existing among hD1R and hD5R. Indeed, hD5R has a slightly shorter IL3 relative to hD1R. In general, deletion mutants produced a more robust effect on receptor functionality, which was partially rescued following alanine substitution.

Interestingly, the single point deletion approach that we have used in this study highlighted the importance of phenylalanine, $F264^{6.27}$ for hD1R functionality and G-protein coupling. Deletion or alanine mutation of F264 located at the far distal region of the IL3 of hD1 has produced a mutant receptor with characteristics resembling the classical features of CAM receptors. These CAM receptors are present mainly in the active state, R*, and display strikingly high basal level of activity. They also exhibit higher agonist-dependent activation and higher agonist affinity and potency **(Fig. 36)**.

Future studies should further explore the impact of the proximal side of the N-terminal region of the IL3/central moiety of hD1-class receptors. Based on our observations from this study, employing a single point deletion or alanine insertion

approach would be helpful to map the specific residues, and potentially uncovering a motif within the proximal terminal part of the IL3/central region, involved in the modulation of distinct ligand binding and G-protein coupling properties of hD1-class receptors. Also, utilizing deletion and alanine replacement approaches to explore the impact of the C-terminal side of IL3/central moiety of hD5R would be of a great importance to pinpoint the structural determinants responsible for the distinguishing properties of hD5R relative to hD1R. These future studies may assist in providing more information about the underlying the structural elements responsible for the distinctive functional behaviors of hD1-class receptors. These studies may prove useful in improving the use of therapeutics targeting in subtype-specific fashion hD1-class receptors, notably agonist-independent and dependent activity of hD5R, which are possibly implicated in illnesses such as Parkinson's disease and hypertension (Baufreton et al., 2003, Baufreton et al., 2005, Asico et al., 2011, Chetrit et al., 2013).

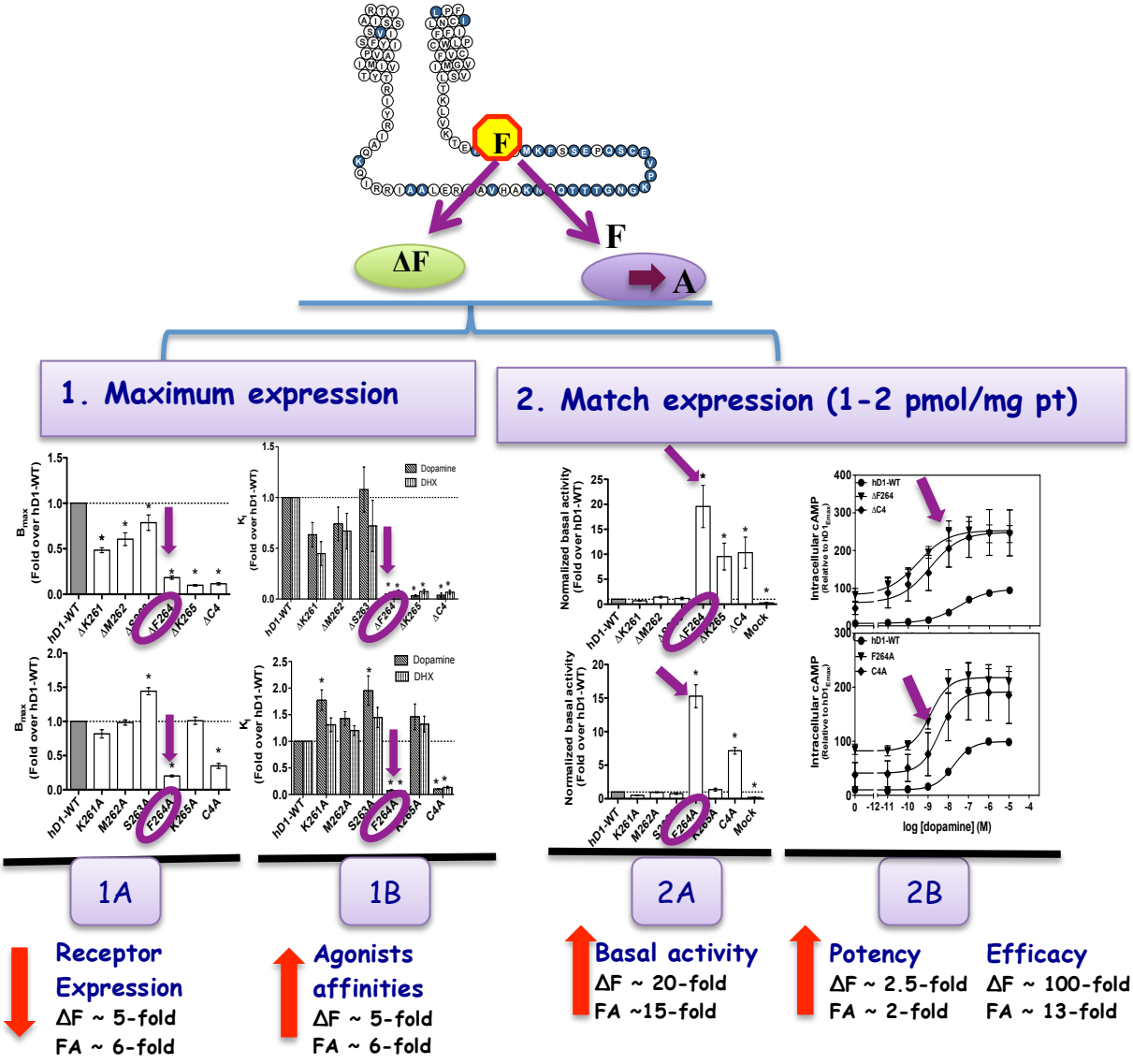


Figure 36. Summary of the effects of deletion and alanine substitution of F264$^{6.27}$ on hD1R expression, G-protein coupling, and receptor activation.

Supplementary Information

Figure S1. Sequence alignment of D1-class receptor.
Sequence alignment of TM5, IL3, and TM6 of D1-class receptors (D1R, D5R) from different species. The most conserved and divergent regions within the IL3 are highlighted with green and yellow, respectively. Sequence alignments were performed using the clustal omega 2.1 (**www.clustal.org**). H: human, M: mouse, R: rat.

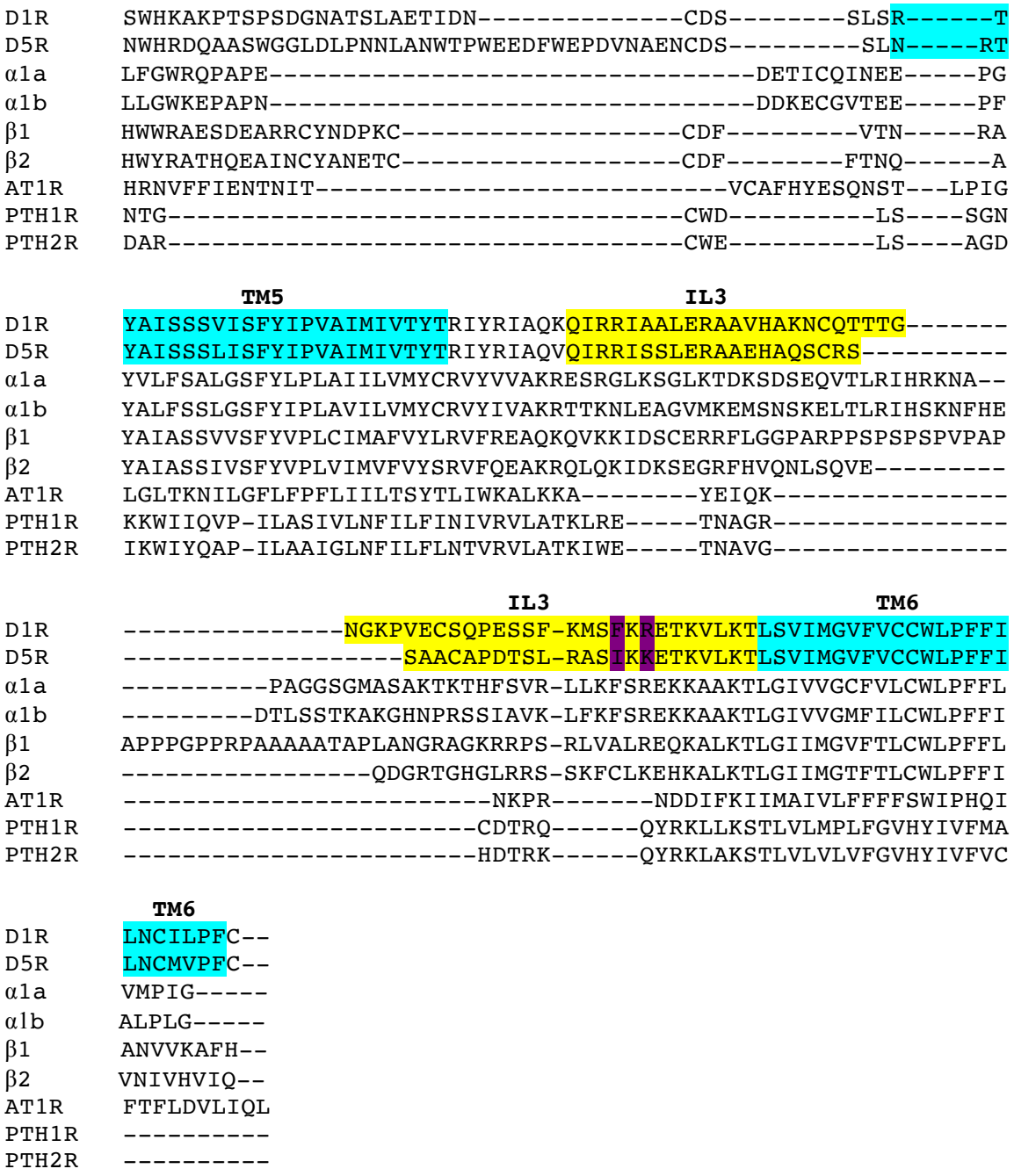


Figure S2. Sequence alignment of D1-class receptor with different GPCRs
Sequence alignment of TM5, IL3, and TM6 of the D1-class receptor (D1R, D5R) with α1-adrenergic receptors (α1a, α1b), β-adrenergic receptors (β1, β2), angiotensin receptor (AT1R), and parathyroid hormone receptors (PTH1R, PTH2R). Two divergent residues between D1R and D5R discussed in the text are highlighted in purpule. Sequence alignments were performed using the clustal omega 2.1 (**www.clustal.org**).

Table S1. Summary of the effects of deletion and alanine substitution of the proximal side of the N-terminal moiety of the IL3 of hD1-class receptors on the receptor expression, G-protein coupling, and receptor activation.

	hD1R		hD5R	
	Deletion	Alanine-mutation	Deletion	Alanine-mutation
	hD1-ΔN1	hD1-N1A	hD5-ΔN1	hD5-N1A
Bmax (functional receptor)	≈ 2 fold	≈ 6 fold	-	≈ 6 fold
Ki (agonist affinity)	≈ 2 fold	≈ 5 fold	≈ 2.5 fold	≈ 2 fold
Basal activity	≈ 2 fold	≈ 2 fold	≈ 10 fold	≈ 3 fold
Emax (Maximal activation)	≈ 1.5 fold	----	≈ 12 fold	≈ 2.5 fold
EC50 (Potency)	≈ 2 fold	----	>2000 fold	≈ 34 fold

Table S2. Summary of the effects of deletion and alanine substitution of the IL3-C-terminal region of hD1-class receptors on the receptor expression, G-protein coupling, and receptor activation.

	Deletions	Alanine-mutations
	ΔC4 (hD1-IL3C/ΔK261-K265)	C4A (hD1-IL3C/K261A-K265A)
Bmax (functional receptor)	≈ 25 fold	≈ 4 fold
Ki (agonist affinity)	≈ 30 fold	≈ 18 fold
Basal activity	≈ 7 fold	≈ 5 fold
Emax (Maximal activation)	≈ 4 fold	≈ 1.8 fold
EC50 (Potency)	≈ 18 fold	≈ 12 fold

Table S3. Summary of the effects of deletion and alanine substitution of F264$^{6.27}$ on hD1R expression, G-protein coupling, and receptor activation.

	Deletions	Alanine-mutations
	ΔF264	F264A
Bmax (functional receptor)	≈ 6 fold	≈ 5 fold
Ki (agonist affinity)	≈ 23-fold (DA) ≈ 12-fold (DHX)	≈ 15-fold
Basal activity	≈ 20 fold	≈15 fold
Emax (Maximal activation)	≈ 2.5 fold	≈ 2 fold
EC50 (Potency)	≈ 100 fold	≈ 14 fold

Chapter 5

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