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Identifying the Roles of the Amino Terminal and first Transmembrane Regions of Human D1-like Dopaminergic Receptors Using Mutagenesis

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IDENTIFYING THE ROLES OF THE AMINO TERMINAL AND FIRST TRANSMEMBRANE REGIONS OF HUMAN D1-LIKE DOPAMINERGIC RECEPTORS USING MUTAGENESIS

by

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Abstract

Although D1-like dopaminergic receptors (D1R and D5R) share similar primary sequences, they possess distinct ligand binding and G protein-coupling properties. Since no ligands discriminate the closely related D1R and D5R, the discovery of their subtype-specific functional and physiological functions is stalled. To provide additional conformational data about the binding pocket of D1-like receptors, we assessed the functional roles of their highly divergent amino terminal cassette. Using a chimerical approach, we swapped the extracellular amino terminal (NT) and first transmembrane (TM1) regions of human D1-like receptors and found that both regions distinctly control dopamine efficacy. Furthermore, chimeras with a swapped TM1 domain have altered affinity and selectivity properties. We further identified two residues in TM1 controlling ligand binding while one of them is also crucial for dopamine efficacy. Our present results highlight structural and functional roles of TM1 and NT of human D1-like dopaminergic receptors.

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List of abbreviations

6-OHDA: 6-hydroxydopamine

AC: Adenylyl cyclase

β1AR: β1-adrenergic receptor

β2AR: β2-adrenergic receptor

B_{max}: Maximal number of membrane-bound receptor

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

COMT: Catechol-*O*-methyl transferase

CT: Carboxy terminal

DA: Dopamine

DAG: Diacylglycerol

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

DAT: Dopamine transporter

D1R: Dopamine D1 receptor

$D2_SR$: Dopamine D2 receptor, short variant

$D2_LR$: Dopamine D2 receptor, long variant

D3R: Dopamine D3 receptor

D4R: Dopamine D4 receptor

D5R: Dopamine D5 receptor

EC_{50}: Effective concentration that elicits 50% of the maximal response

E_{max}: Maximal effect

EL: Extracellular loop

Fab: Fragment, antigen binding

FBS: Fetal bovine serum

GAP: GTPase activating protein

GEF: Guanine nucleotide exchange factor

GIP: G protein-coupled receptor kinase-interacting protein

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

H8: Intracellular helix 8

HEK293: Human embryonic kidney 293

HVA: Homovanillic acid

IL: Intracellular loop

IP_3: Inositol triphosphate

MAO: Monoamine oxidase

MAP: Mitogen-activated protein

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NE: Norepinephrine

NHE: Sodium-hydrogen exchanger

NMDA: N-methyl-D-aspartate

NT: Amino terminal

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PDE: Phosphodiesterase

PET: Positron emission tomography

PH: Pleckstrin homology

PI3K: Phosphoinositide-3-kinase

PIP_2: Phosphatidylinositol bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PDS-95: Post-synaptic density-95 protein

RGS: Regulator of G protein signaling

TM: Transmembrane

TRL: Terminal receptor locus

VMAT: Vesicular monoamine transporter

VTA: Ventral tegmental area

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Chapter 1. Introduction

1.1 The basic principles of dopamine neurotransmission

1.1.1 Neurotransmitter synthesis, storage and release

In the late 1950s, Arvid Carlsson elegantly showed that dopamine (DA) was not only a precursor of noradrenaline, but a neurotransmitter itself (Carlsson 1959). This landmark discovery and his studies about DA throughout his career earned him a Nobel Prize in Physiology and Medicine in 2000. In fact, we now know that DA is part of a cycle ultimately leading to the synthesis of epinephrine (Fig. I). Since catecholamines (DA, norepinephrine (NE) and epinephrine) cannot cross the blood-brain barrier, amino acids L-tyrosine and L-DOPA trigger their synthesis in the brain. The sequential actions of tyrosine hydroxylase on L-tyrosine triggering L-DOPA formation and L-DOPA decarboxylase (also named L-aromatic amino acid decarboxylase) effect on L-DOPA lead to the synthesis of DA (Fig. I, step 1) (Verheij and Cools 2008). In cytoplasm, DA is actively transported and stored into synaptic vesicles through a vesicular monoamine transporter (VMAT) (Fig. I, step 2). NE is synthesized inside vesicles by the actions of dopamine β-hydroxylase on DA in neurons producing this enzyme (Verheij and Cools 2008). Following an action potential and Ca^{+2} influx, synaptic vesicles move towards the synaptic terminal, fuse with the neuron plasma membrane and release their pool in the synapse (Fig. I, step 3) (Wojcik and Brose 2007). Once released, DA can bind to post-synaptic receptors on a second neuron to propagate a signal (Fig. I, step 4). Additionally, as a negative feedback mechanism, DA can activate pre-synaptic DA receptors involved in controlling vesicle release (Fig. I, step 4), be actively transported back (reuptake) in the pre-synaptic terminal by DA transporter (DAT) (Fig. I, step 5), diffuse in the

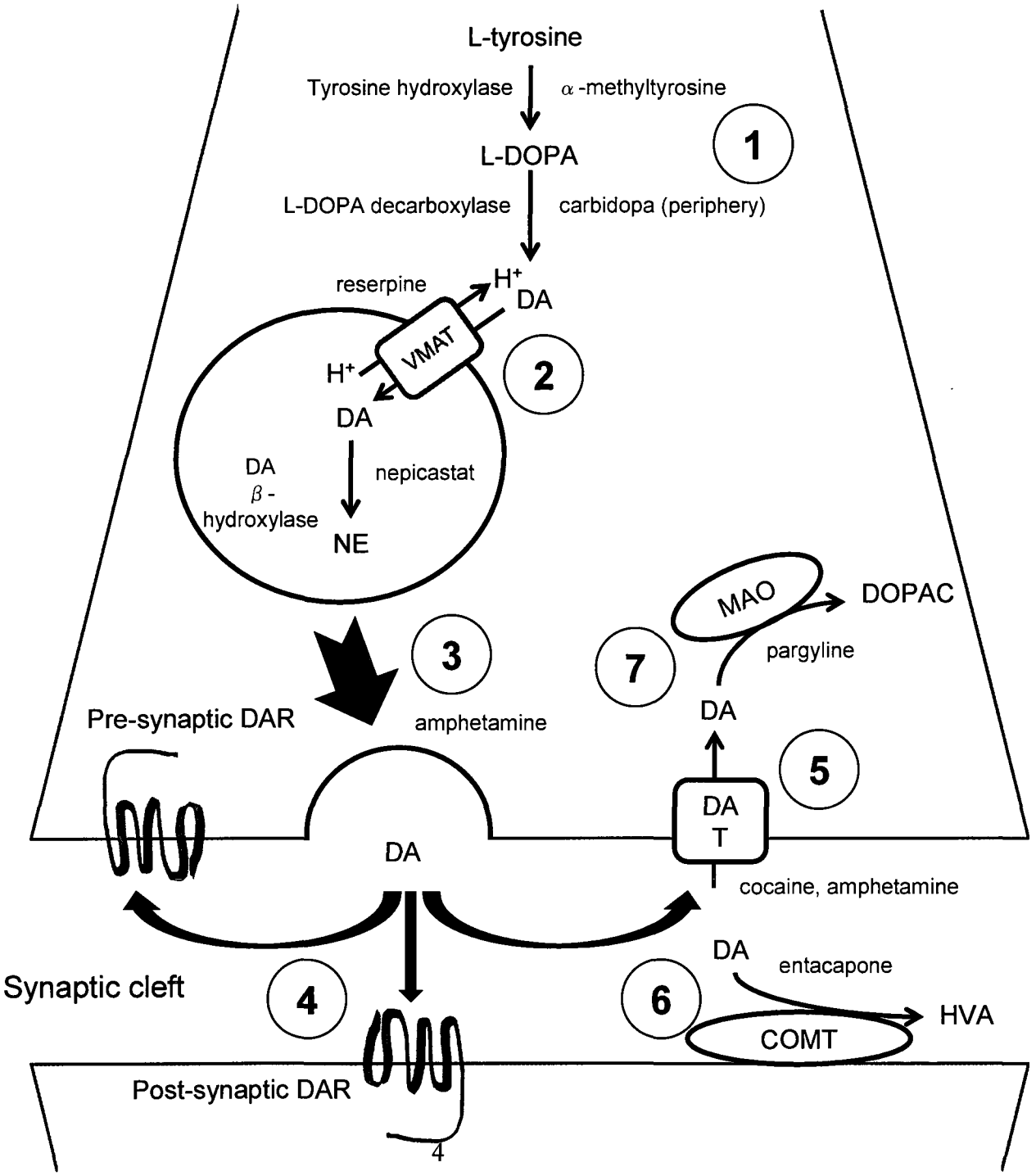
extracellular matrix or be degraded into homovanillic acid (HVA) by a post-synaptic catechol-*O*-methyltransferase (COMT) (Fig. I, step 6). Once inside the pre-synaptic neuron, DA can be stored again in vesicles, where it undergoes the same regulatory process, or be metabolized into DOPAC by monoamine oxidase (MAO) (Fig. I, step 7) (Elsworth and Roth 1997). Every step of this process (synthesis, storage, release, uptake and degradation) has been extensively studied and drugs modulating these steps are available. Blocking the rate-limiting enzyme tyrosine hydroxylase with α-methyltyrosine inhibits the synthesis of catecholamines and blockade of L-DOPA decarboxylase is achieved with carbidopa, which do not cross the blood-brain barrier. Reserpine interferes with the vesicular storage of bioamines whereas amphetamine increases the synaptic release of stored DA and NE. In addition, amphetamine and cocaine block the synaptic reuptake of DA by inhibiting the activity of DAT. Blockers of COMT (entacapone) and MAO (pargyline) can also be used to study DA functions.

After its synaptic release, the inhibitory or stimulatory actions of DA are mediated by pre- and post-synaptic receptors. Molecular cloning has identified a total of five DA receptors (six with alternative splicing) in humans. In the next section, some characteristics of the DA receptors will be discussed.

1.1.2 The family of dopamine receptors

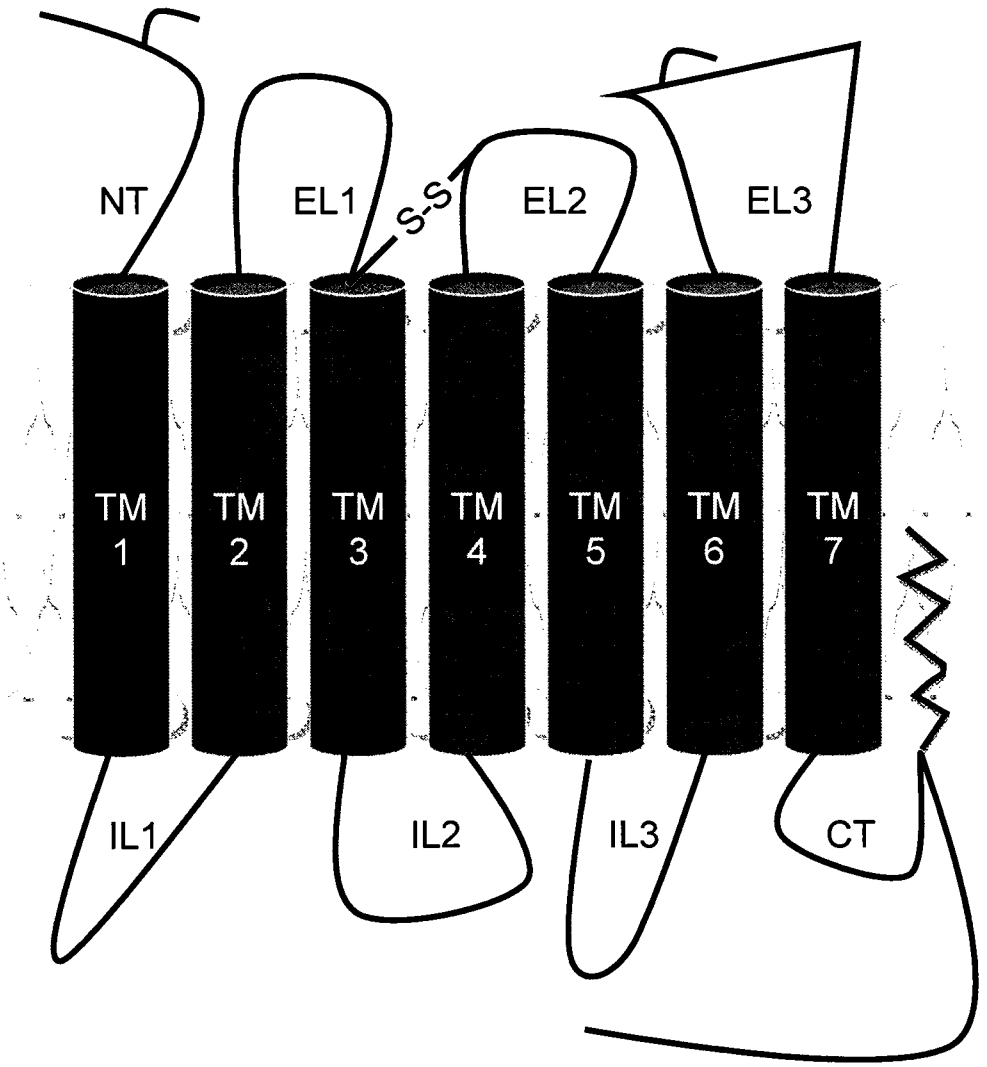
Historically, DA receptors were distinguished by biochemical and pharmacological studies. In 1979, Kebabian and Calne proposed for the first time that two different classes of DA receptors existed based on their association to adenylyl cyclase (AC), an enzyme converting ATP into cyclic AMP (cAMP) (Kebabian and Calne 1979). It was only after the emergence of genetics that research groups identified five

Figure I. Synthesis, storage, release and reuptake of dopamine. After the actions of tyrosine hydroxylase on L-tyrosine generating L-DOPA, and of L-DOPA decarboxylase on L-DOPA (step 1), DA is transported into synaptic vesicles by VMAT (step 2). An action potential triggers the release of synaptic vesicles into the synaptic cleft (step 3). Released DA can activate post-synaptic or pre-synaptic receptors (step 4), diffuse in the extracellular space or be transported back into the terminal by DAT (step 5). DA is degraded by COMT (step 6) or by MAO (step 7). Pharmacological blockers of enzymatic reactions (synthesis, transport, degradation) are shown in red. The release of synaptic vesicles is increased with amphetamine (step 3, in green).



different DA receptors, named D1 to D5, that were present in different species including rats and humans (Bunzow et al. 1988; Grandy et al. 1989; Dearry et al. 1990; Monsma et al. 1990; Sokoloff et al. 1990; Sunahara et al. 1990; Sunahara et al. 1991; Tiberi et al. 1991; Van Tol et al. 1991). DA receptors belong to the superfamily of G protein-coupled receptors (GPCR), which are characterized by their seven-membrane-spanning domains (Fig. II) (Missale et al. 1998; Neve et al. 2004). DA receptors are classified into the D1-like (D1R, D5R) or D2-like (D2R, D3R, D4R) family based on their ability to stimulate or inhibit AC, respectively (Missale et al. 1998; Neve et al. 2004). The structure of DA receptors is homologous, although some differences are seen between and within families (Jarvie and Caron 1993). Seven transmembrane (TM) domains shape their core structure, three intracellular (IL) and three extracellular (EL) loops connect the TM domains and an extracellular amino terminal (NT) region as well as an intracellular carboxy terminal (CT) tail delimit the secondary structure (Fig. II). Like other GPCRs, DA receptors share a disulphide bridge linking the first and second EL stabilizing the receptor structure (Missale et al. 1998). Moreover, post-translational modifications in the Golgi apparatus add sugars to the structure. Glycosylation sites are found on extracellular domains of DA receptor: D1-like receptors have two potential glycosylation sites while D2R, D3R and D4R have four, three and one potential sites respectively (Fig. II, in red) (Missale et al. 1998). Also, a cysteine residue is palmitoylated, which anchors the CT to the membrane (Fig. II, in green). Members of the D1-like family share 80% identity in their TM domains whereas members of the D2-like family share between 54-78% identity in their TM domains (Jarvie and Caron 1993). The three-dimensional structure of GPCRs has been elucidated by the crystallization of a few receptors and will be discussed shortly.

Figure II. Schematic representation of a G protein-coupled receptor. GPCRs have seven membrane-spanning α-helices (TM1-TM7), an extracellular amino terminal (NT) region, an intracellular carboxy terminal (CT) tail, three extracellular loops (EL1-EL3) and three intracellular loops (IL1-IL3). GPCRs can be glycosylated on their extracellular domains (shown in red) and disulphide bonds shape the receptor structure. GPCRs also have an intracellular helical region (H8, not shown) between TM7 and the cysteine-linked palmitoylation (in green) of the CT.



Another important feature distinguishing DA receptors is gene structure. Genes of the D2-like family contain several introns whereas coding sequences of D1-like receptors are intronless (Civelli et al. 1993; Wong et al. 2000). Introns in the D2R gene allow for alternative splicing which generates 2 different receptors, $D2_SR$ and $D2_LR$ with a 29 amino acid difference in IL3 responsible for coupling to specific G proteins (Grandy et al. 1989; Missale et al. 1998).

The involvement of DA in various physiological processes and diseases has been the focus of an abundance of papers over the last fifty years; an overview of the physiological actions of DA is therefore required. As a major neurotransmitter in the mammalian brain, DA has been associated with numerous psychiatric and degenerative diseases. Furthermore, DA is also found in peripheral tissues like kidney and blood vessels where it exerts important functions. A few physiological processes will be discussed below, with a particular interest in DA receptors involved, known activation pathways and therapeutic strategies employed in DA-related dysfunctions.

1.1.3 The nigrostriatal pathway and Parkinson's disease

In his historical study, Carlsson found high levels of DA in caudate nucleus and putamen, regions associated with extrapyramidal symptoms and motor activity (Carlsson 1959). A few years later, a nigrostriatal pathway originating in the substantia nigra pars compacta and leading to the neostriatum (caudate nucleus and putamen) was identified (Anden et al. 1964). This suggested that the degeneration of nigrostriatal DA neurons, thus leading to a deficit in striatal DA, caused Parkinson's disease (Anden et al. 1964). Although DA neurons degenerate with ageing in normal subjects, an accelerated degeneration is observed in parkinsonism (Agid 1991). Regression analysis estimated

that about 50-60% neuronal loss in substantia nigra is already achieved when the first parkinsonian symptoms (akinesia, rigidity of movement, tremors) are observed (Agid 1991). To alleviate these symptoms, the initial and most common therapeutic strategy employed is the administration of DA precursor L-DOPA followed by treatment with DA agonists and MAO-B inhibitors (Rao et al. 2006; Olanow 2009).

DA receptor agonists clinically used include bromocriptine, pergolide, pramipexole and ropinirole, which are mostly active at D2R and are often used in combination with L-DOPA (Rao et al. 2006). Although agonists selective for the D1-like receptor family are currently not available clinically for the treatment of Parkinson's disease, studies suggest that they represent a promising alternative to L-DOPA (Lewis et al. 2006). D1-like receptor agonists A-77636 and dihydrexidine ameliorated locomotion in animals that have been treated with 6-OHDA or MPTP, drugs that selectively destroy neurons of the substantia nigra (Taylor et al. 1991; Kebabian et al. 1992). In addition, a D1-like receptor prodrug (ABT-431) that is quickly converted into A-86929 demonstrated a full antiparkinsonian effect in humans but induced dyskinesias to a similar level than L-DOPA (Rascol et al. 1999; Rascol et al. 2001).

Since Parkinson's disease is neurodegenerative, the optimal treatment would be to block neuronal degeneration. However, there are currently no marketed neuroprotective agents preventing the progression of the disease although some drugs have shown potential benefits *in vitro* and in clinical studies (Le and Jankovic 2001; Lewis et al. 2006; Yu et al. 2008; Olanow 2009).

1.1.4 The mesocortical and mesolimbic pathways and schizophrenia

While DA was associated with Parkinson's disease, other groups considered the role of DA in schizophrenia. Studies in the 1960s showed that a metabolite of DA was found in high concentrations in the urine of schizophrenic patients (Friedhoff and Van Winkle 1962a; Friedhoff and Van Winkle 1962b). Additional studies suggested that blockade of DA receptors alleviated symptoms of schizophrenia (Snyder et al. 1974; Creese et al. 1976). The therapeutic effects of these DA receptor blockers were not related to the nigrostriatal pathway but rather to the mesolimbic and mesocortical pathways originating in the ventral tegmental area (VTA) and projecting to the ventral striatum (nucleus accumbens) and cortex, respectively (Abi-Dargham 2004; Lieberman 2004). Negative and cognitive symptoms of schizophrenia such as social withdrawal and emotional flattening are caused by a hypodopaminergia in the cortex while hyperdopaminergic neurotransmission in the ventral striatum induces positive symptoms like hallucinations and delusions (Davis et al. 1991; Emilien et al. 1999; Abi-Dargham 2004; Lieberman 2004). Commonly used DA antagonists to treat schizophrenia block D2R and alleviate hallucinations but do not ameliorate the negative symptoms (Lieberman 2004; Lieberman et al. 2008). In order to ameliorate cognitive and social impairments, prefrontal DA neurotransmission must be increased. Notably, an inverted-U relationship exists between D1R stimulation in prefrontal cortex and working memory (Williams and Castner 2006). In other words, a controlled D1R stimulation is critically needed in order to have efficient cognitive processes (Goldman-Rakic et al. 2004). In this regard, dihydrexidine, a full D1R agonist having some agonistic activity at D2R,

increased prefrontal perfusion in schizophrenic patients suggesting that it could improve negative symptoms and cognitive impairments (Mu et al. 2007).

Drug reward and addiction mechanisms also require the participation of DA. In fact, an increased activity of the mesolimbic pathway leads to pleasure or reward (Adinoff 2004). Many drugs of abuse (amphetamine, cocaine, alcohol) and other rewarding stimuli (food, sex) increase concentrations of DA in the nucleus accumbens, thus the pleasurable experience of reward (Koob 1992). In parallel, addiction is defined by the need to experience a constant and pleasurable stimulus, in other words the need to increase mesolimbic DA over a long length of time (Adinoff 2004). Therefore, users become addicted to the stimulus and will abusively need stimulation to prevent a depletion of mesolimbic DA. All five DA receptors have been associated with reward, although the roles of D1R and D2R are more established (Foll et al. 2009).

1.1.5 The hypothalamo-pituitary pathway

Dopaminergic neurons also regulate neuroendocrine secretion of prolactin, a peptide stimulating lactogenesis in breasts. Neurons from the hypothalamus release DA in the anterior lobe of the pituitary gland where it stimulates D2R, inhibiting as a result secretion of prolactin and α-melanocyte-stimulating hormone in the bloodstream (Albert et al. 1990; Ben-Jonathan and Hnasko 2001). D2R agonists are clinically used to treat hyperprolactinemia while D2R antagonists increase plasma levels of prolactin (Ben-Jonathan and Hnasko 2001). Although D1R are not found in the anterior pituitary, administration of a D1R agonist increased prolactin release in rats (Cocchi et al. 1987; Durham et al. 1998). Activation of D1R may decrease DA release by the hypothalamus thereby diminishing the DA-mediated tonic inhibition of prolactin release.

1.1.6 Roles of dopamine outside the central nervous system

In addition to the role of DA in the nervous system, many studies highlighted its role in cardiovascular and renal functions (Amenta et al. 2002; Zeng et al. 2008). The use of DA and DA agonists underlined the role of the dopaminergic system in the regulation of mesenteric, coronary and cerebral vascular bed blood pressure where it caused hypotension (Amenta et al. 2002). It was later demonstrated that two types of DA receptors existed in the periphery: prejunctional (D2-like) receptors inhibiting the sympathetic vasoconstriction and postjunctional (mainly D1-like but also D2-like) receptors causing a direct vasodilatation. In the kidney, DA can be synthesized from L-DOPA in proximal tubules and act in an autocrine/paracrine manner to cause vasodilatation, diuresis and natriuresis (Contreras et al. 2002; Jose et al. 2003; Zeng et al. 2004). Secreted DA activates DA receptors, which decreases sodium transport under salt excess by acting at multiple transporters and exchangers (Na^+/K^+/ATPase, Na^+/HCO_3^-, Na^+/Pi, NHE1, NHE3) (Wang et al. 2008; Zeng et al. 2008). Unfortunately, the paracrine/autocrine functions of DA are regulated by tubular mechanisms, thus systematically administered dopaminergic agonists are useless (Zeng et al. 2008). Given their role in the homeostasis of sodium, DA receptors are crucial components of blood pressure regulation. More specifically, D1-like receptor dysfunction has been associated with essential hypertension (Jose et al. 2003; Zeng et al. 2004; Wang et al. 2008). For instance, fenoldopam, a selective D1-like receptor agonist, has impaired ability to increase cAMP in human renal proximal tubular cells from hypertensive subjects (Sanada et al. 1999). Moreover, D1R are hyperphosphorylated at basal state in renal cells from spontaneously hypertensive rats (SHR), are not localized at the cell surface and respond

less to fenoldopam (Yu et al. 2006). The hyperphosphorylation status of D1R is most likely due to a G protein-coupled receptor kinase (GRK) involved in the desensitization of the receptor, GRK4, which has increased activity in hypertensive subjects (Felder et al. 2002). Therefore, a reduced activation of D1-like receptor due to the desensitization would attenuate the inhibition of sodium transport and lead to hypertension (Albrecht et al. 1996; Jose et al. 1996).

Physiologically, the natriuretic effects of DA are counterbalanced by angiotensin II, an antinatriuretic hormone responsible for 50% of total renal sodium reabsorption (Gildea et al. 2008). Interestingly, stimulation of D5R but not D1R induced a degradation of angiotensin II type I receptor (Gildea et al. 2008; Li et al. 2008). These results suggest that a selective D5R agonist could be used to treat hypertension by preventing the antinatriuretic actions of angiotensin II.

But how does DA bind to its receptors in order to produce all the effects described above? Tremendous effort has been deployed in the past years to provide the scientific community with three-dimensional models of GPCRs. In the next chapter, a summary of the crystal structures obtained so far and the differences between them will be presented.

1.2 Crystallographic studies of G protein-coupled receptors

1.2.1 Rhodopsin, the first crystallized GPCR

Unlike other GPCRs, rhodopsin is unique as it is covalently bound at basal state to its ligand 11-*cis*-retinal, an inverse agonist. Upon the activation by a photon of light, the isomerization of the 11-*cis*-retinal to all-*trans*-retinal changes the conformation of the receptor from bathorhodopsin to lumirhodopsin, metarhodopsin I and finally to the fully

activated receptor state metarhodopsin II, which is covalently bound to all-*trans*-retinal and activates G protein transducin (Ruprecht et al. 2004). Characteristic UV/visible absorption maxima differentiate these intermediate conformational states (Ruprecht et al. 2004). It is believed that the hydrolysis of all-*trans*-retinal from metarhodopsin II allows the binding of a new molecule of 11-*cis*-retinal to ligand-free opsin receptor (Park et al. 2008). The first crystallization of a GPCR, bovine rhodopsin, in 2000 had a profound impact in the field. Bovine rhodopsin, located exclusively in the retina, was crystallized from mixed micelles and diffraction data at 2.8 Å were obtained (Palczewski et al. 2000). From an extracellular view, TM helices are organized anti-clockwise where phenyl rings of residues in TM7 make contacts with TM1 (not seen in Fig. III). The extracellular NT region (Fig. III, grey) and all three EL are compactly associated. The NT region extends to EL3 (Fig. III, purple) but otherwise sits closer to EL1 (Fig. III, pink). Deeply folded between TM domains, EL2 (Fig. III, forest green) acts as a 'plug' to rhodopsin. A disulphide bond links the extracellular portion of TM3 (Fig. III, yellow) to EL2. Proline residues located in TM1, TM4, TM6 and TM7 (Fig. III, red, magenta, orange, cyan, respectively) bend those helices while TM2 (Fig. III, blue) is kinked to be closer to TM3. All TM domains are tightly connected to other TM domains through a number of hydrogen bonds and hydrophobic interactions. The three IL are not as packed as the EL, suggesting that these regions are flexible in order to activate G proteins. Of interest, a region beneath TM7 named helix 8 (H8) (Fig. III, white) is arranged as a α-helical domain that can be attached to the lipid membrane through cysteine-linked palmitoylation. The chromophore is mainly bound to residues in TM3, TM5 (Fig. III, brown) and TM6 (Palczewski et al. 2000).

Figure III. Three-dimensional structure of a GPCR, created from the crystal structure of bovine rhodopsin (Protein Data Bank accession number: 1F88). As demonstrated by crystallographic studies, TM regions form an anti-parallel bundle. TM1 (red) sits close to TM7 (cyan) and TM2 (blue) while TM3 (yellow), TM4 (magenta), TM5 (brown) and TM6 (orange) are at the other end. The H8 region (white) is located after TM7 and before CT (green, not fully resolved). The EL2 (forest green) forms the retinal plug. The NT (grey) makes contacts with all EL. EL1 (pink), EL3 (purple), IL1 (salmon), IL2 (pale green) and IL3 (pale yellow).

Recently, the crystal structure of opsin receptor, rhodopsin in the absence of its bound ligand, was resolved by creating a mutant receptor with increased thermal stability (Park et al. 2008). The three-dimensional structure of a segment encompassed by TM1 to TM4 is conserved compared with rhodopsin, suggesting that this segment is part of a rigid core and that movement of this core is not essential for receptor activation. The structure of NT and EL domains are comparable to ligand-bound rhodopsin, as is the retinal plug closing the receptor. TM5 appears to be protruding in the cytoplasm due to an extended helical domain, as seen in squid rhodopsin (Murakami and Kouyama 2008). The most noticeable difference is a 6-7 Å outward tilt of TM6 in opsin compared with rhodopsin (Park et al. 2008). This allows the formation of an opening between TM5 and TM6 that may correspond to the entry path of 11-*cis*-retinal. It also causes rearrangements of IL2 and IL3, regions important for signal transduction. In addition, another smaller opening between TM1 and TM7, caused by a 120° rotation of a residue in TM7, may correspond to the exit path of all-*trans*-retinal.

1.2.2 Problems with the crystallization other GPCRs and their resolution

Crystallization of other GPCRs has been challenging. Unlike rhodopsin, many GPCRs display a ligand-independent activity, a condition that has been associated with structural instability. Also, rhodopsin can be extracted in large quantities from bovine retina, which is not the case for other GPCRs in various tissues (Shukla et al. 2008). Therefore, these GPCRs must be overexpressed in a cellular system, purified extensively and grown in lipid environments that maintain the receptor in a native conformation (Kobilka and Schertler 2008; Shukla et al. 2008). In addition, mutation in the primary sequence of the receptor and additional purification steps had to be done in order to

enhance the crystallization efficiency. Accordingly, crystallized β2-adrenergic receptor (β2AR) has a 48-amino acid truncation of the CT and bind to carazolol, a partial inverse agonist, to increase the efficiency of crystallization (Cherezov et al. 2007; Rasmussen et al. 2007).

Two ingenious technical breakthroughs led to the crystallization of human β2AR. A first technique employed a purified antibody fragment (Fab) recognizing the three-dimensional structure of IL3 of β2AR and stabilizing the receptor (Day et al. 2007; Rasmussen et al. 2007). This Fab-linked β2AR had normal binding affinities for agonist and antagonist but could not couple efficiently to G protein because of the steric hindrance caused by the Fab fragment. Nevertheless, using bimane fluorescence, they showed that receptor conformational changes induced by an agonist were protected (Day et al. 2007). Bimane fluorescence relies on the fact that the amino acid tryptophan quenches the fluorescence emitted by a bimane molecule (Yao et al. 2006). A mutation inserting a cysteine residue near the cytoplasmic end of TM6 in β2AR was labeled with monobromobimane while another mutation at the cytoplasmic end of TM3 introduces a tryptophan. At basal state, these residues are not interacting because of an 'ionic lock' caused by residues in TM3 and TM6 constraining the receptor in its inactive conformation. Following receptor activation by an agonist, this ionic lock is broken thus allowing tryptophan to quench the fluorescence emitted by the excited monobromobimane molecule on the cysteine residue (Yao et al. 2006).

In parallel, T4 lysozyme, a soluble and highly crystallizable protein, was used to replace the entire IL3 of β2AR. This mutation increased receptor stability and allowed crystallization of β2AR (Cherezov et al. 2007). Removal of a glycosylation site on EL2

also improved this crystal structure. The T4 lysozyme mutant β2AR had normal binding affinity for antagonists and inverse agonists but increased affinity for agonists (Rosenbaum et al. 2007). As observed with the Fab-linked mutant receptor, G protein-coupling properties were deficient. Using the same bimane fluorescence technique, this group showed that the conformational changes induced by agonists are similar for wild type and T4 lysozyme-fused β2AR (Rosenbaum et al. 2007).

1.2.3 Differences between the new three-dimensional structures of mutant GPCRs and rhodopsin

In the β2AR crystal structure resolved with the Fab fragment, the cytoplasmic ends of TM domains are well resolved (Rasmussen et al. 2007). However, all extracellular domains and side chains of TM residues facing the lipid environment are unresolved. Overall, the conformation of β2AR is more open but very similar to rhodopsin. Interestingly, the ionic lock formed by residues in TM3 and TM6 is less compact. The more open structure of the ionic lock in β2AR may be due to the constitutive activity of the receptor. β2AR residues involved in binding to carazolol are similar to those in rhodopsin binding to 11-*cis*-retinal, suggesting that the ligand binding pocket is well conserved among the rhodopsin-like family.

The T4 lysozyme stabilized β2AR allowed the resolution of extracellular domains but not the NT region (Cherezov et al. 2007). The EL2 of β2AR differs greatly from rhodopsin in which it acted as a 'plug'. Instead, it contains a short helical domain with two disulphide bonds constraining EL2 outside the pocket formed by TM domains

thereby creating an entry site for ligands. Additionally, the fact that TM1 is straighter in β2AR could allow the entry or exit of a ligand.

Soon thereafter, another research group presented a crystal structure of turkey β1-adrenergic receptor (β1AR) (Warne et al. 2008). This time, six point mutations increased the receptor thermostability, one mutation increased the receptor functional expression and a final mutation eliminated the cysteine-linked palmitoylation site in H8. Furthermore, truncations in the NT, IL3 and CT yielded a more stable receptor. The antagonist cyanopindolol was used to maintain the receptor in an inactive state. The crystal structure shows a conformation of EL2 reminiscent of what was observed in β2AR that may represent a "*common feature in those GPCRs that bind their ligands rapidly and reversibly*" (Warne et al. 2008). As observed with β2AR, the ionic lock formed by residues in TM3 and TM6 is loose in β1AR, further questioning the existence of such a lock in other GPCRs. Residues involved in binding to cyanopindolol in β1AR are similar to those making contacts with carazolol in β2AR. According to the authors who crystallized β-adrenergic receptors, the binding pocket as described for inverse agonists must be tightened upon binding to the natural ligand epinephrine in order to have simultaneously two serine residues in TM5 binding the hydroxyl groups of the catechol ring and an aspartic acid residue in TM3 binding the amine group of epinephrine (Rosenbaum et al. 2007; Warne et al. 2008). This suggests that completely different residues are involved in binding to agonists and antagonists.

Finally, the crystal structure of human adenosine A_{2A} receptor was resolved using the T4 lysozyme fusion technique (Jaakola et al. 2008). Additionally, the antagonist ZM241385 stabilized the receptor in an inactive state, the CT was removed and the

receptor was deglycosylated to improve crystallization. This mutant receptor had similar affinity for antagonist but increased affinity for agonist compared with wild type receptor (Jaakola et al. 2008). The wild type adenosine A_{2A} receptor does not have a palmitoylation site and the structure shows that the intracellular H8 is stabilized by interactions with the TM1. The tip of EL2 could not be determined due to a lack of resolution. However, EL2 makes three disulphide bridges with EL1 while EL3 makes an 'intraloop' disulphide bond constraining the EL3 at the top of the binding site. Strikingly, ZM241385 is located almost perpendicular to the membrane plane, thus binds the receptor in a completely different fashion than what was observed for β-adrenergic receptors and rhodopsin.

The human genome contains over 800 genes coding for a GPCR and ligands recognizing those receptors are diverse (Fredriksson et al. 2003). Typically, amines, peptides, glycoproteins, nucleotides, Ca^{+2} ions, odorants, pheromones and even a photon on light can bind to and activate a GPCR (Fredriksson et al. 2003; Kristiansen 2004). An estimated 30% of currently marketed drugs are targeted to influence the functions of GPCRs (Hopkins and Groom 2002; Landry and Gies 2008). Therefore, it is critical to study the functional aspects of GPCRs to ameliorate the therapeutic effects of those drugs.

1.3 Regulation of G protein-coupled receptor functions

1.3.1 Families of G protein-coupled receptors under the GRAFS system

Assiduous work to identify and separate human genes coding for GPCR has lead to the classification of GPCRs in five main families forming the GRAFS system:

Glutamate, **R**hodopsin-like, **A**dhesion, **F**rizzled/Taste2 and **S**ecretin (Fredriksson et al. 2003).

The glutamate family consists of receptors with a long NT (280-580 amino acids), which is crucial for ligand binding. Metabotropic glutamate and GABA receptors, calcium-sensing receptor and type I taste receptors (TAS1) form this family.

The rhodopsin-like family is by far the largest family with its 701 members. Receptors in this family share a NPxxY motif in TM7 and a (E/D)RY motif in TM3. Most ligands bind the receptor in a cavity formed between TM domains although glycoproteins bind the NT of receptors in this family. Prostaglandin receptor, amine receptor (DA, NE, histamine, muscarinic, serotonin), opsin receptor, melatonin receptor, cannabinoid receptor, peptide (CCK, neurotensin, neuropeptide Y) receptor, somatostatin receptor, chemokine receptor, angiotensin receptor, leukotriene receptor, MAS1 oncogene receptor and olfactory receptors are examples of members of this family (Fredriksson et al. 2003).

The adhesion family contains receptors with an extra long NT (200-2800 amino acids) that possesses structural motifs likely to participate in cell adhesion, such as EGF-like repeats, mucin-like repeats and conserved cysteine-rich motifs (Fredriksson et al. 2003).

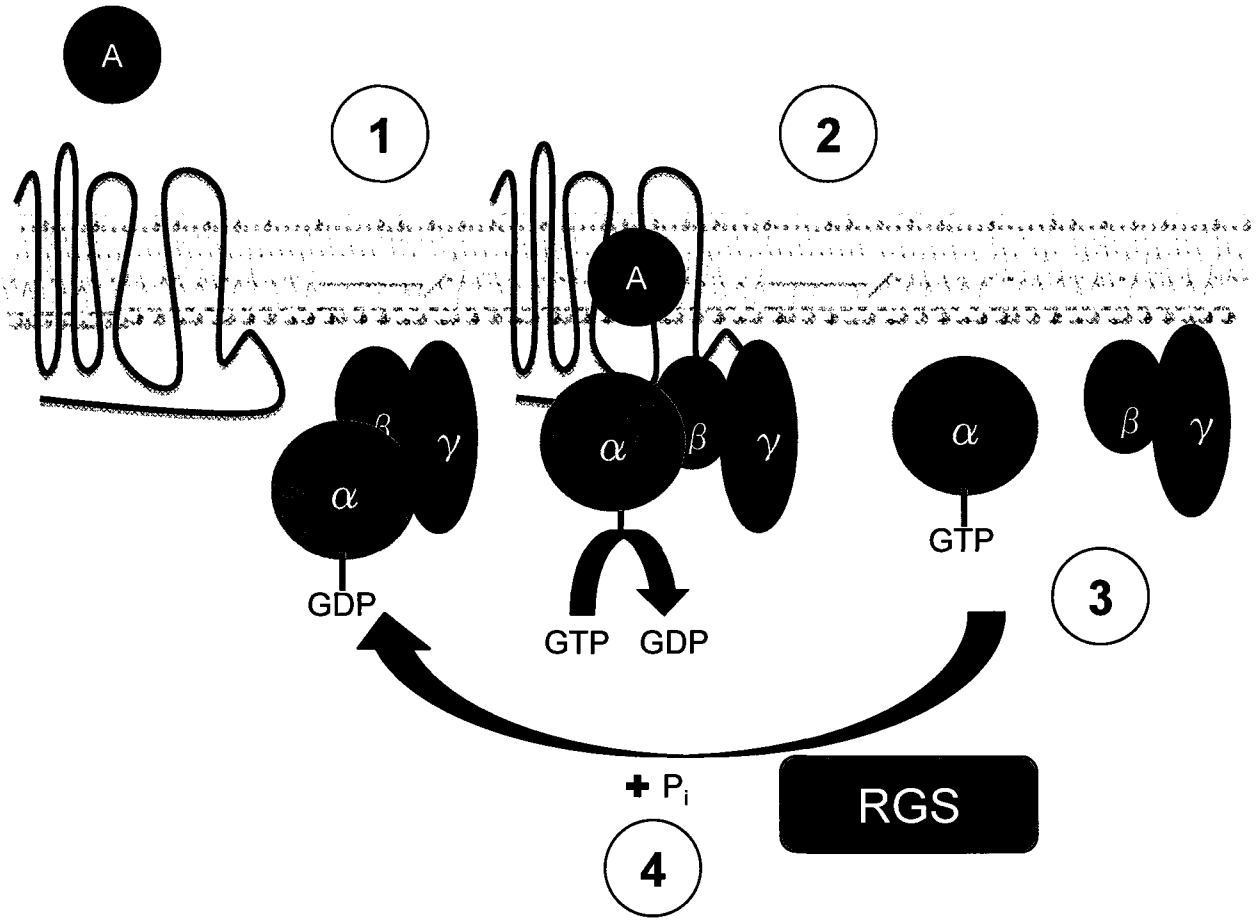
The frizzled/taste2 family is composed of type II taste receptors (TAS2), which have a short NT and are completely different than TAS1 receptors, and frizzled receptors that control cell-fate and proliferation and bind to glycoproteins named Wnt. Frizzled receptors have a long NT (~200 amino acids) involved in Wnt binding (Fredriksson et al. 2003).

Finally, members of the secretin family bind large peptides and have a fairly long NT domain (60-80 amino acids) important for ligand binding. The calcitonin receptor, corticotropin-releasing hormone receptor, glucagon receptor, gastric inhibitory polypeptide receptor, glucagon-like peptide receptor, growth hormone-releasing hormone receptor, pituitary AC-activating protein receptor, parathyroid hormone receptor, secretin receptor and vasoactive intestinal peptide receptor constitute the members of this family (Fredriksson et al. 2003).

1.3.2 The heterotrimeric G proteins and their effectors

Recognition of a ligand by a receptor triggers conformational changes that promote coupling to a G protein (Fig. IV). A G protein is composed of a GTP-binding α-subunit and an undissociable βγ dimer (Wettschureck and Offermanns 2005). A number of each subtypes has been identified which all have their specificity of expression and effector proteins. The guanine exchange factor (GEF) property of the bound-receptor promotes the exchange of GDP for a GTP on the α-subunit (Fig. IV, step 1), which provokes the dissociation of the α-subunit from the βγ-dimer (Fig. IV, step 2) (Luttrell 2008). The dissociation of the heterotrimeric G protein has been challenged and there is proof that it does not always occur (Lambert 2008). Nevertheless, free GTP-bound α-subunit and βγ-dimer regulate the activity of effector proteins, such as an enzyme or ion channel (Fig. IV, step 3). The activated effector protein generates second messengers that activate protein kinases. Signaling continues until the hydrolysis of GTP to GDP by the inherent GTPase activating protein (GAP) property of the α-subunit (Fig. IV, step 4) (Wettschureck and Offermanns 2005; Luttrell 2008). In addition, proteins called

Figure IV. Activation and signal termination of heterotrimeric G proteins. Following the binding of an agonist to the receptor, GDP-bound α-subunit bound to βγ-dimer (G protein) is recruited to the bound receptor. The guanine exchange factor (GEF) activity of the bound receptor triggers the exchange of the GDP for a GTP on the α-subunit (step 1). Activated α-subunit is released from the βγ-dimer (step 2) and both components of the G protein can stimulate effector proteins (step 3). The activity of GTP-bound α-subunit is shut down by the intrinsic GTPase activating protein (GAP) activity of the α-subunit, which can be increased by regulator of G protein signaling (RGS) proteins (step 4).



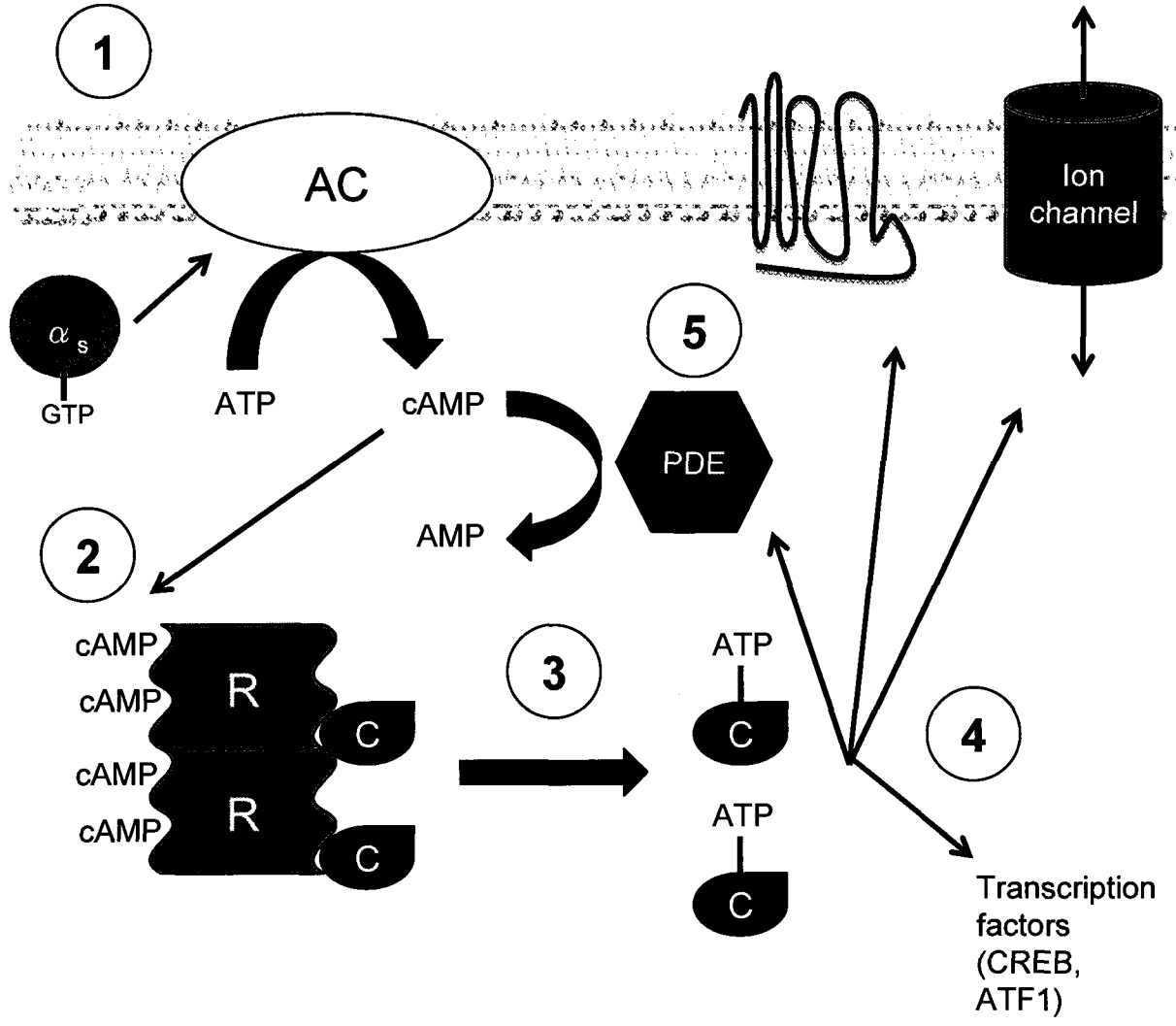
regulators of G protein signaling (RGS) also exhibit a GAP function that accelerates the hydrolysis of GTP (Fig. IV, step 4) (Wettschureck and Offermanns 2005).

Four families of α-subunit, which defines the basic functions of a heterotrimeric G protein, are defined by their structural homology and functional properties: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Wettschureck and Offermanns 2005; Luttrell 2008).

$G\alpha_s$ family consists of two subunits, α_s and α_{olf}, that stimulate the activity of the AC resulting in an increase of intracellular concentration of cAMP (Fig. V, step 1). It is pharmacologically possible to activate $G\alpha_s$ family with cholera toxin that transfers an ADP-ribose to $G\alpha_s$ (Milligan and Kostenis 2006). The increase of cAMP will activate cAMP-dependent protein kinase A (PKA), a tetramer protein with two regulatory and two catalytic subunits (Fig. V, step 2) (Tasken and Aandahl 2004). Following its activation by four cooperative cAMP molecules binding the regulatory subunits, the catalytic subunits will dissociate from the regulatory subunits and bind ATP (Fig. V, step 3). ATP-bound catalytic subunits will phosphorylate a wide range of proteins, like transcription factors, ion channels, receptors and phosphodiesterases (Fig. V, step 4) (Murray 2008). Cyclic nucleotide phosphodiesterase (PDE) transforms cAMP into AMP, thus shutting down cAMP-mediated signaling and can be activated by PKA in a negative feedback mechanism (Fig. V, step 5) (Tasken and Aandahl 2004; Murray 2008).

Members of $G\alpha_{i/0}$ family have various effectors. The $\alpha_{i1\text{-}3}$ and α_z subunits inhibit AC whereas the α_{gust}, $\alpha_{t\text{-}r}$ and $\alpha_{t\text{-}c}$ (gustducin and transducin) subunits increase the actions of PDE, which regulates the degradation of cyclic guanosine monophosphate (cGMP). Direct effectors of α_o have not been identified although the βγ-dimer released from the GTP-bound α_o-subunit hampers voltage-dependent Ca^{+2} channels while increasing the

Figure V. The cAMP-PKA signaling pathway. GTP-bound α_s-subunit stimulates the activity of adenylyl cyclase (AC), which converts ATP into cAMP (step 1). The cooperative binding of four cAMP molecules to the two regulatory (R) subunits of PKA (step 2) induces a conformational change causing the dissociation of the two catalytic (C) subunits of PKA and their binding to ATP (step 3). Activated catalytic subunits phosphorylate a wide variety of proteins such as receptors, ion channels and transcription factors. Phosphodiesterase (PDE) cleaves the phosphodiester bond of cAMP, generating AMP and shutting down the signaling pathway.



activity of G protein-regulated inward rectifier K^+ channel. Use of pertussis toxin, which ADP-ribosylate α_i and α_o, hinders the coupling of the G protein with the receptor (Milligan and Kostenis 2006).

The role of the members of $G\alpha_{q/11}$ family, composed of α_q, α_{11}, α_{14} and $\alpha_{15/16}$ subunits, is to increase the activity of phospholipase C (PLC), an enzyme catalyzing the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). Inhibition of $\alpha_{q/11}$ is achieved with YM-254890, an inhibitor of the GDP for GTP exchange (Milligan and Kostenis 2006). DAG in cooperation with Ca^{+2} and phosphatidylserine will recruit and activate protein kinase C (PKC) to the membrane, where it phosphorylates receptors, transcription factors and enzymes (Newton 1995). The other product of PIP_2 hydrolysis, IP_3, activates IP_3 receptors which act as Ca^{+2} channels at the surface of endoplasmic reticulum (Kockskamper et al. 2008).

Finally, α_{12} and α_{13} can activate other G proteins but mostly RhoGEF, which controls cytoskeletal assembly (Wettschureck and Offermanns 2005; Luttrell 2008). Post-translational modifications (myristoylation or palmitoylation) anchor these α-subunits to plasma membrane (Milligan and Kostenis 2006).

Five β-subunits and 12 γ-subunits have been identified and almost all combinations are possible. Stable $\beta\gamma$-dimers can form heterotrimeric proteins with most α-subunits and thus have a wide variety of functions, which are not fully understood. Interestingly, some $\beta\gamma$-dimers released from specific α-subunits regulate K^+ and Ca^{+2} ion channels, AC, PLC and phosphoinositide-3-kinase (PI3K) isoforms (Wettschureck and Offermanns 2005; Luttrell 2008). Different combinations of β and γ-subunits can induce distinct or even opposite responses (Bayewitch et al. 1998a; Bayewitch et al. 1998b). $\beta\gamma$-dimers can also

inhibit GAP activity of proteins such as $PLC_{\beta 1}$ or $PLC_{\beta 3}$, RGS4 and RGS-Z1 (Tang et al. 2006). Farnesylation and geranylgeranylation of γ-subunits attach the βγ-dimer to the plasma membrane (Milligan and Kostenis 2006).

As mentioned before, stimulation of DA receptors ultimately modulates the activity of AC. There are ten identified mammalian AC, nine membrane-bound and one soluble form, which have specific tissue distribution (Patel et al. 2001; Pierre et al. 2009). Membrane-bound ACs have twelve membrane-spanning domains separated in two sets of six (M1 and M2) with an intracellular NH_2-terminal and two large cytoplasmic domains (C1 and C2). C1 is located between M1 and M2 whereas C2 is situated after M2. The production of cAMP is induced by the interaction of both AC cytoplasmic domains, which can be increased and stabilized with the use of forskolin (Patel et al. 2001). All membrane-bound ACs are stimulated by α_s, inhibited by P-site analogs, which are non-competitive blockers like adenine nucleoside 3' polyphosphates, and all but ACIX are stimulated by forskolin. The soluble AC, not stimulated by α_s or forskolin, acts as a bicarbonate sensor in the testes, kidney and choroid plexus. The nine membrane-bound AC are divided into four groups according to their activators and inhibitors.

Ca^{+2} and calmodulin regulate the activity of the members of group I (ACI, ACIII and ACVIII). ACI is inhibited by α_i, βγ-dimer and the phosphorylation induced by calmodulin kinase IV while phosphorylation by calmodulin kinase II blocks the activity of ACIII. The βγ-dimer released from heterotrimeric G protein stimulates members of group II (ACII, ACIV and ACVII) only in the presence of α_s. Phosphorylation of ACII and ACVII by PKC activates their cAMP production. ACV and ACVI, forming the third group, are predominantly expressed in the heart and are inhibited by α_i and Ca^{+2}.

Moreover, their activity is inhibited by PKA. However, PKC-mediated phosphorylation of ACIV and ACVI decreases α_s-dependent cAMP production. Finally, calcineurin inhibits the activity of ACIX (Patel et al. 2001).

Termination of GTP-bound α-subunit signaling occurs after the hydrolysis of GTP to GDP. The α-subunit possesses an intrinsic GAP function that can be enhanced by RGS proteins (De Vries et al. 2000). An increasing number of RGS proteins are being discovered and they all share a structural RGS box responsible for binding specific α-subunits. Other regions of RGS proteins have characteristic structural motifs, which cause specificity of action (Siderovski and Willard 2005). For example, the NH_2-terminal end of RGS proteins is responsible for membrane localization and recognition of GPCRs, ion channels and effectors (Xie and Palmer 2007). Other regions can interact with β-subunit (GGL domain), GDP-bound $\alpha_{i/o}$-subunit (GoLoco domain) or signaling complexes (PDZ domain) (Siderovski and Willard 2005; Xie and Palmer 2007).

Once the signaling machinery is engaged after receptor activation, the receptor also undergoes a series of events ultimately leading to its internalization and recycling or degradation.

1.3.3 Receptor trafficking, from the endoplasmic reticulum to lysosomes

Like other proteins, receptors are synthesized in the endoplasmic reticulum and processed in the Golgi apparatus. The quality control system of cells prevents misfolded proteins to reach cell surface. This control is particularly important since retention of receptors causes diseases like retinitis pigmentosa, X-linked nephrogenic diabetes insipidus, hypogonadotropic hypogonadism, Leydig cell hypoplasia, familial

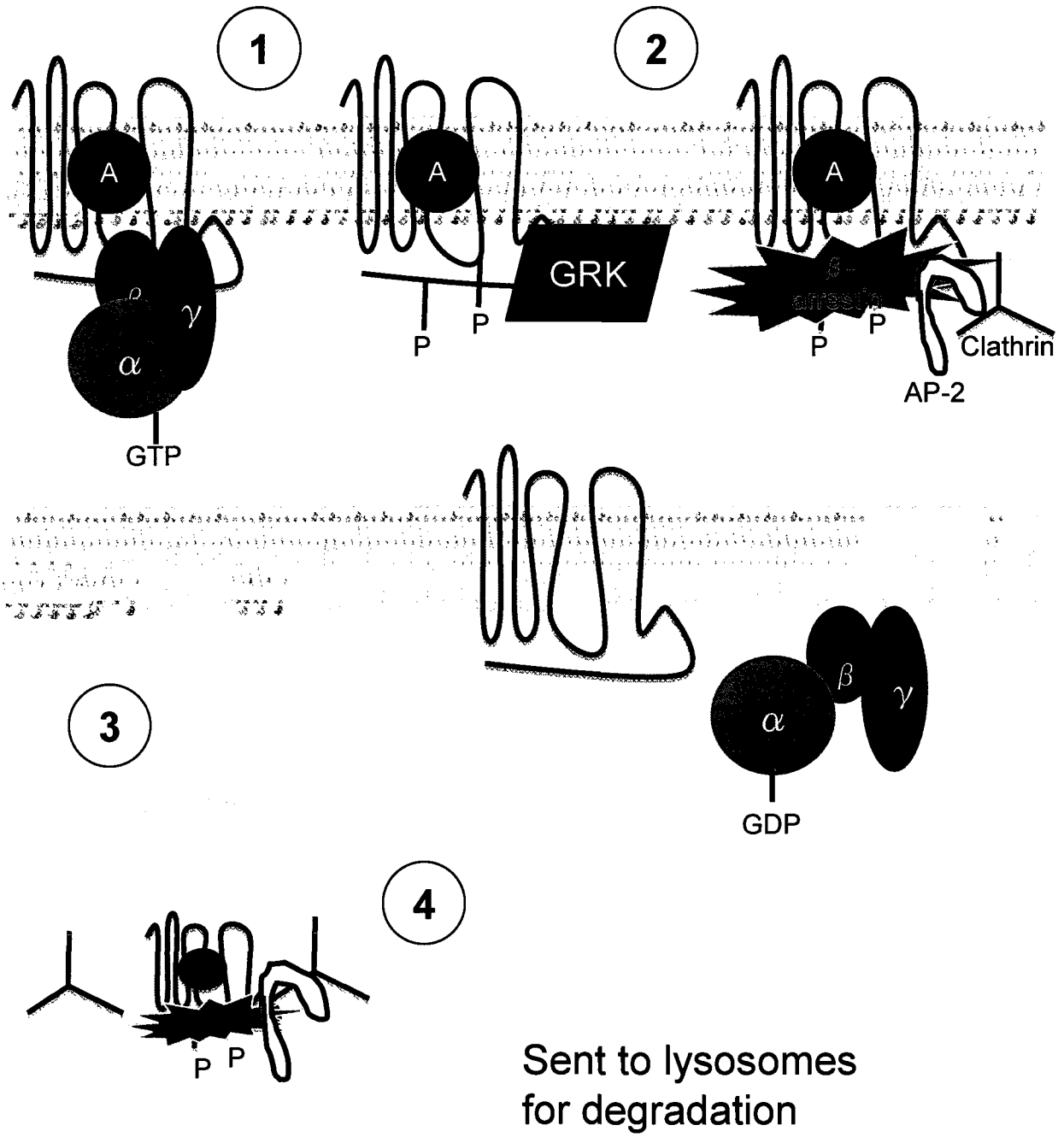
hypocalciuric hypercalcemia, morbid obesity and Hirschsprung's disease (Conn et al. 2007). Pharmacological chaperones have been used to correct the folding of retained receptors and rescue cell surface expression of gonadotropin hormone-releasing hormone receptor, vasopressin type 2 receptor and rhodopsin (Conn et al. 2007). Post-translational modifications occurring in the Golgi apparatus influences cell surface expression of GPCRs. The most studied modification, *N*-linked glycosylation at the consensus Asn-X-Ser/Thr site, regulates cell surface expression of some GPCRs (Duvernay et al. 2005).

It is also believed that the association of two or more GPCRs in the endoplasmic reticulum can lead to proper protein folding and cell surface expression (Terrillon and Bouvier 2004). Indeed, various mutant GPCRs that are not properly expressed exert a dominant-negative influence on wild type receptor cell surface expression, thus providing proof of an early formation of receptor dimers (Colley et al. 1995; Grosse et al. 1997; Zhu and Wess 1998; Karpa et al. 2000; Shioda et al. 2001). Dimerization of GPCR can also affect ligand binding, signal transduction and receptor trafficking (Rios et al. 2001; Angers et al. 2002; Terrillon and Bouvier 2004). Different regions are thought to act as dimerization interface of GPCRs, such as the NT, TM and CT domains (Bouvier 2001; Rios et al. 2001; Milligan 2007).

Once the receptor reaches cell surface and is exposed to the extracellular milieu, it can recognize and bind specific ligands and become activated. Then, the heterotrimeric G protein is recruited, activated through the GEF property of the bound-receptor, released from the receptor after which it modulates the activity of its effectors. In parallel, a series of events lead to the desensitization and internalization of the receptor. Firstly, activated receptors are phosphorylated by GRKs or second messenger-dependent kinases (PKA,

PKC) (Fig. VI, step 1) (Ferguson 2001). GRK specifically phosphorylates serine or threonine residues of the IL3 and CT of activated GPCRs and induces a loss of response to the agonist that activated the receptor, a phenomenon also known as homologous desensitization (Kelly et al. 2008). Following GRK-mediated phosphorylation, proteins known as arrestins bind with high affinity the agonist-bound phosphorylated receptor thereby inhibiting G protein coupling (Fig. VI, step 2) (Ferguson 2001). The GPCR/arrestin complex is targeted to clathrin-coated pits for internalization/endocytosis (Fig. VI, step 3) (Moore et al. 2007). The GPCR can then be dephosphorylated, freed from the ligand and recycled to the cell surface (Fig. VI, step 4). Otherwise, the receptor is sent to lysosomes for degradation (Fig. VI, step 4) (Ferguson 2001; Kelly et al. 2008). The different processes of receptor sorting largely depends on the strength of the interaction between the phosphorylated receptor and arrestin (Lefkowitz and Shenoy 2005). When arrestin binds weakly to the receptor, arrestin will dissociate as the receptor internalize and the receptor will return to the cell surface. Alternatively, when a strong binding occurs, arrestin stays attached to the receptor upon internalization where the complex can slowly be recycled to the cell surface or be sent to degradation pathways (Lefkowitz and Shenoy 2005). In contrast, second messenger-dependent phosphorylation of GPCR probably desensitizes the receptor by hindering G protein coupling (Kelly et al. 2008). Furthermore, PKA and PKC can phosphorylate unoccupied receptor through a mechanism causing a generalized loss of responsiveness of multiple GPCR, also known as heterologous desensitization (Kelly et al. 2008). It should be noted however that second messenger-dependent kinase can also be specific for the activated receptor, hence a homologous desensitization. Second messenger-dependent kinase can also

Figure VI. Trafficking of G protein-coupled receptors following activation. Upon the activation of G proteins, G protein-coupled receptor kinase (GRK) phosphorylates serine and threonine residues of IL3 or CT of the receptor (step 1). This phosphorylation increases the affinity of β-arrestin for the receptor (step 2) and hinders the coupling of the receptor with another G protein. The endocytic machinery (AP-2 and clathrin) attached to β-arrestin causes the internalization of the receptor (step 3). Once inside the cell, the receptor can be dephosphorylated, freed from its ligand and recycled to the cell surface or sent to lysosomes for degradation (step 4).



phosphorylate GRKs, thus modulating the activation of GRKs (Kelly et al. 2008).

Seven GRK (1-7) and four arrestins have been identified. GRK1 and GRK7 are found in retinal rods and cones, respectively, and are attached to the membrane through farnesylation. GRK2 and GRK3 share a pleckstrin homology (PH) domain, which associates with PIP_2 at the membrane, and a βγ-dimer recognizing domain attracting these GRKs at the membrane. GRK4, GRK5 and GRK6 are constitutively connected with the membrane (Reiter and Lefkowitz 2006). Arrestin 1 and arrestin 4 (X arrestin) are found in retinal rods and cones, respectively. In contrast, arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2) are ubiquitously expressed (Reiter and Lefkowitz 2006). In addition to their role in desensitization, GRKs and arrestins are also signaling molecules. β-arrestins can serve as adaptor proteins, interacting with clathrin, clathrin adaptor AP-2, c-Src, c-Jun NH_2-terminal kinase, mitogen-activated protein (MAP) kinase signaling modules and many other proteins involved in chemotaxis and apoptosis (Lefkowitz and Shenoy 2005; Reiter and Lefkowitz 2006). GRKs can also mediate phosphorylation-independent signaling processes by interacting with α_q, βγ-dimers, PI3K, clathrin, G protein-coupled receptor kinase-interacting protein (GIT) and caveolin (Reiter and Lefkowitz 2006).

From ligand binding to signal transduction and receptor trafficking, regulation of GPCRs is fairly complex and involves many proteins. Therefore, to better understand the physiological actions of DA, a focus on the mechanisms of activation and regulation of DA receptors is essential. The five known DA receptors are divided in two distinct families based on their signaling properties. The high degree of identity between members of the D1-like receptor family is a key feature that will help highlight the

structural determinants crucial for ligand binding and G protein coupling properties. Therefore, a particular emphasis on the functional and physiological characteristics of D1R and D5R will be placed.

1.4 The dopamine D1-like receptor sub-family

1.4.1 Polymorphisms occurring in D1-like receptors

A few genetic polymorphisms have been described for D1-like receptors. However, the polymorphisms in the D1R gene are not associated with any amino acid change as they are located in the 5' untranslated region or cause a silent mutation (Wong et al. 2000). These polymorphisms are not linked to any neuropsychiatric diseases but a genetic association between D1R gene and essential hypertension has been made. The polymorphism A-48G in the 5' untranslated region was found more often in patients with essential hypertension than in normotensive Japanese subjects (Sato et al. 2000). Two polymorphisms (A-48G and T1403C) were also associated with alcohol dependence (Batel et al. 2008; Foll et al. 2009).

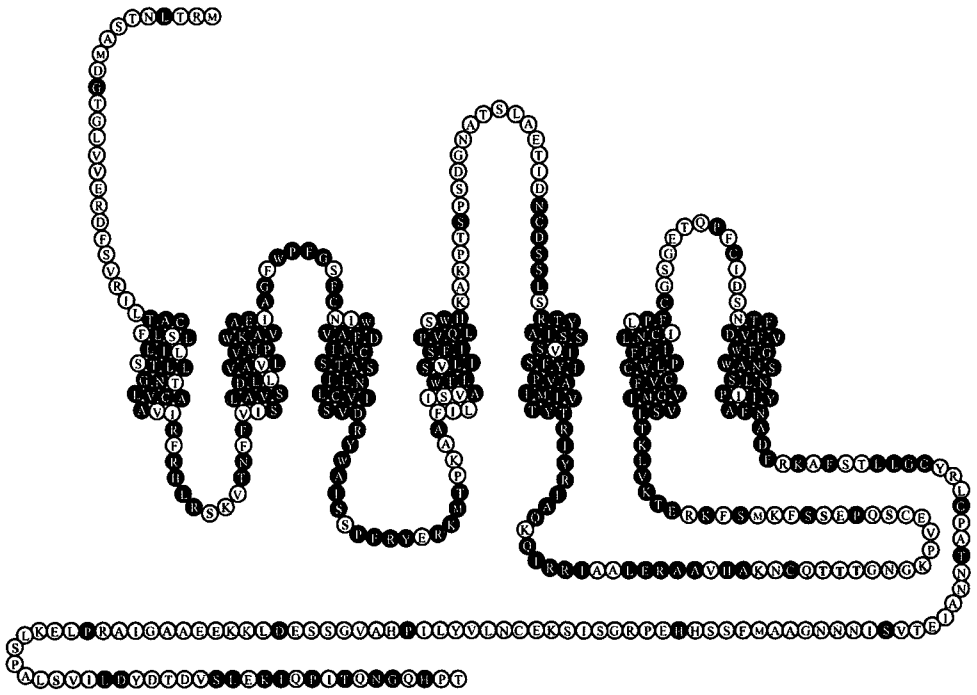
A total of ten polymorphisms are associated with D5R gene and six of them result in an amino acid change. Despite that, no diseases have been linked to polymorphisms in the D5R gene (Wong et al. 2000). Additionally, mutations slightly affect ligand binding (Cravchik and Gejman 1999). Very few studies have attempted to link D5R gene polymorphisms and diseases (Foll et al. 2009). Nevertheless, an association between D5R gene polymorphisms and an earlier age of onset of attention deficit hyperactivity disorder has been shown (Lasky-Su et al. 2007).

1.4.2 Characteristics of the D1-like receptor sub-family

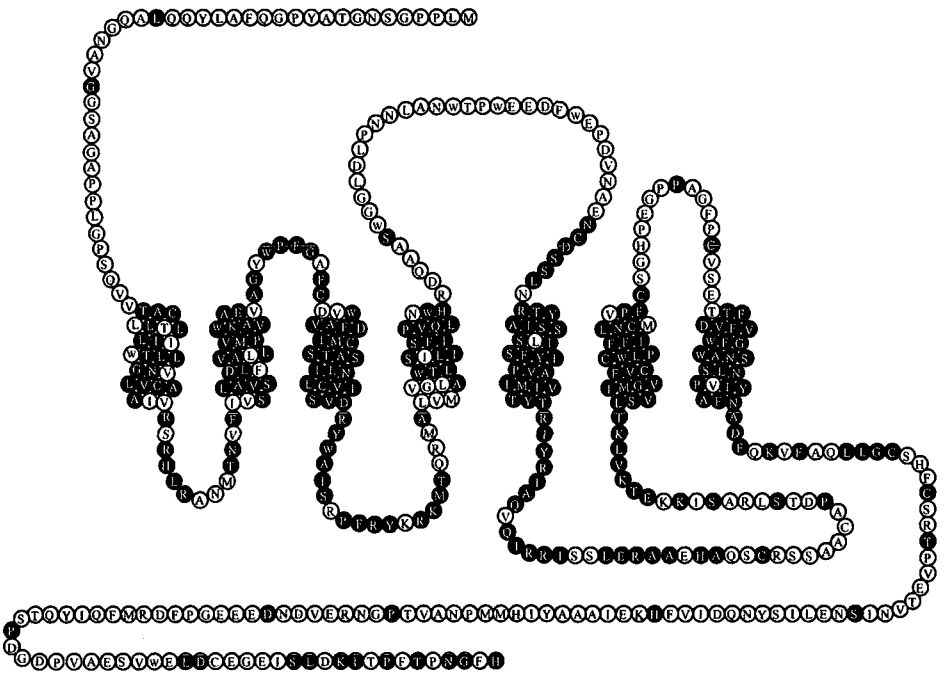
The high degree of identity between the members of the D1-like family has been a challenge in the discovery of selective ligands but also in the attribution of specific roles of these receptors (Huang et al. 2001). Human D1-like receptors share 60% of their amino acid sequences, thus the small differences must account for the subtype-specific signaling and ligand binding properties. For instance, human embryonic kidney 293 (HEK293) cells expressing D5R display higher constitutive activity than D1R and membranes containing D5R bind with higher affinity DA and other agonists but with lower affinity antagonists and inverse agonists (Tiberi and Caron 1994), features evoking those of constitutively active mutant receptors (Ren et al. 1993; Samama et al. 1993).

Different structural components of D1R and D5R could account for the aforesaid subtype-specific characteristics (Fig. VII). For example, the CT of D1-like receptors is almost the same length but the sequence is much diverse, which causes important conformational constraints affecting DA-independent signaling and DA binding affinity (Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al. 2003; Tumova et al. 2004). Residues located in IL3 also dictate DA affinity and responsiveness (Charpentier et al. 1996). Extracellular loops of D5R, especially EL2 and EL3, contain more residues than D1R. The NT region of D5R is long (42 amino acids) and contains many glycine (7), proline (6) and glutamine (5) residues compared with D1R (25; 2; 0; 0, respectively). Although TM domains are 80% identical, TM3, TM5, TM6 and TM7 are nearly identical whereas TM1, TM2 and TM4 are more divergent.

Figure VII. Topology of human D1-like dopaminergic receptors. Conserved residues are shown in black and divergent residues are circled (white). Size differences can be observed in the NT, EL2, EL3 and IL3 regions. TM3, TM5, TM6 and TM7 helices are highly conserved between hD1R and hD5R while TM1, TM2 and TM4 are more divergent. Intracellular and extracellular regions display a low degree of identity between D1-like receptors.



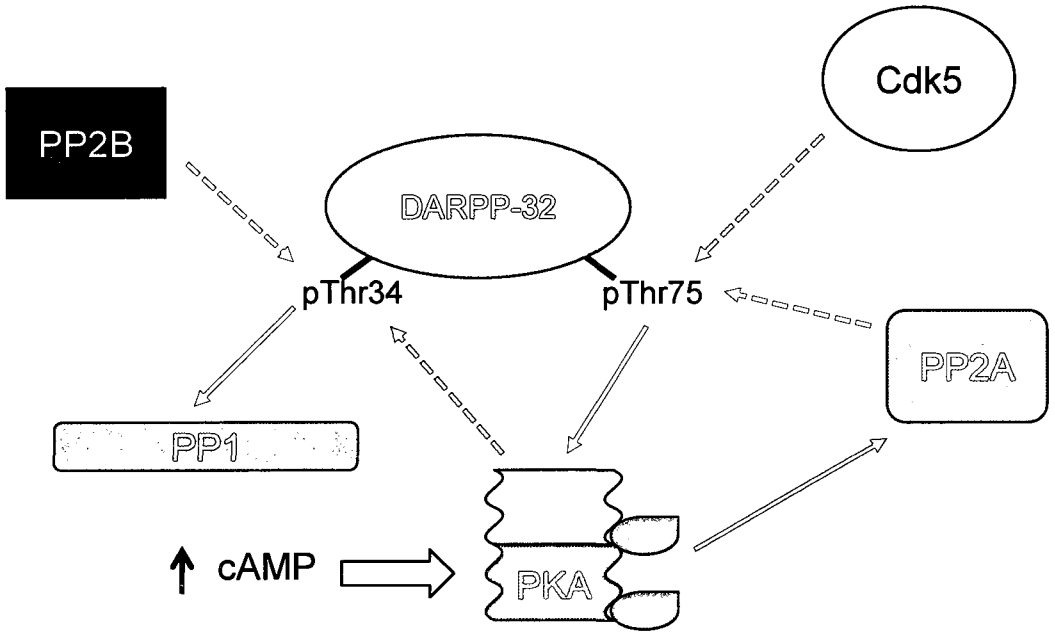
human D1R



human D5R

The D1-like receptor family is essentially coupled to the $\alpha_{s/olf}$-subunit (Huang et al. 2001; Neve et al. 2004). The α_o and α_q subunits can also be coupled to D1R in specific cell lines (Kimura et al. 1995; Jin et al. 2001) while the α_q and α_z subunits can be coupled to D5R (Sidhu et al. 1998; Rashid et al. 2007; So et al. 2009). Little is known about the $\beta\gamma$-dimer associated with $\alpha_{s/olf}$-subunit. Depletion of γ7-subunit using a ribozyme approach in HEK293 cells dampens agonist-induced D1R and D5R stimulation of AC (Wang et al. 2001). The intracellular cAMP increase following D1-like receptor stimulation leads to activation of PKA, which in turn phosphorylates many different proteins. One of them is the dopamine and cAMP-regulated phosphoprotein of 32 kDA (DARPP-32), a protein that inhibits protein phosphatase-1 (PP1) or PKA, depending on the localization of its phosphorylated threonine residue (Fig. VIII). It should be noted that PP1 counteracts PKA by dephosphorylating proteins that are activated by PKA. DARPP-32 phosphorylated on Thr34 by PKA inhibits PP1 while phosphorylation of Thr75 by cyclin-dependent kinase 5 (Cdk5) inhibits PKA (Greengard 2001; Neve et al. 2004). PKA also stimulates protein phosphatase-2A (PP2A) resulting in the dephosphorylation of Thr75 of DARPP-32. Therefore, PKA-dependent phosphorylation of PP2A, dephosphorylation of DARPP-32 (Thr75) by PP2A and inhibition of PP1 by PKA-dependent phosphorylation of DARPP-32 (Thr34) potentiate D1-like receptor signaling (Greengard 2001; Neve et al. 2004). Conversely, a scaffolding protein of the post-synaptic density, PSD-95, was found to constitutively reduce cell surface expression of D1R and consequently reduce D1R-mediated cAMP accumulation (Zhang et al. 2007). Moreover, D1 signaling modifies the activity of many receptors and ion channels. D1-like receptor stimulation can modulate $GABA_A$ receptor responsiveness, potentiate

Figure VIII. DARPP-32 signaling cascade. The increase of cAMP activates PKA, which, in turn, phosphorylates Thr34 of DARPP-32. When phosphorylated at that position, DARPP-32 inhibits PP1, a phosphatase protein counteracting the activity of PKA. However, when phosphorylated at Thr75 by Cdk5, DARPP-32 inhibits PKA. To counteract this negative influence, PKA activates PP2A, which specifically dephosphorylates DARPP-32 at Thr75. Thus, the disinhibition of PKA and the inhibition of PP1 potentiate the cAMP-mediated signaling. Red arrows indicate inhibition and green arrows indicate activation. Dotted lines show the addition (green) or removal (red) of phosphorylation.



NMDA and AMPA receptors, attenuate K^+ currents, increase L-type and decrease N, P/Q-type Ca^{+2} currents and regulate Na^+ channels. Activation of D1-like receptors can also inhibit Na^+/K^+/ATPase and activate MAP kinases (Neve et al. 2004). Although D1R was originally thought to associate with the protein calcyon to increase Ca^{+2} concentration following stimulation of D1R, the authors retracted this report because of a sequencing error (Lezcano et al. 2000; Lezcano et al. 2006).

Signaling of D1-like receptors in brain and periphery is still under extensive investigation in order to discern molecular differences in the activation process of both receptors. To gain insight into subtype-specific physiological roles of D1R and D5R signaling, the localization and distribution of D1-like receptors in brain and periphery will be discussed.

1.4.3 The distribution of D1-like dopamine receptors, in brain and in periphery

DA receptors are widely expressed in brain and periphery. D1R mRNA was found in human caudate nucleus, putamen, cortex, cerebellum, hippocampus, VTA, nucleus accumbens and olfactory tubercle (Dearry et al. 1990). It was also found in rat caudate nucleus, putamen, olfactory tubercle, amygdala, different regions of the cortex (prefrontal, perirhinal, entorhinal) and hippocampus (Weiner et al. 1991). D1R immunostaining appears in rat striatum, substantia nigra pars reticulata, olfactory bulb, amygdala, cortex, hippocampus and in human and monkey basal ganglia and globus pallidus (Levey et al. 1993). In addition, D1R were found in rat hypothalamus, lateral mammillary nucleus, olfactory tubercle, cerebellum, reticular nucleus and VTA (Huang et al. 1992). Electron microscopy identified D1R in monkey and rat dendritic spines and shafts, post-synaptic densities and to a lesser extent in axon terminals (Levey et al. 1993;

Smiley et al. 1994). Similarly in rhesus monkey brain, D1R immunolabeling was identified in pyramidal neurons of cortex, striatum, hippocampus, substantia nigra pars reticulata, globus pallidus, thalamus and amygdala (Bergson et al. 1995). In addition to probing receptor through mRNA or immunoblots, positron emission tomography (PET) studies also allow the detection of receptors. Using PET, higher levels of D1R were found in rat striatum, olfactory tubercle and frontal cortex than in cerebellum (DaSilva et al. 1999). However, due to a lack of radioligand selectivity for D1R or D5R, the identification of subtype-specific receptor expression with PET is not possible, unless a receptor knockout approach is used.

D1R are also detected in the periphery where they modulate cardiovascular and renal functions. D1R are located in the endothelium of pulmonary arteries (Amenta 1997). They are also present in various segments of the kidney, such as the proximal tubule, medullary ascending loop of Henle, collecting duct and macula densa (Zeng et al. 2008). The widespread distribution of D1R in various species supports the notion that impaired D1R signaling is associated with many pathophysiological conditions. In contrast, D5R expression is fainter and more limited than D1R, a feature that does not exclude its importance in the regulation of physiological functions.

D5 mRNA was located in rat hippocampus, lateral mammillary nucleus and parafascicular nucleus (Tiberi et al. 1991; Meador-Woodruff et al. 1992). Immunostaining of D5R in rat brain was found in cortex, hypothalamus, thalamus, striatum, hippocampus, substantia nigra pars compacta and pars reticulata, VTA, globus pallidus, cerebellum and lateral mammillary nucleus (Ciliax et al. 2000; Khan et al. 2000). In monkey brain, D5R immunostaining was notably detected in cortex,

hippocampus, globus pallidus, thalamus, amygdala, large cholinergic interneurons of caudate nucleus but not in substantia nigra pars reticulata (Bergson et al. 1995). The striatum was also stained with D5R antibody, although there are more D1R in this region (Bergson et al. 1995). Coexpression of D1-like receptors in prefrontal cortex of monkey brain suggest that both receptors can act simultaneously to control working memory (Bordelon-Glausier et al. 2008). Although both D1-like receptors were located pre- and post-synaptically, D5R were mostly found on dendritic shafts whereas D1R were spotted on dendritic spines, suggesting distinct roles for both subtypes in synaptic transmission (Bergson et al. 1995). In the periphery, D5R are located in smooth muscle of systemic arteries (Amenta 1997), and proximal tubule, medullary ascending loop of Henle and collecting duct of kidneys (Zeng et al. 2008).

Levels of D1R and D5R vary over time. Low levels of D1-like receptors are found during the embryonic development of mice relative to D2-like receptors (Araki et al. 2007). D1R and D5R mRNA expression increase in striatum and cingulate cortex until postnatal day 60 while D1R and D5R mRNA levels decrease and increase respectively in frontal cortex over time (Araki et al. 2007). A specific upregulation of D1-like receptors is observed during the adolescence period in rat striatum, nucleus accumbens and prefrontal cortex (Andersen et al. 2000). However, levels of D1-like receptors decrease in prefrontal cortex and striatum with age (Andersen et al. 2000). In humans, a correlation between decreased D1-like binding in caudate nucleus, putamen and occipital cortex and decreased motor function scores has been observed (Wang et al. 1998). These studies highlight subtle changes in D1-like receptor expression that have profound impact for the physiological roles of these receptors.

The widespread expression patterns in brain and periphery of D1-like receptors explain the physiological importance of DA receptors. A greater appreciation of the functions of D1-like receptors was achieved with the use of gene knockout mice models.

1.4.4 What have we learned from D1-like receptor knockout mice models?

Development and characterization of genetically altered mice models provide well-founded clues about the roles of the targeted protein. Two techniques, one using antisense probes preventing mRNA transcription and the other using gene alteration by homologous recombination, have served research groups to elucidate the physiological significance of D1R and D5R (Sibley 1999). The use of antisense probes offers a temporal and reversibility approach, but the efficacy of this technique in terms of protein synthesis inhibition is empirical. A gene knockout model has the advantage of completely nullifying the protein but allows physiological changes such as compensatory mechanisms masking the real consequences of eliminating a specific protein.

Antisense treatments to reduce the expression of D1R in mice resulted in D1-like agonist specific reduction of grooming, which could be reversed eight days after the last treatment (Zhang et al. 1994). However, levels of D1R were not quantified in this study. Another study reported that the increased locomotor activity induced by a D1-like agonist in mice treated with 6-OHDA was blocked by D1R antisense but not by D5R antisense (Dziewczapolski et al. 1998). Surprisingly, levels of D1R and D5R, as assessed by autoradiography using a ^{3}H-labeled D1-like receptor antagonist, were similar in treated and control mice. The lack of evidence showing a reduction of D1R or D5R protein demonstrates clearly the major issue concerning the use of antisense probes.

Two independent groups generated mice lacking D1R almost simultaneously (Drago et al. 1994; Xu et al. 1994). D1R deficient mice were growth retarded but bone and brain architecture were normal albeit smaller. Both transgenic mice had reduced levels of the neuropeptide substance P in the striatum, which is preferentially expressed on neurons expressing D1-like receptors. In addition, the neuropeptide dynorphin, also coexpressed with D1-like receptors in striatum, was less abundant in striatum but not in cortex or hippocampus (Xu et al. 1994). Interestingly, one knockout mouse showed a hyperlocomotive behavior and were not responsive to D1-like receptor agonist or antagonist (Xu et al. 1994) whereas the other mouse had lower to normal locomotion compared with wild type littermates (Drago et al. 1994; Smith et al. 1998). These marked differences are probably due to the genetic background of the transgenic mice. In addition, spatial memory of D1R knockout mice was seriously deficient (Smith et al. 1998). Memory deficits of D1R knockout mice are caused by loss of early and late-phase of long-term potentiation and by a lack of induction of two genes, arc and zif268, which are required for the consolidation of long-term memory (Matthies et al. 1997; Granado et al. 2008). Finally, mice lacking D1R are hypertensive supporting the notion that DA receptor function is linked to essential hypertension (Albrecht et al. 1996).

Mice lacking D5R were also generated using a gene-targeting technology (Hollon et al. 2002). The mice were normal in size and body architecture. Old-aged mice (3-9 months) had increased arterial blood pressure and heart weights. These altered parameters were also observed in younger mice (< 2 months) although the small number of younger mice used (n = 3) does not allow an appropriate evaluation of those parameters. Blocking α-adrenergic receptors resulted in a greater reduction of mean arterial blood pressure in

D5R knockout mice compared with wild type mice, suggesting an elevated activity of the sympathetic nervous system in D5R knockout mice. Moreover, administration of centrally active glutamatergic AMPA/kainate receptor and V_1 vasopressin receptor antagonists reduced the mean arterial blood pressure of D5R knockout mice only but the effects were not synergistic. Importantly, pretreatment with an oxytocin receptor antagonist abrogated the effects of these two antagonists, suggesting that oxytocin increased the activity of central vasopressin and non-NMDA pathways in D5R-null mice (Hollon et al. 2002). Recently, it was demonstrated that D5R knockout mouse had increased levels of angiotensin II type I receptor and that blockade of this receptor restored blood pressure to normal levels (Li et al. 2008). Therefore, abnormalities in the central nervous system and in the kidney are responsible for the increased blood pressure measured in D5R knockout mice.

Further studies looked at the response to cocaine in D1-like receptor knockout mice to assess the roles of these receptors in cocaine-induced locomotion. In one study, although the rewarding properties of cocaine were conserved in D1R-null mice, its potential to increase locomotor activity was lost (Miner et al. 1995). Similarly, following cocaine exposure, D1R knockout mice traveled less than their wild type and D5R knockout littermates (Karlsson et al. 2008). In contrast, another study reported that D1R knockout mice did not find cocaine reinforcing as demonstrated by their lack of self-administration (Caine et al. 2007). These data suggest that D1R and not D5R are responsible for cocaine-induced locomotion and that the D1R-mediated rewarding properties of cocaine are still controversial. It has been proposed that these aberrant cocaine-induced motor behaviors are caused by abnormal cellular responses to

psychostimulants. Indeed, the induction of cFos and junB expression in striatum is lost in D1R knockout mice following cocaine and amphetamine treatment (Moratalla et al. 1996). Moreover, disruption of D1R impaired the alcohol-seeking behavior of mice suggesting that D1R activation is crucial for the reinforcing mechanism of alcohol (El-Ghundi et al. 1998).

A different way to assess the subtype-specific characteristics of D1-like receptors is to examine the consequence on ligand binding and G protein coupling properties of mutating residues of the primary sequence of D1-like receptors. Accordingly, many studies over the years examined the impact of mutating residues of the alleged ligand binding pocket of D1-like receptors. In addition, the roles of divergent structural components of D1-like receptors were examined.

1.4.5 The binding pocket of dopamine D1 receptor

It was hypothesized in the early 1980s that 11-*cis*-retinal bound to rhodopsin within a binding pocket located between TM domains (Thomas and Stryer 1982). A few years later, it was proposed for the first time that the hydrophobic domains – the TM domains – of the hamster β2AR were involved in ligand binding (Dixon et al. 1987). Then, mutagenesis studies helped identify the role of specific receptor regions and binding sites in GPCRs. A highly conserved aspartic residue in TM3 of bioamine GPCRs was established as a binding site for the amino group of catecholamines (Strader et al. 1988). Then, conserved serine residues in TM5 of bioamine GPCRs were implicated in binding the hydroxyl groups of catecholamines (Strader et al. 1989). The conclusions drawn from these studies done with β-adrenergic receptors were extended to all

catecholamine GPCRs since only those GPCRs have an aspartic acid in TM3 and serine residues in TM5 (Strader et al. 1994).

In the case of DA D1R, mutating serine residues to alanine residues in TM5 induced major changes in binding and intracellular signaling (Pollock et al. 1992). Ser199 in TM5 of D1R was found to be involved in the binding of all compounds independently of their intrinsic activity. Mutating Ser199 to an alanine residue also decreased the potency of DA in a dose-response curve suggesting that binding to this residue is critical for proper signal transduction. Mutating Ser202 of TM5 into an alanine residue decreased the binding affinity and potency of DA by 48-fold (Pollock et al. 1992). Interestingly, the affinity and potency of SKF38393, a D1R agonist, were mostly affected when mutating only Ser199. Changing Ser202 for alanine caused a 2-fold decreased affinity for SKF38393 while it increased its potency by 3-fold (Pollock et al. 1992). These results suggested that different residues are responsible for binding DA or synthetic D1-like agonists. Mutation of Ser198 to alanine in TM5 of D1R caused an absence of binding sites in the receptor like it was observed for β-adrenergic receptor, suggesting that this residue is critical for the stability of the receptor (Strader et al. 1989; Pollock et al. 1992). However, this mutant D1R increased the production of cAMP after DA stimulation, indicating that the receptor is present at the cell surface but the drastically altered ligand-binding site can no longer bind to SCH23390 (Pollock et al. 1992). Another study reported that mutating serine residues at positions 199 and 202 (TM5) of D1R affected DA binding (Tomic et al. 1993). Moreover, it showed that Asp70 in TM2 and Cys106 and Ser107 in TM3 of D1R are involved in agonist and antagonist binding (Tomic et al. 1993). Finally, mutating Trp321 in TM7 of D1R only decreased the affinity of antagonist

(Tomic et al. 1993). In the absence of a D1R three-dimensional model, we cannot rule out the possibility that these residues are not binding site per se. Rather, mutating those residues could have induced conformational changes that affected ligand binding. Accordingly, changing residues immediately before proline residue in TM5 and TM6 of D1R altered ligand binding (Cho et al. 1996). Since proline residues induce a kink in TM domains, it is more likely that these mutations changed the receptor conformation thereby affecting ligand binding instead of being true ligand binding site.

1.4.6 Elucidating the functional properties of structural components of D1-like receptors

Studies comparing D1R and D5R binding affinity values and G protein coupling properties reported important differences that were assessed with mutagenesis studies. The most distinguishing features between human or rat D1R and D5R are the ~10-fold higher affinity of DA for D5R over D1R, the higher affinity of agonists for D5R and the higher constitutive activity of D5R (Sunahara et al. 1991; Tiberi et al. 1991; Tiberi and Caron 1994; Charpentier et al. 1996; Iwasiow et al. 1999; Demchyshyn et al. 2000; Jackson et al. 2000; Tumova et al. 2003; Tumova et al. 2004). In addition, D1R has a higher affinity for antagonists and inverse agonists and exhibit higher DA-mediated stimulation of AC activity. The constitutive activity of D5R and its higher affinity for agonists correspond to properties of constitutively activated mutant adrenergic receptors according to the 'extended ternary complex model' (Ren et al. 1993; Samama et al. 1993; Samama et al. 1994).

Over the years, our lab and others have participated in the elucidation of the functions of structural components of D1-like receptors. As a result, various segments of

D1-like receptors have been implicated in the distinguishing features mentioned above. A terminal receptor locus (TRL), consisting of the TM6-EL3-TM7-CT region, contains structural determinant dictating DA binding but not antagonist or inverse agonist binding (Iwasiow et al. 1999). DA has increased affinity for the chimerical D1R with the TRL^{D5} whereas DA loses affinity for the chimerical D5R counterpart. Swapping only the EL3 caused a smaller decrease and increase in DA affinity for D5R and D1R, respectively (Iwasiow et al. 1999). However, swapping only the CT caused a complete reversal in DA affinity, having then 9 times more affinity for the D1-CT^{D5} chimera than for D5-CT^{D1} (Jackson et al. 2000). Another group reported that swapping the CT domain between D1-like receptors increased and decreased the affinity of various agonists for D1-CT^{D5} and D5-CT^{D1}, respectively (Demchyshyn et al. 2000). More specifically, exchanging the H8 region between D1-like receptors led to an almost complete swap of DA affinity (Tumova et al. 2004). Furthermore, swapping both EL3 and CT simultaneously between D1R and D5R resulted in a complete reversal of DA affinity compared with wild type receptors without affecting antagonist binding (Tumova et al. 2003). In addition to these structures, exchanging a specific residue of the carboxy-terminal end of IL3 between D1-like receptors results in changes in DA binding but not in antagonist binding (Charpentier et al. 1996). These results suggest that IL3, EL3 and CT contain structural features that are crucial for DA binding. However, since DA is known to bind within TM domains, these regions presumably do not possess ligand binding sites but must control the three-dimensional conformation of the binding pocket of D1-like receptors.

In addition to these chimerical studies, truncations of the CT of D1R were done to delineate segments of the tail implicated in DA responsiveness. Results indicated that

truncating half of the CT (position 379) was sufficient to increase DA affinity whereas truncating closer to the TM region (position 351) caused an even more pronounced increase in DA affinity (Chaar et al. 2001). Importantly, residue 351 corresponds to the cysteine-linked palmitoylation site of D1R and the last residue of H8. Although an increase in DA affinity was observed after the truncation at position 351, this increase is not as dramatic as it was observed after swapping the whole CT (D1-CT^{D5}). Therefore, other important residues in the CT must affect the three-dimensional structure of the orthosteric binding site.

The TRL swap also changed ligand-independent and dependent activation of D1-like receptors. Indeed, chimerical D1R behaved as D5R and vice versa in terms of constitutive activity and DA potency (Iwasiow et al. 1999). More precisely, swapping only EL3 changed the constitutive activity of both chimeras and reversed DA-mediated maximal activation of AC (Iwasiow et al. 1999). The exchange of CT reversed the agonist-independent activity of the chimeras and negatively affected DA-mediated activity as DA had decreased potency with both chimeras (Jackson et al. 2000). When EL3 and CT were jointly swapped, the constitutive activity of chimeras was switched while there was no effect on DA-dependent activation of AC (Tumova et al. 2003). In addition, the H8 region appears to control the subtype-specific constitutive activity of D1-like receptors while it modulates DA-mediated stimulation of AC and DA potency (Tumova et al. 2004). Interestingly, the truncated D1R at position 351 had increased constitutive activity and DA-mediated stimulation of AC activity suggesting that the CT is a critical component of D1R signaling (Chaar et al. 2001). As well, permuting residues

in IL3 modulated the constitutive activity of the chimeras and DA responsiveness (Charpentier et al. 1996).

These results with chimerical receptors have highlighted the importance of particular domains of D1-like receptors in the control of subtype-specific characteristics. As mentioned however, none of these swapped regions (except CT) seemed to affect the binding affinity of synthetic ligands as only the affinity of DA was modified. Furthermore, the intracellular and extracellular location of those domains most likely affect the three-dimensional conformation of the receptor that allows a proper folding for the binding of DA. Consequently, a focus on TM domains must be taken in order to shape the ligand binding pocket of D1-like receptors. Mutagenesis of residues in TM domains has pinpointed residues in TM2, TM3, TM5 and TM7 that modulate ligand binding of D1R. However, since those residues are found in both D1-like receptors, they cannot modulate subtype-specific binding properties. In addition, primary sequences of TM3, TM5 and TM7 are almost identical between D1R and D5R.

1.5 Issues, hypotheses and objectives

There are currently no ligands able to discriminate D1R and D5R. This constitutes an obstacle to the assignment of specific physiological roles for these highly identical receptors. Knockout mice models provided clues about the physiological functions of D1-like receptors and mutagenesis studies highlighted the importance of specific structural regions of the receptors. However, these studies also showed that the lack of a discriminating ligand interferes with a more detailed appreciation of each D1-like receptor subtype.

In an attempt to provide additional structural and molecular clues, we decided to assess the role of an amino terminal cassette, encompassed by the amino terminal (NT) and first transmembrane (TM1) regions of human D1-like receptors, a region with low sequence identity between D1R and D5R. Biochemical and crystallographic studies suggested that residues of TM domains form the ligand binding pocket of GPCRs. Importantly, the TM1 of D1R and D5R represents the TM region displaying the lowest degree of identity within the primary structure, suggesting that TM1 potentially harbors some structural determinants shaping the subtype-specific affinity and selectivity properties of DA and dopaminergic ligands. Additionally, little is known about NT region, the 'extracellular extension' of TM1. Crystallographic studies stressed the role of the NT in rhodopsin and opsin structure, forming a compact 'retinal plug' with ELs (Palczewski et al. 2000; Park et al. 2008). However, the absence of an extracellular plug in β-adrenergic and adenosine receptors and the unresolved crystallographic structure of their NT do not permit an identification of the functions of NT in those GPCRs.

Therefore, the hypotheses of my Master's research project are that the amino terminal cassette of human D1 and D5 dopaminergic receptors encompassing the NT and TM1 regions **(1)** controls the affinity and selectivity of dopaminergic ligands and **(2)** contributes to the distinct G protein coupling properties of D1-like receptors. To address these issues, my research project objectives are **(1)** to probe the role of the NT and TM1 of D1-like receptors in regulating subtype affinity and selectivity using a chimerical receptor approach; **(2)** to evaluate the potential role of NT and TM1 in regulating G protein coupling properties; **(3)** to delineate the specific amino acids of TM1 regulating

the subtype affinity, selectivity and G protein coupling properties of D1-like receptors using multiple and single point mutations.

In the first manuscript, I address the first two objectives. The construction and characterization of chimerical receptors in which the NT and TM1 are simultaneously or separately swapped between D1R and D5R highlights the importance of these two receptor structures in distinctly shaping ligand affinity and efficacy. In addition, the use of a variety of benzazepine ligands delineates the role of specific functional groups in ligand efficacy. Then, the second manuscript addresses the third objective of my Master's studies by identifying crucial residues within the TM1 of D1-like receptors controlling ligand binding, membrane-bound receptor expression and DA responsiveness.

Chapter 2. Manuscript #1

The extracellular amino terminus and first membrane-spanning helix of dopamine D1 and D5 receptors shape distinct structural interplays for ligand selectivity and efficacy

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The abbreviations used are: B_{max}, maximal binding capacity, CT, cytoplasmic tail, DA, dopamine, GPCR, G protein coupled receptor, HEK293, human embryonic kidney 293, NT, amino terminus, TM, transmembrane domain

ABSTRACT

Transmembrane (TM) helices of human D1-like dopaminergic receptors (hD1R and hD5R) harbor the same residues implicated in ligand binding and activation of catecholamine G protein-coupled receptors (GPCRs). Yet, hD1R and hD5R naturally display the distinct functional properties commonly shared by wild type and constitutively active mutant GPCRs, respectively. Here, we explore the role of distinct extracellular amino terminus (NT) and TM1 primary sequences of hD1R and hD5R in regulating D1-like functionalities. Our mutational study shows that TM1 shapes D1-like conformations for ligand affinity and selectivity. We also demonstrate that NT and TM1 of hD1R and hD5R differentially modulate dopamine-mediated responsiveness while having no role in receptor constitutive activity or antipsychotic-mediated inverse agonism. However, the TM1 exchange between hD1R and hD5R mediated drastic changes in intrinsic efficacy and activity displayed by D1-like benzazepine drugs. Benzazepines were converted into strong partial agonists or full agonists in cells expressing hD1R-TM1^{D5} chimera. In contrast, benzazepines were switched from full agonists to partial agonists and partial agonists to antagonists in cells expressing hD5R-TM1^{D1} chimera. Interestingly, we noticed that benzazepine agonists harboring a methyl on the azepine ring exhibited a lower affinity for the more constitutively activated hD5R. This finding opposes the "allosteric ternary complex model" postulating a higher agonist affinity for constitutively active GPCRs. In summary, our study shows that NT and TM1 of D1-like receptors play a major role in ligand binding and agonist-induced activation, poising these regions as important structural determinants for GPCR function, which could not be appreciated from crystallized catecholamine receptors.

INTRODUCTION

Cells can sense a wide variety of photonic and chemical cues (ligands) from the outside environment using specialized surface proteins called G protein-coupled receptors (GPCRs). This sensing process is initiated by a series of ligand binding and activation steps taking place in GPCR extracellular and transmembrane (TM) domains. These steps trigger intracellular signaling through a structural rearrangement of receptor cytoplasmic interface facilitating recruitment of heterotrimeric GTP-binding (G) proteins and non-G protein partners. Earlier studies provided functional insight into a role of the predicted seven TM domains of GPCRs in the formation of binding pockets for diffusible ligands (1-3). Crystallographic data have now supported this view and provided insights into how TM and extracellular regions may regulate ligand binding to rhodopsin-like (class A) GPCRs (4-8).

Identification of TM residues of GPCRs contributing to binding to various natural and synthetic diffusible ligands has been challenging (9,10). This is an important issue as numerous pathologies are treated with pharmaceuticals targeting compromised GPCRs (11). Thus, a further understanding of the molecular principles underlying the structural basis of different GPCRs is required to develop new therapeutic compounds selectively targeting individual members of a GPCR subclass. This issue is best highlighted by dopamine (DA) receptors, which belong to rhodopsin-like (class A) GPCRs and play a crucial role in the clinical manifestations of several brain disorders (11-13). Five distinct genes encode D1-like (D1R and D5R) and D2-like ($D2R_{short/long}$, D3R and D4R) receptors. D1-like receptors are positively linked to adenylyl cyclase (AC) activation

through a direct coupling to stimulatory heterotrimeric $G_{s/olf}$ proteins (12). D1-like receptors display high degree of amino acid sequence (>80%) identity within their TM regions (14). Yet, DA binds to D5R with noticeably higher affinity. In fact, the pharmacological and functional properties distinguishing D5R from D1R are reminiscent of those reported for constitutively active mutant and wild type forms of GPCRs, respectively (15-21).

Evidence suggest that D1-like subtypes are potentially important therapeutic targets (13,22). Meanwhile, development of subtype-selective ligands for D1R and D5R has been very difficult (13,22). Studies have shown that the third intracellular loop (IL3) and cytoplasmic tail (CT) regions of D1-like receptors contain structural determinants regulating subtype-specific DA affinity, DA-independent and dependent G protein-coupling properties of D1R and D5R (16,23-27). Because of the intracellular localization of these regions, it is unlikely that IL3 and CT sequences bind to DA. Instead, intramolecular constraints underlying the three-dimensional orientation of TM regions of D1R and D5R for ligand binding are likely modulated by IL3 and CT -specific sequences of D1-like subtypes. Residues in TM3, TM5, TM6 and TM7 have been implicated in ligand binding and activation of D1R (12). However, sequences of these TM regions virtually display full identity with those of D5R (14). This suggests a minor role of these membrane-spanning helices in promoting D1-like subtype-specific TM interplays for ligand affinity and selectivity.

Studies have highlighted the role of the extracellular amino terminus (NT) in regulating ligand binding and responsiveness of GPCRs (4,28-39). The NT regions of human D1R and D5R display a very low degree of primary structure identity (< 20%)

and drastically different number of residues (25 for D1R vs. 42 for D5R). Furthermore, the NT of human D5R contains a higher number of glycine (7) and proline (6) residues than that of the human D1R, which has only two glycine residues and no proline. These amino acids are associated with secondary structure beta turns, which may have a role in shaping distinct TM bundle interplays for D1-like subtype-specific ligand binding properties. Both NT regions possess one conserved *N*-linked glycosylation site playing a determinant role in the plasma membrane expression of D5R while not affecting ligand binding (40). Studies have also demonstrated that TM1 can regulate intramolecular interactions and compatibility between TM regions for the binding function and conformational state stabilization of GPCRs (41-50). Interestingly, TM1 of D1R and D5R displays the most amino acid differences spread out along the whole helical domain when compared to other TM regions.

In the present study, we investigated the specific roles played by NT and TM1 regions of human D1R and D5R in regulating their subtype-specific ligand binding and G protein coupling properties. Our results using chimerical receptors support the notion that NT and TM1 regions regulate distinct structural changes that impart subtype-specific ligand binding and activation properties to D1R and D5R. Moreover, our D5R binding data obtained with synthetic benzazepines ligands suggest that GPCRs with greater constitutive activity relative to their more "silent" forms can also bind agonists with considerably lower affinity; a finding somewhat contrary to the idea put forth by the "allosteric ternary complex model" (19-21).

EXPERIMENTAL PROCEDURES

Materials- (+)-*N*-[methyl-^{3}H]-SCH23390 (65-91 Ci/mmol) and [^{14}C]-cAMP (250-275 mCi/mmol were from GE Healthcare. [2, 8-^{3}H]-adenine (24-27 Ci/mmole) was purchased from PerkinElmer. Dopamine (DA) hydrochloride, (+)-butaclamol hydrochloride, *cis*-flupenthixol dihydrochloride, fluphenazine dihydrochloride, trifluoperazine dihydrochloride, thioridazine hydrochloride, thiothixene hydrochloride, SKF82526 (fenoldopam) hydrobromide, (+)-SCH23390 hydrochloride, (+)-SKF38393 hydrochloride, SKF75670 hydrobromide, SKF83566 hydrochloride, SKF82958 (chloro-APB) hydrobromide and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich. Dihydrexidine hydrochloride, SKF83959 hydrobromide and (+)-SKF81297 hydrobromide were acquired from Tocris. Taq DNA polymerase, gentamicin, minimal essential media (MEM), phosphate buffered saline (PBS) and fetal bovine serum (FBS) were obtained from Invitrogen.

Expression constructs- We used a site-directed polymerase chain reaction-based overlap extension approach to construct six chimerical receptors: hD1R-NTTM1^{D5}, hD1R-NT^{D5}, hD1R-TM1^{D5}, hD5R-NTTM1^{D1}, hD5R-NT^{D1} and hD5R-TM1^{D1} (Fig. 1). Custom DNA oligonucleotides were from Sigma Genosys. PCR primer sequences (P1-P6) are listed in *Supplementary Information* (Table S1). Wild type human D1 (hD1R) and D5 receptor (hD5R) DNA sequences cloned in the pCMV5 expression vector were used as templates. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively. An overlapping region (15-18 nucleotides) between A and B allowed the amplification of the final PCR product using primers P5-P6. PCR

reactions were carried out with Taq DNA polymerase in an Eppendorf thermal Mastercycler using the following conditions: 1 cycle (94°C for 3 min, 46-55°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 46-55°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 the following conditions: 1 cycle (94°C for 3 min, 46-55°C for 1 min, 72°C for 10 min), 25 cycles (94°C for 45 s, 46-55°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). Purified DNA bands were digested with appropriate restriction enzymes and ligated in-frame with the expression vector pCMV5 containing wild type human D1R or D5R DNA sequences. Chimerical constructs hD1R-NTTM1^{D5} and hD1R-NT^{D5} were first subcloned in linearized (*Bgl*II-*EcoR*I) pBlueScript II SK+ (Stratagene). The expression construct containing the full coding sequence of chimerical hD1R-TM1^{D5} was generated via a 3-piece ligation reaction using linearized pCMV5 (*EcoR*I-*Xba*I), hD1R-TM1^{D5} (*EcoR*I-*Bgl*II) and wild type hD1R (*Bgl*II-*Xba*I) fragments. Linearized hD5R-pCMV5 construct (*EcoR*I-*Bsm*I) was utilized to generate hD5R-NTTM1^{D1}, hD5R-NT^{D1} and hD5R-TM1^{D1} chimeras. Integrity of the coding sequences was confirmed by automated fluorescent DNA sequencing (Applied Biosystems 3730 DNA Analyzer) performed at the StemCore Laboratories of the Ottawa Genomics Innovation Centre (Ottawa, ON).

Cell culture and transfection- Human embryonic kidney 293 (HEK293) cells (American Tissue Culture Collection, CRL-1573) were cultured at 37°C with 5% CO_2 atmosphere in MEM with Earle's salts supplemented with 10% v/v heat-inactivated FBS and 20 μg/mL gentamicin. Cells were seeded in 100-mm dishes (2.5 x 10^6 cells/dish) and transiently

transfected by a modified calcium-phosphate method described previously using a total amount of 5 μg of receptor DNA/dish (26). Empty pCMV5 vector was used to adjust DNA amounts when less than 5 μg of receptor DNA was needed for transfections. All experiments were performed with cells from 41 to 52 passages.

Membrane preparation- After 18-24h incubation with the DNA-calcium-phosphate precipitate, HEK293 cells were washed with PBS, trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Then, HEK293 cells were washed with cold PBS, scraped in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA, pH 8.0) and centrifuged at 40,000 x *g* for 20 min at 4°C. Pellets were then washed in lysis buffer using a Brinkmann Polytron (17,000 rpm for 15 s) and centrifuged at 4°C (40,000 x *g* for 20 min). The washing procedure was repeated once. The final pellets were resuspended in lysis buffer and crude membranes were frozen in liquid nitrogen and stored at -80°C until used. Prior to freezing, an aliquot of different receptor membrane preparations were utilized for saturation studies as described in the next section.

Radioligand binding assays- Fresh or frozen membranes (thawed on ice) were mixed in a resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA, pH 8.0) using a Brinkmann Polytron (17,000 rpm for 15 s). Radioligand saturation and competition assays were performed 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) with 100 μL of membranes in a total volume of 500 uL using *N*-[methyl-^{3}H]-SCH23390 as radioligand. Saturation studies were done using increasing concentrations of *N*-[methyl-^{3}H]-SCH23390 ranging from 0.02 to 8 nM. Non-specific binding was delineated using 10 μM of *cis*-flupenthixol. For competition studies, frozen membranes were thawed on

ice, mixed in resuspension buffer and incubated with a constant concentration of *N*-[methyl-^{3}H]-SCH23390 (0.5-1 nM) and increasing concentrations of cold competing ligand. Competition studies using DA were done in the presence of 0.1 mM ascorbic acid (final in assays). Binding assays were incubated for 90 min at room temperature and terminated using rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed three times with 5 ml of cold washing buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS6500). Protein concentration was determined using the Bio-Rad assay kit with bovine serum albumin (BSA) as standard. To determine the equilibrium dissociation constant (K_d, nM), the inhibitory dissociation constant (K_i, nM) and receptor number (Bmax, pmol/mg of membrane proteins) values, binding isotherms were analyzed using the non-linear curve-fitting program GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Whole cell cAMP assays- G protein-mediated activation of adenylyl cyclase by wild type and chimerical receptors was assessed using a whole cell cAMP assays as described previously (26,51). Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12-well dishes. The following day, the reseeding medium was replaced with fresh MEM containing FBS (5% v/v), gentamicin (20 µg/mL) and [^{3}H]-adenine (1-2 µCi/mL), and HEK293 cells metabolically labeled overnight at 37°C and 5% CO_2. The next day, the labeling medium was removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing IBMX (1 mM) in the presence or absence of drugs for 30 min at 37°C DA was tested in the presence of ascorbic acid at a final concentration of 0.1 mM. After the incubation period,

the medium was aspirated and each well filled with 1 mL of lysis solution (2.5% (v/v) perchloric acid, 0.1 mM cAMP and [^{14}C]cAMP (5 μCi, 8000-12,000 dpm)) for 30 min at 4°C. The lysates were then transferred to tubes containing 0.1 mL of a neutralizing solution (4.2 M KOH), mixed by vortex and subjected to a low-speed centrifugation (1500 rpm, 15 min) at 4°C to eliminate salt precipitates. [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex and alumina columns as described before (51). The amount of [^{3}H]-cAMP (CA) over the total amount of intracellular [^{3}H]-adenine (TU) was calculated to determine the relative adenylyl cyclase activity (expressed as CA/TU x 1000). Dose-response curves to DA were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02. Receptor expression (Bmax values) were determined using a saturating concentration (~7 nM) of *N*-[methyl-^{3}H]-SCH23390.

Statistics- Equilibrium and inhibitory dissociation binding constants (K_d and K_i) are expressed using the geometric mean with the 95% lower and upper confidence interval. All other data are reported as arithmetic means ± standard error unless stated otherwise. Dose-response curves to DA were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02 using constrained or unconstrained parameters (bottom or constitutive activity, top or maximal stimulation, log of EC_{50} or log of effective concentration that elicits 50% of maximal stimulation, slope factor). This analysis was performed to establish whether differences observed between best-fitted values were statistically different, i.e., whether constraining a specific curve parameter to a shared or fixed value worsens the goodness of fit. Student's t test and analysis of variance (one-way ANOVA) with Newman-Keuls multiple comparison test were used to

compare two or multiple groups, respectively. Two-sided tests were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) with a critical level of probability set at $\alpha = 0.05$.

RESULTS

Role of NT and TM1 regions of D1-like receptors in controlling functional ligand binding- To probe the role of the extracellular NT and TM1 regions of hD1R and hD5R in controlling functional ligand binding properties, a series of chimerical receptors between D1-like receptors were constructed as depicted in Fig. 1. Results obtained from saturation studies using *N*-[methyl-^{3}H]-SCH23390 in membrane preparations made from wild type and chimerical D1-like receptors are shown in Table I. All chimerical receptors retain their ability to bind with high affinity to *N*-[methyl-^{3}H]-SCH23390 indicating that there was no major alteration in the folding conformation of ligand binding pocket for this compound. The maximal expression (B_{max}) values of wild type and chimerical D1-like receptors attainable in HEK293 cells were also determined (Table I). Statistically detectable differences were only noted between B_{max} values of TM1 chimeras and their respective wild type parent receptors. B_{max} values of hD1R-TM1^{D5} and hD5R-TM1^{D1} were increased and decreased by ~35%, respectively (Table I).

There were also statistically detectable differences between equilibrium dissociation constant values (K_d) of *N*-[methyl-^{3}H]-SCH23390 obtained with wild type and chimerical receptors (Table I). These differences suggest that NT and TM1 of hD1R and hD5R may play an opposite role in regulating the binding to this classical D1-like benzazepine.

Figure 1. Primary sequence of the extracellular amino terminus (NT) and first transmembrane domain (TM1) of human D1-like receptors and schematic representation of wild type and chimerical receptors. *A.* Alignment of the primary structure of the NT and TM1 regions of human D1-like receptors. Identical residues are indicated by an asterisk. *B.* The topology of wild type D1R (black circles), D5R (grey circles) and all chimerical receptors is illustrated.

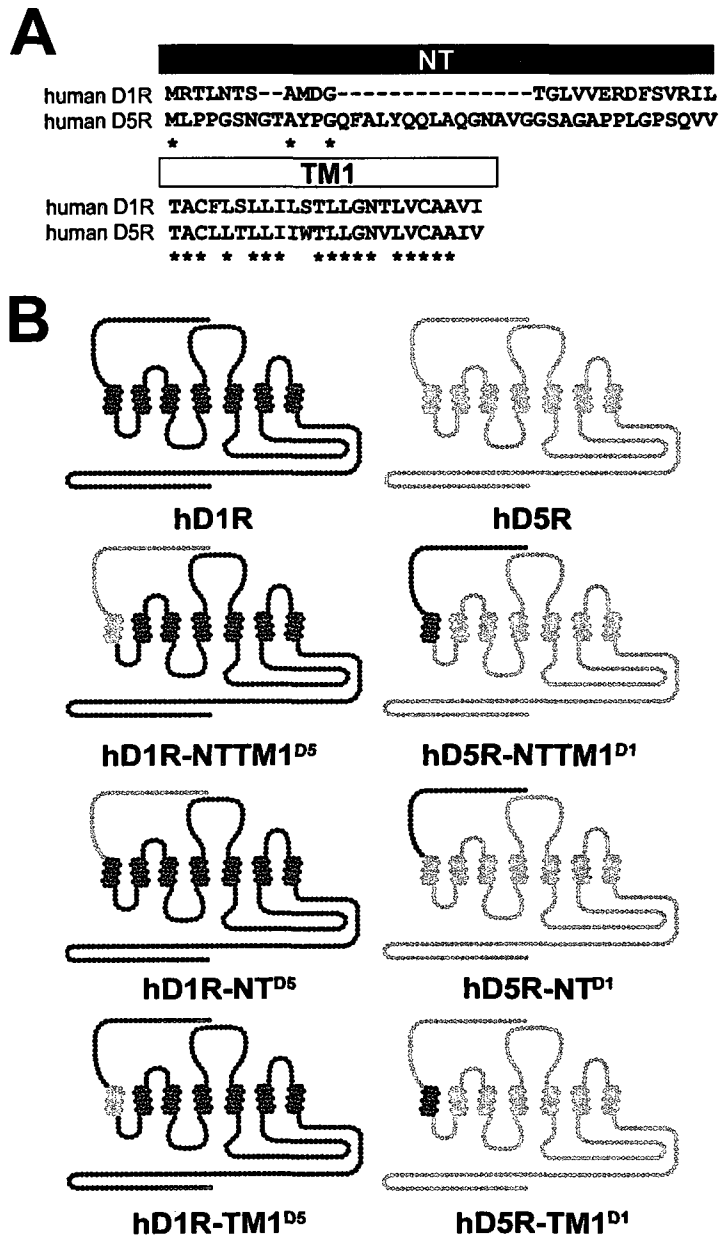


Table I. Dissociation constants (K_d) and maximal binding capacity (B_{max}) values of *N*-[methyl-^{3}H]-SCH23390 for wild type and chimerical D1-like receptors. K_d (nM) are expressed as geometric means and fold over parent wild type receptor K_d value from six to eleven experiments done in duplicate determinations with the lower and upper 95% confidence interval in brackets. The receptor expression (pmol/mg of membrane proteins, B_{max}) are expressed as arithmetic means with the lower and upper 95% confidence interval from six to eleven experiments and are also expressed as fold over parent wild-type receptor expression. *, $p<0.05$ when compared with hD1R; #, $p<0.05$ when compared with hD5R.

Receptor	K_d (nM)	Fold over parent wt	B_{max} (pmol/mg prot.)	Fold over parent wt
hD1R	**0.60** (0.50-0.70)	**1**	**13.5** (9.7-17.3)	**1**
hD1R-NTTM1^{D5}	**0.70** (0.52-0.94)	**1.23** (0.99-1.48)	**13.8** (8.4-19.2)	**1.01** (0.85-1.18)
hD1R-NT^{D5}	**0.50** (0.43-0.59)	**0.85*** (0.78-0.92)	**11.8** (9.4-14.1)	**0.92** (0.78-1.05)
hD1R-TM1^{D5}	**0.80** (0.70-0.91)	**1.36*** (1.22-1.50)	**17.1** (13.6-20.6)	**1.34*** (1.12-1.55)
hD5R	**1.12** (0.85-1.46)	**1**	**15.8** (12.8-18.9)	**1**
hD5R-NTTM1^{D1}	**0.43** (0.30-0.62)	**0.45#** (0.36-0.53)	**13.0** (7.4-18.5)	**0.88** (0.76-1.01)
hD5R-NT^{D1}	**0.88** (0.72-1.09)	**0.80#** (0.71-0.89)	**15.0** (11.9-18.2)	**0.95** (0.87-1.02)
hD5R-TM1^{D1}	**0.58** (0.47-0.72)	**0.53#** (0.46-0.59)	**10.3** (8.2-12.3)	**0.66#** (0.58-0.74)

Indeed, the full D1R chimera (hD1R-NTTM1^{D5}) showed an affinity loss for *N*-[methyl-^{3}H]-SCH23390 in comparison with hD1R. An opposing trend was observed with the full D5R chimera (hD5R-NTTM1^{D1}) relative to hD5R. This divergent effect was further delineated using chimeras in which NT or TM1 regions were individually swapped between hD1R and hD5R (Table I). Saturation studies reveal that *N*-[methyl-^{3}H]-SCH23390 binds hD5R-TM1^{D1} with higher affinity than hD5R, a result comparable to that observed with hD5R-NTTM1^{D1} (Table I). In a similar fashion to hD1R-NTTM1^{D5}, the *N*-[methyl-^{3}H]-SCH23390 affinity at hD1R-TM1^{D5} was reduced relative to wild type hD1R (Table I). In the next series of experiments, the functional roles of NT and TM1 regions in ligand binding were further explored using competition studies with DA (natural ligand) and three chemically unrelated synthetic heterocyclic compounds: SKF82526 (benzazepine), *cis*-flupenthixol (thioxanthene) and (+)-butaclamol (dibenzocycloheptene).

NT and TM1 regions of D1-like receptors differentially modulate binding to DA and synthetic drugs- Our competition studies show that DA binds to hD1R-NTTM1^{D5} (full chimera) with an affinity undistinguishable from the parent wild type hD1R (Table II). Meanwhile, hD1R-NT^{D5} and hD1R-TM1^{D5} both display a small increase in DA affinity as compared with wild type hD1R, although this increase was not detectable statistically (Table II). These results suggest that the lower DA affinity at wild type hD1R is dependent on a molecular interplay between specific NT and TM1 sequences. Different observations were made with synthetic compounds. Indeed, SKF82526 and (+)-butaclamol had a lower affinity for hD1R-NTTM1^{D5} relative to hD1R. This effect was mediated by the exchange of TM1 region as suggested by our data obtained with hD1R-

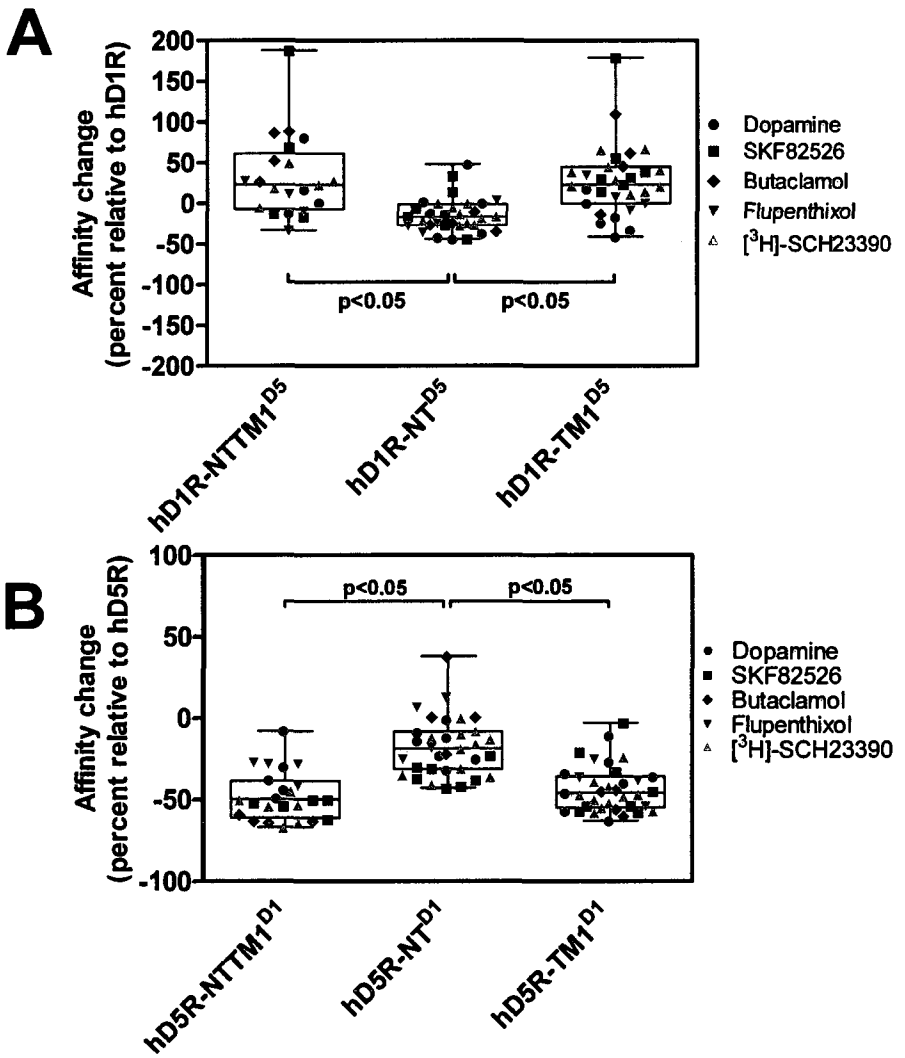
$TM1^{D5}$ (Table II). In a similar fashion to DA, the affinity of *cis*-flupenthixol for hD1R-$NTTM1^{D5}$ remains unchanged (Table II). However, when compared with wild type hD1R and hD1R-$NTTM1^{D5}$, the affinity of *cis*-flupenthixol was increased and decreased at hD1R-NT^{D5} and hD1R-$TM1^{D5}$, respectively (Table II). These results are distinct from the increase in DA affinity detected at these two chimeras. Potentially, competition studies done using synthetic drugs and chimerical hD1R harboring NT and/or TM1 of hD5R point at the existence of different modes of binding for DA and synthetic ligands.

Conversely, studies using hD5R chimeras show that the modes of binding for DA and synthetic drugs at hD5R share more similarities. The hD5R-$NTTM1^{D1}$ exhibits higher affinity for DA relative to its parent wild type hD5R. The hD5R-$TM1^{D1}$ also displays an increased DA affinity when compared with wild type hD5R (Table II). The DA affinity for hD5R-NT^{D1} chimera was not statistically distinguishable from that measured at hD5R (Table II). Similar results were obtained with synthetic drugs. Indeed, our studies suggest that the synthetic compounds tested in this series of experiments generally bind to hD5R-$NTTM1^{D1}$ with higher affinities than those calculated for hD5R (Table II). These findings were fully recapitulated with hD5R-$TM1^{D1}$. While NT-mediated effects on ligand binding conformation were detected, they were somehow mitigated relative TM1-mediated effects. The general trend of individual affinity changes of DA and synthetic drugs at chimerical receptors relative to their respective wild type parent receptors is depicted in Fig. 2. Our data indicate that TM1 is the prime determinant within the amino terminal GPCR cassette (NT+TM1) of hD1R and hD5R in regulating ligand affinity. We next assessed how TM1-mediated effects on affinity can influence the ability of ligands to discriminate between D1-like receptors.

Table II. Inhibitory dissociation constants (K_i) values of dopaminergic ligands for wild type and chimerical D1-like receptors. K_i (nM) are expressed as geometric means and fold over wild type receptor K_i value from four to eight experiments done in duplicate determinations with the lower and upper 95% conconfidence interval in brackets. *, $p<0.05$ when compared with hD1R; #,$p<0.05$ when compared with hD5R.

	Dopamine		SKF82526		Flupenthixol		Butaclamol	
Receptor	K_i (nM)	Fold over parent wt	K_i (nM)	Fold over parent wt	K_i (nM)	Fold over parent wt	K_i (nM)	Fold over parent wt
hD1R	**6208** (5214-7391)	**1**	**41.4** (34.8-49.3)	**1**	**5.88** (3.79-9.13)	**1**	**3.81** (2.23-6.48)	**1**
hD1R-NTTM1^{D5}	**6305** (3879-10249)	**1.22** (0.56-1.87)	**60.6** (38.9-94.3)	**1.57** (0.05-3.09)	**5.71** (2.69-12.2)	**1.00** (0.57-1.42)	**6.15** (4.51-8.38)	**1.64*** (1.17-2.11)
hD1R-NT^{D5}	**4990** (3996-6232)	**0.88** (0.57-1.18)	**37.2** (29.4-46.9)	**0.92** (0.68-1.16)	**4.62** (2.19-9.75)	**0.80** (0.52-1.07)	**2.89** (2.00-4.17)	**0.77*** (0.61-0.92)
hD1R-TM1^{D5}	**4873** (4048-5865)	**0.84** (0.61-1.07)	**60.7** (47.0-78.4)	**1.53*** (1.01-2.06)	**6.34** (3.64-11.0)	**1.09** (0.79-1.39)	**5.49** (3.85-7.83)	**1.51** (0.71-2.32)
hD5R	**629** (501-789)	**1**	**24.9** (17.6-35.1)	**1**	**9.96** (5.54-17.9)	**1**	**38.7** (25.7-58.1)	**1**
hD5R-NTTM1^{D1}	**381** (262-553)	**0.66**$^{\#}$ (0.46-0.86)	**10.8** (6.6-17.8)	**0.46**$^{\#}$ (0.40-0.53)	**6.84** (3.21-14.6)	**0.69**$^{\#}$ (0.58-0.80)	**14.6** (10.6-20.1)	**0.38**$^{\#}$ (0.34-0.41)
hD5R-NT^{D1}	**542** (409-719)	**0.83**$^{\#}$ (0.74-0.93)	**16.7** (11.9-23.7)	**0.65**$^{\#}$ (0.58-0.72)	**9.22** (4.32-19.7)	**0.94** (0.65-1.24)	**39.6** (32.9-47.7)	**1.05** (0.65-1.44)
hD5R-TM1^{D1}	**370** (245-558)	**0.61**$^{\#}$ (0.47-0.75)	**14.1** (11.2-17.9)	**0.59**$^{\#}$ (0.43-0.76)	**6.07** (2.46-14.9)	**0.62**$^{\#}$ (0.43-0.81)	**18.7** (14.7-23.9)	**0.49**$^{\#}$ (0.36-0.61)

Figure 2. Affinity change (in %) of dopaminergic ligands for chimerical receptors relative to parent wild type receptor. For each drug tested, percent change in affinity relative to wild type receptor was calculated from K_d and K_i values reported in Table I and II. Each box represents the 25th and 75th percentiles and whiskers show the minimum and maximum values of ligand affinity changes of each chimerical receptor relative to parent wild type receptor. $p<0.05$, one-way ANOVA followed by Newman-Keuls. No differences are observed between full chimeras and TM1-swapped receptors. All values are different from 0; one-sample t test ($p<0.05$). (DA, red circle; [^{3}H]-SCH23390, grey triangle; SKF82526, blue square; Butaclamol, purple diamond; Flupenthixol, green inverted triangle) *A.* Box-and-whisker plot of chimerical D1R relative to wild type hD1R. *B.* Box-and-whisker plot of chimerical D5R relative to wild type hD5R.



D1-like ligand selectivity is mediated by TM1- We computed selectivity ratios of drugs having a higher affinity for hD1R (hD5R/hD1R > 1) or hD5R (hD1R/hD5R > 1) using K_d or K_i values determined in our radioligand binding studies. Selectivity ratios of drugs displaying higher affinity for hD5R were considerably augmented following the exchange of NT and TM1 regions (Fig. 3*A*). In stark contrast, selectivity ratios of drugs with a higher affinity for hD1R were drastically reduced when probed with NTTM1 chimeras (Fig. 3*B*). Notably, the effects on drug selectivity ratios measured with NTTM1 chimeras were recapitulated with TM1 chimeras. These data highlight the functional importance of TM1 of hD1R and hD5R in the regulation of drug selectivity at these D1-like subtypes. We further tested the idea that TM1 is the prime structural determinant for synthetic drug selectivity using competition studies with the TM1-swapped chimeras and a broader range of benzazepine compounds and antipsychotics. Our data show that affinity of synthetic drugs was globally reduced at hD1R-$TM1^{D5}$ while being increased at hD5R-$TM1^{D1}$ relative to their respective parent wild type receptors (Table III, Fig. 4). These data provide additional evidence for a role of the TM1 in modulating ligand binding properties of hD1R and hD5R. In agreement with data shown in Fig. 3, the selectivity ratios of drugs exhibiting higher affinity for hD5R were greatly increased following the exchange of TM1 regions (Fig. 5*A*). Meanwhile, drugs displaying a greater selectivity for hD1R did not discriminate anymore between hD1R-$TM1^{D5}$ and hD5R-$TM1^{D1}$ (Fig. 5*B*). Thus, TM1 of D1-like receptors controls subtype-dependent ligand selectivity. We next explored whether the GPCR amino cassette (NT and TM1) of hD1R and hD5R regulates ligand-independent and dependent regulation of intracellular cAMP formation.

Figure 3. Selectivity ratios of dopaminergic ligands for wild type and chimerical D1-like receptors. For each drug tested, selectivity ratio was calculated from K_d and K_i values reported in Table I and II. Bars represent the arithmetic mean ± S.E. of four to eleven experiments done in duplicate determinations. *A.* Selectivity ratios of DA and SKF82526 were calculated for each experiment by dividing the affinity for wild type or chimerical hD1R by the affinity for their respective hD5R counterpart. *, $p<0.05$ when compared with wild type hD1R/hD5R selectivity ratio. *B.* Selectivity ratios of *N*-[methyl-^{3}H]-SCH23390, flupenthixol and butaclamol were calculated for each experiment by dividing the affinity for wild type or chimerical hD5R by the affinity for their respective hD1R counterpart. *, $p<0.05$ when compared with wild type hD5R/hD1R selectivity ratio.

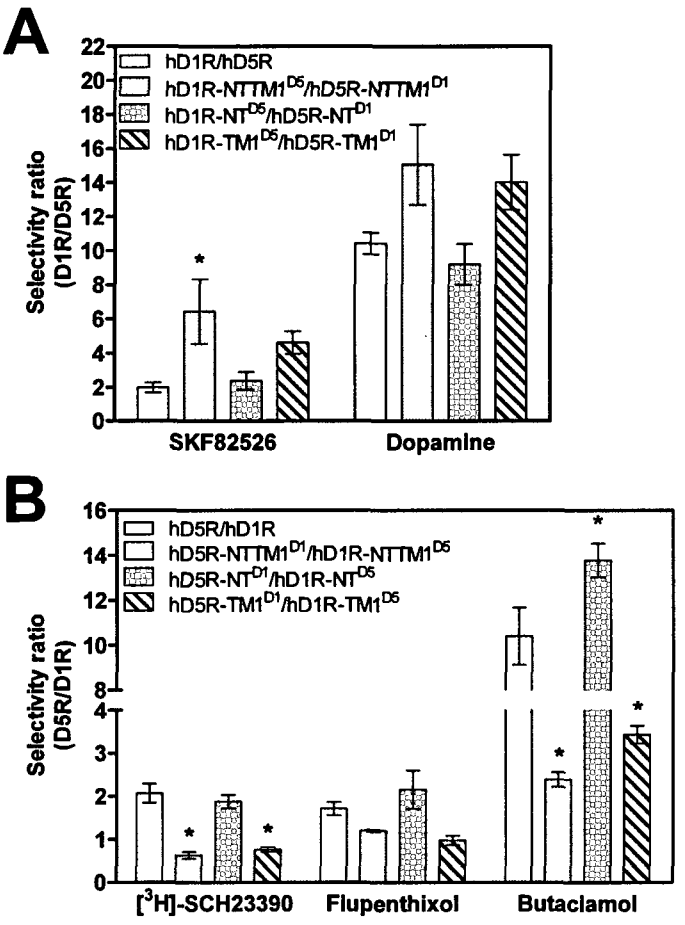


Table III. Inhibitory dissociation constants (K_i) values of dopaminergic ligands for wild type and TM1-swapped D1-like receptors. K_i (nM) are expressed as geometric means and fold over parent wild type receptor K_i value from three to six experiments done in duplicate determinations with the lower and upper 95% confidence interval in brackets. *, $p<0.05$ when compared with hD1R; #, $p<0.05$ when compared with hD5R.

	hD1R	hD1R-TM1^{D5}		hD5R	hD5R-TM1^{D1}	
Ligands	K_i (nM)	K_i (nM)	Fold over parent wt	K_i (nM)	K_i (nM)	Fold over parent wt
SKF38393	**102** (80.8-128)	**92.2** (71.6-119)	**0.94** (0.68-1.20)	**102** (82.4-125)	**56.0** (48.1-65.1)	**0.56**# (0.46-0.65)
SKF75670	**17.2** (11.7-25.3)	**16.7** (12.2-22.9)	**0.99** (0.70-1.27)	**28.7** (13.8-59.6)	**14.8** (10.2-21.5)	**0.53**# (0.28-0.78)
SKF81297	**42.1** (29.5-60.1)	**48.3** (31.6-74.0)	1.15* (1.07-1.23)	**11.8** (6.45-21.5)	**8.12** (4.85-13.6)	**0.69**# (0.60-0.79)
SKF82958	**23.6** (13.8-40.2)	**31.3** (21.5-45.6)	**1.36** (0.79-1.93)	**13.1** (8.34-20.4)	**5.81** (4.04-8.35)	**0.45**# (0.35-0.54)
SKF83566	**1.99** (1.40-2.83)	**2.52** (2.04-3.12)	**1.29** (0.93-1.64)	**3.26** (2.46-4.31)	**1.62** (1.22-2.14)	**0.51**# (0.32-0.69)
SKF83959	**1.59** (1.14-2.21)	**1.88** (1.46-2.43)	**1.19** (0.84-1.54)	**4.72** (3.20-6.95)	**2.82** (1.85-4.30)	**0.61**# (0.38-0.83)
Dihydrexidine	**513** (413-637)	**530** (326-862)	**1.08** (0.49-1.67)	**45.8** (27.4-76.7)	**28.1** (14.6-54.2)	**0.62**# (0.49-0.74)
Fluphenazine	**25.4** (15.4-42.1)	**32.8** (19.8-54.3)	**1.31** (0.91-1.70)	**54.3** (31.3-94.3)	**31.7** (19.8-50.6)	**0.59**# (0.50-0.67)
Thioridazine	**74.4** (55.0-101)	**143** (111-186)	1.98* (1.20-2.75)	**316** (243-411)	**131** (98.5-174)	**0.42**# (0.34-0.50)
Trifluoperazine	**28.6** (17.1-48.0)	**36.8** (13.6-99.9)	**1.33** (0.72-1.94)	**60.3** (27.6-132)	**33.5** (16.9-66.2)	**0.56**# (0.50-0.61)
Thiothixene	**92.3** (81.4-105)	**132** (99.0-177)	**1.44** (0.96-1.93)	**482** (316-735)	**272** (244-303)	**0.57**# (0.36-0.77)

Figure 4. Affinity change (in %) of dopaminergic ligands for TM1-swapped receptors relative to parent wild type receptor. For each drug tested, percent change in affinity relative to wild type receptor was calculated from K_i values reported in Table III. Each box represents the 25th and 75th percentiles and whiskers show the minimum and maximum values of ligand affinity changes of each TM1-swapped receptor relative to parent wild type receptor. Values are different from 0; one-sample t test, $p<0.05$. ((+)-SKF38393, red circle; SKF75670, blue square; (+)-SKF81297, purple diamond; SKF82958, green inverted triangle; SKF83566, grey triangle; SKF83959, +; dihydrexidine, X; fluphenazine, open circle; thioridazine, *; thiothixene, open square; trifluoperazine, brown star)

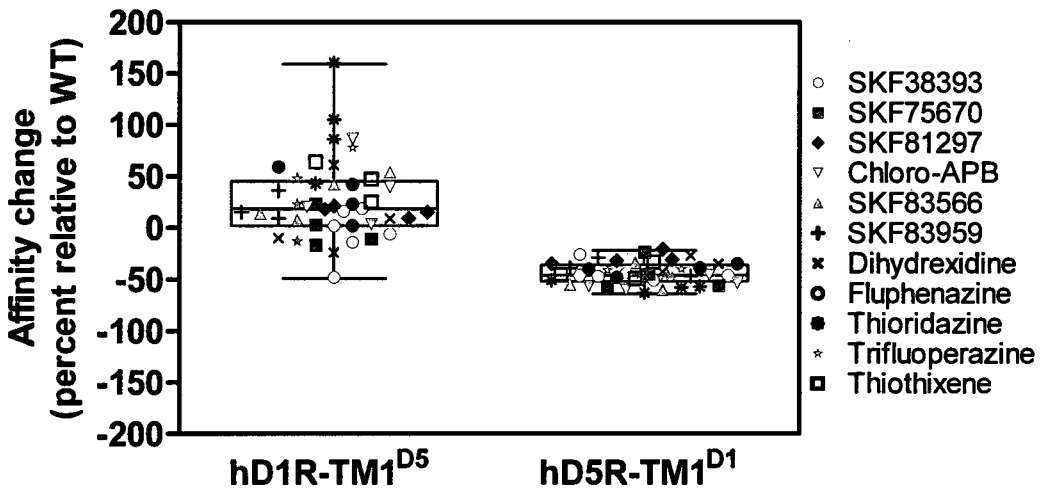
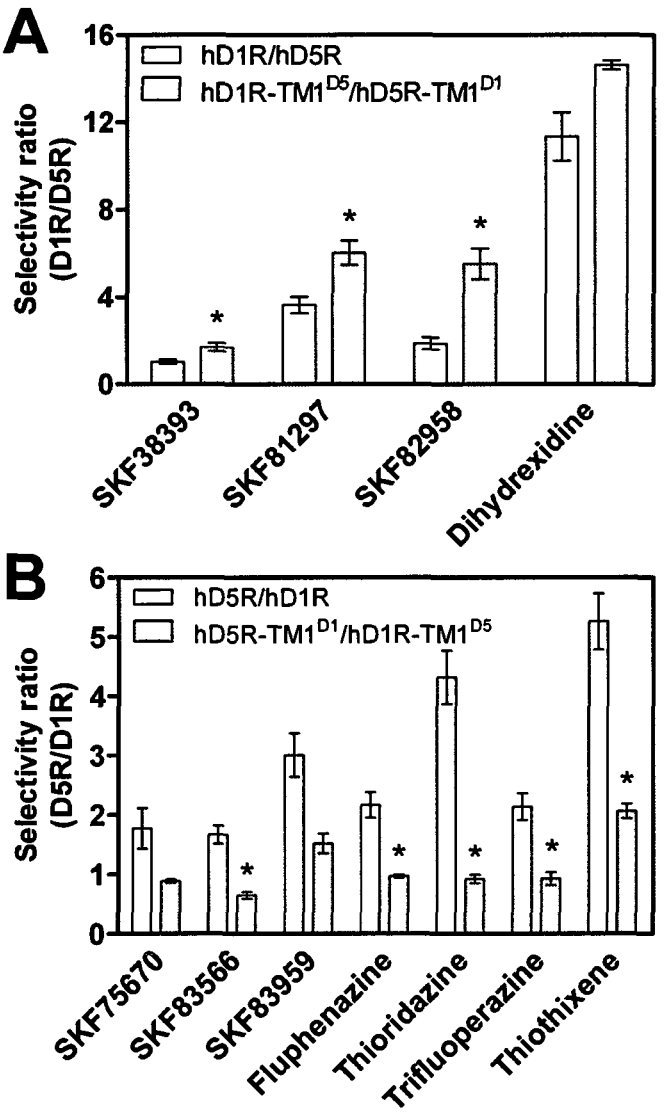


Figure 5. Selectivity ratios of dopaminergic ligands for wild type and TM1-swapped D1-like receptors. For each drug tested, selectivity ratio was calculated from K_i values reported in Table III. Bars represent the arithmetic mean ± S.E. of three to six experiments done in duplicate determinations. *A.* Selectivity ratios of SKF38393, SKF81297, SKF82958 and dihydrexidine were calculated for each experiment by dividing the affinity for hD1R or hD1R-TM1^{D5} by the affinity for their respective hD5R counterpart. *, $p<0.05$ when compared with wild type hD1R/hD5R selectivity ratio. *B.* Selectivity ratios of SKF75670, SKF83566, SKF83959, fluphenazine, thioridazine, trifluoperazine and thiothixene were calculated for each experiment by dividing the affinity for hD5R or hD5R-TM1^{D1} by the affinity for their respective hD1R counterpart. *, $p<0.05$ when compared with wild type hD5R/hD1R selectivity ratio.



Constitutive activity of D1-like receptors is not controlled by NT and TM1 sequences- HEK293 cells expressing similar high levels of wild type and chimerical D1-like receptors were used to assess their agonist-independent activity in a whole cell cAMP assay (Fig. 6). As reported before, cells expressing hD5R displayed higher constitutive activity than those expressing hD1R (15,16,23-27). All chimeras displayed similar constitutive activity as compared with their parent wild type receptors. Overall, we show that the extent of constitutive activation of hD1R and hD5R is not regulated by NT and TM1. In the next series of experiments, we assessed the role of NT and TM1 of D1-like receptors in DA-dependent stimulation of AC.

NT and TM1 regions of hD1R and hD5R regulate dopamine-dependent stimulation of AC- Dopamine dose-response curves were performed in whole HEK293 cells transfected with similar low expression levels of wild type and chimerical D1-like receptors. Curves were plotted as the percentage of the maximal activation elicited by their respective parent wild type receptor. Averaged curves were analyzed simultaneously to determine EC_{50} (potency) and E_{max} values for DA-mediated stimulation of AC (Fig. 7). The 10-fold difference between EC_{50} values of wild type hD1R and hD5R is in agreement with previous studies (15,23,25,27). Differences between EC_{50} values of DA measured in cells expressing chimerical receptors and respective parent wild type receptors were not statistically detectable (see legend of Fig. 7). Cells harboring hD1R-NTTM1^{D5} exhibit an increase (133 ± 6%; $p<0.05$ vs. hD1R) in E_{max} value statistically distinguishable from that measured in cells expressing hD1R (Fig. 7*A*). When NT and TM1 regions were individually swapped, our results show that cells expressing hD1R-NT^{D5} (134 ± 6%; $p<0.05$ vs. hD1R) showed an increased DA efficacy whereas it remains unchanged (99 ±

4%; p>0.05 vs. hD1R) in cells transfected with hD1R-TM1^{D5} (Fig. 7*A*). Cells expressing hD5R-NTTM1^{D1} had also a higher DA efficacy in comparison with cells harboring wild type hD5R (113 ± 5%; p<0.05 vs. hD5R). However, the increase in E_{max} of cells expressing hD5R-NTTM1^{D1} was not recapitulated following the exchange of NT of hD5R (96 ± 5%, p>0.05 vs. hD5R) (Fig. 7*B*). In contrast to cells expressing hD1R-TM1^{D5}, an increase in E_{max} of DA was detected in cells expressing hD5R-TM1^{D1} (133 ± 4%, p < 0.05) suggesting that DA efficacy in cells expressing D1-like receptors is dependent on distinct interplays between NT and TM1 sequences (Fig. 7*B*). These findings were corroborated by transducer ratio τ values indicating that coupling efficiency of full chimeras was increased relative to their respective parent wild type receptors (Fig. 7, *C* and *D*). The contribution of TM1 to DA-mediated coupling efficiency of hD1R and hD5R was drastically distinct. We next assessed whether maximal responses produced by synthetic agonists are regulated in similar fashion by TM1 of hD1R and hD5R.

TM1 of D1-like receptors control maximal responsiveness produced by synthetic agonists- We first probed the intrinsic efficacy of synthetic drugs (i.e. capacity of ligands to be agonist, antagonist or inverse agonist) with respect to the regulation of cAMP production in intact HEK293 cells (Figs. 8 & 9). Our data indicate that the ligands tested at wild type receptors can be grouped into inverse agonists or full and partial agonists. Several antipsychotic drugs (Fig. S2) have been shown to behave as inverse agonists on D1-like receptors (15,52,53). Our studies show that all antipsychotic drugs tested were inverse agonists in intact HEK293 cells (Fig. 8). In fact, we have identified two additional antipsychotic drugs (trifluoperazine and thiothixene) that behave as inverse

Figure 6. DA-independent activity of wild type and chimerical D1-like receptors expressed in HEK293 cells. HEK293 cells were transfected with 5 μg per dish of wild type and chimerical receptor DNA. Intracellular cAMP levels were measured in 6-well dish in the absence of DA. Each bar represents the arithmetic mean ± S.E. of six experiments done in triplicate determinations and expressed as [^{3}H]-cAMP formed (CA) over the total amount of [^{3}H]-adenine uptake (TU) X 1000. The receptor expression in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) are as follows: 11.7 ± 1.8 (hD1R); 10.9 ± 1.8 (hD1R-NTTM1^{D5}); 9.9 ± 1.2 (hD1R-NT^{D5}); 13.2 ± 2.3 (hD1R-TM1^{D5}); 9.4 ± 1.9 (hD5R); 9.1 ± 1.5 (hD5R-NTTM1^{D1}); 9.0 ± 1.7 (hD5R-NT^{D1}); 7.1 ± 1.0 (hD5R-TM1^{D1}). *A.* For a graphical representation, a representative example of the constitutive activity of wild type and chimerical D1-like receptors using raw data is shown. *B.* Constitutive activity of chimerical and wild type D1-like receptors are plotted as relative to the constitutive activity of hD5R for six experiments done in triplicate determinations. *, $p<0.05$ when compared with hD5R (value of 1).

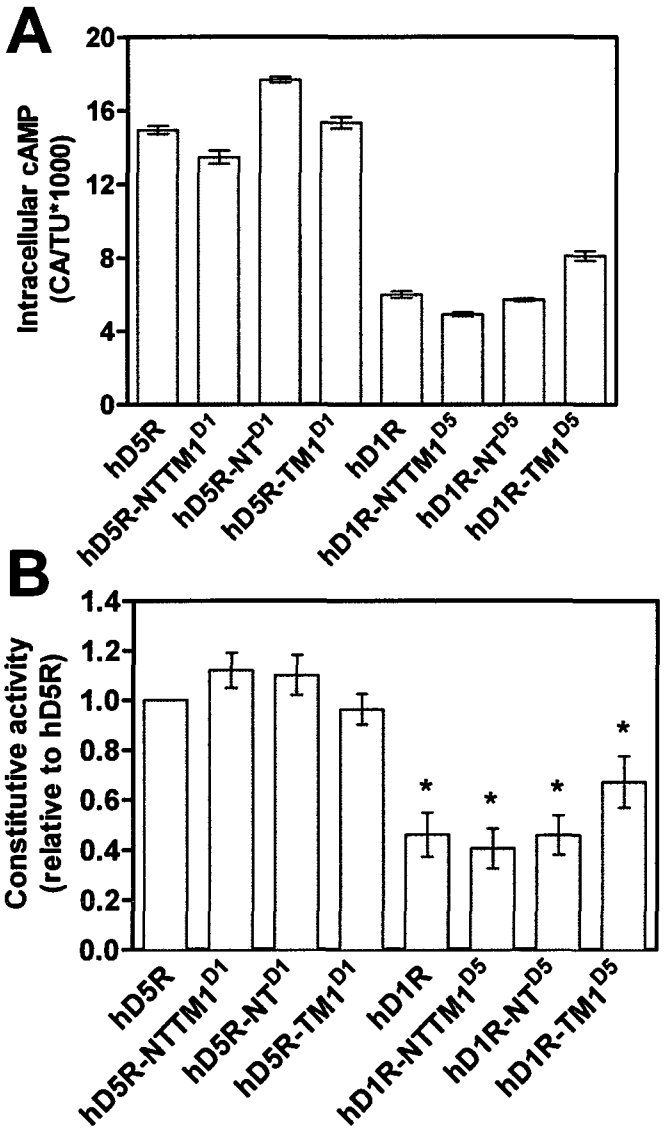
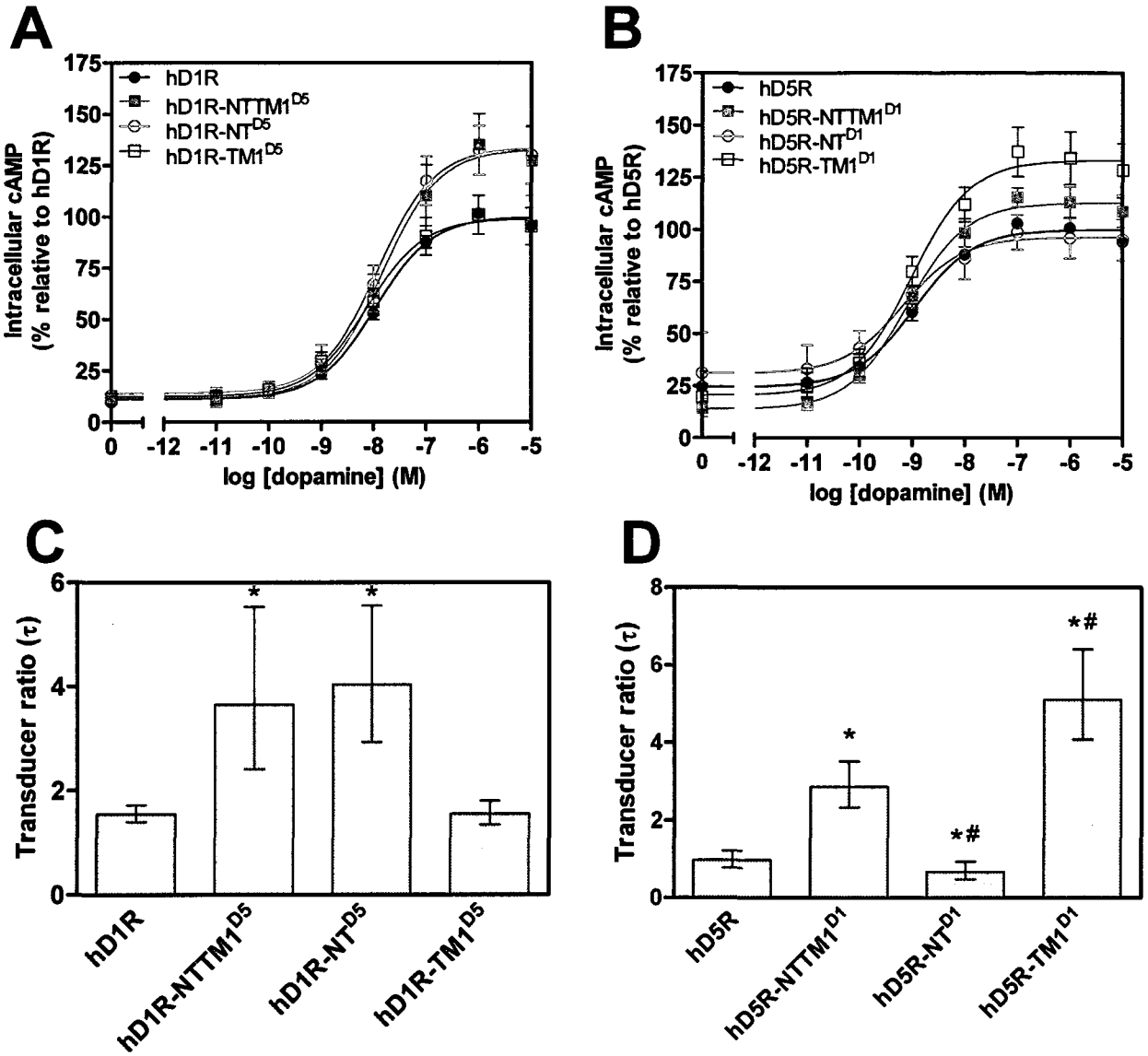


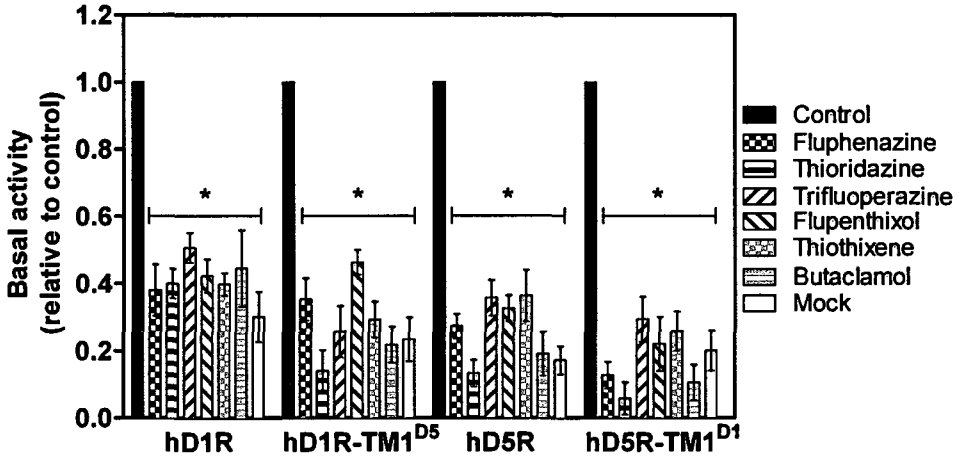
Figure 7. DA-mediated stimulation of adenylyl cyclase activity by wild type and chimerical D1-like receptors expressed in HEK293 cells. HEK293 cells were transfected with wild type or chimerical receptor using the following amount of DNA per dish: 0.02-0.038 μg of hD1R, 0.025-0.05 μg of hD1R-NTTM1^{D5}, 0.025-0.05 μg of hD1R-NT^{D5}, 0.02-0.038 μg of hD1R-TM1^{D5}, 0.02-0.04 μg of hD5R, 0.025-0.038 μg of hD5R-NTTM1^{D1}, 0.02-0.038 μg of hD5R-NT^{D1}, 0.03-0.038 μg of hD5R-TM1^{D1}. The receptor expressions in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) are as follows: 2.0 ± 0.4 (hD1R), 1.8 ± 0.4 (hD1R-NTTM1^{D5}), 2.0 ± 0.7 (hD1R-NT^{D5}), 2.1 ± 0.5 (hD1R-TM1^{D5}), 2.3 ± 0.3 (hD5R), 2.8 ± 0.4 (hD5R-NTTM1^{D1}), 2.8 ± 0.3 (hD5R-NT^{D1}), 2.2 ± 0.3 (hD5R-TM1^{D1}). Intracellular cAMP levels were measured in 12-well dish in the presence of increasing concentration of DA (10^{-11} to 10^{-5} M). Each point represents the arithmetic mean ± S.E. of four to eight experiments done in triplicate determinations and plotted as the percentage of maximal activation obtained with parent wild type receptor. Dose-response curves to DA were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02 using constrained or unconstrained parameters. Calculated EC_{50} values (nM) with the 95% lower and upper confidence interval are as follows: 10.9 (5.8-20.5) for hD1R, 14.5 (7.5-28.2) for hD1R-NTTM1^{D5}, 11.9 (6.1-23.1) for hD1R-NT^{D5}, 7.5 (3.9-14.3) for hD1R-TM1^{D5}, 1.11 (0.48-2.59) for hD5R, 0.81 (0.31-2.12) for hD5R-NTTM1^{D1}, 0.68 (0.15-3.03) for hD5R-NT^{D1}, 0.97 (0.51-1.83) for hD5R-TM1^{D1}. *A and C.* Dose-response curves to DA and transducer value τ of wild type and chimerical hD1R. *B and D.* Dose-response curves to DA and transducer value τ of wild type and chimerical hD5R.


A
hD1R
hD1R-NTTM1D5
hD1R-NTD5
hD1R-TM1D5
Intracellular cAMP
(% relative to hD1R)
log [dopamine] (M)
B
hD5R
hD5R-NTTM1D1
hD5R-NTD1
hD5R-TM1D1
Intracellular cAMP
(% relative to hD5R)
log [dopamine] (M)
C
Transducer ratio (τ)
hD1R
hD1R-NTTM1D5
hD1R-NTD5
hD1R-TM1D5
D
Transducer ratio (τ)
hD5R
hD5R-NTTM1D1
hD5R-NTD1
hD5R-TM1D1

agonists (Fig. 8). The exchange of TM1 between hD1R and hD5R had no detectable effect on the ability of antipsychotic drugs to mediate inverse agonism in HEK293 cells displaying similar receptor levels (Fig. 8).

We next measured the net E_{max} (i.e. maximal responses minus basal levels) produced by synthetic agonists with respect to DA-mediated cAMP production in intact HEK293 cells expressing similar low levels of wild type and chimerical receptors (Fig. 9). As previously reported (15), E_{max} values of DA were higher in HEK293 cells expressing hD1R relative to those transfected with hD5R (Fig. 9*A*). Higher E_{max} in hD1R-expressing cells were also observed with synthetic drugs behaving like full agonists (Fig. 9*A*). Maximal responses mediated by synthetic full agonists in cells expressing hD1R-TM1^{D5} were unchanged when compared with those obtained in cells harboring hD1R (Fig. 9*A*). In contrast, higher E_{max} values for synthetic full agonists were generally detected in cells harboring hD5R-TM1^{D1} in comparison with cells expressing hD5R but lower than those mediated by hD1R-TM1^{D5} (Fig. 9*A*). This trend was in line with the increased E_{max} of DA measured in cells transfected with hD5R-TM1^{D1} using full dose-response curves (Fig. 7*B*) or a single high DA concentration (Fig. 9*A*). However, the increased E_{max} values in cells expressing hD5R-TM1^{D1} were only observed with full synthetic agonists (Fig. 9*A*). Indeed, SKF83959, which is a partial agonist at both D1-like subtypes, produced a similar E_{max} value in cells expressing hD5R-TM1^{D1} and parent wild type receptor (Fig. 9*A*). Three other benzazepines (SKF75670, SCH23390 and SKF83566) displaying varying degrees of agonist activity in cells expressing wild type receptors were also tested (Fig. 9*B*). With respect to E_{max} of DA measured in cells transfected with wild type receptors, SKF75670 mediates sub-maximal responses in cells expressing hD1R

Figure 8. Basal activity of antipsychotic drugs on HEK293 cells expressing wild type or TM1-swapped D1-like receptors relative to the basal activity of respective receptor. HEK293 cells were transfected with 5 μg per dish of wild type and TM1-swapped receptor DNA. The receptor expression in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) are as follows: 11.1 ± 0.9 (hD1R), 12.7 ± 0.9 (hD1R-TM1^{D5}), 9.9 ± 0.6 (hD5R), 7.2 ± 0.6 (hD5R-TM1^{D1}). Bars represent the arithmetic mean ± S.E. of three to four experiments done in triplicate determinations. *, $p<0.05$ when compared with basal activity of each receptor (value of 1). The activity of antipsychotics in cells expressing wild type or chimerical receptors was not different than the activity observed in mock transfected cells.

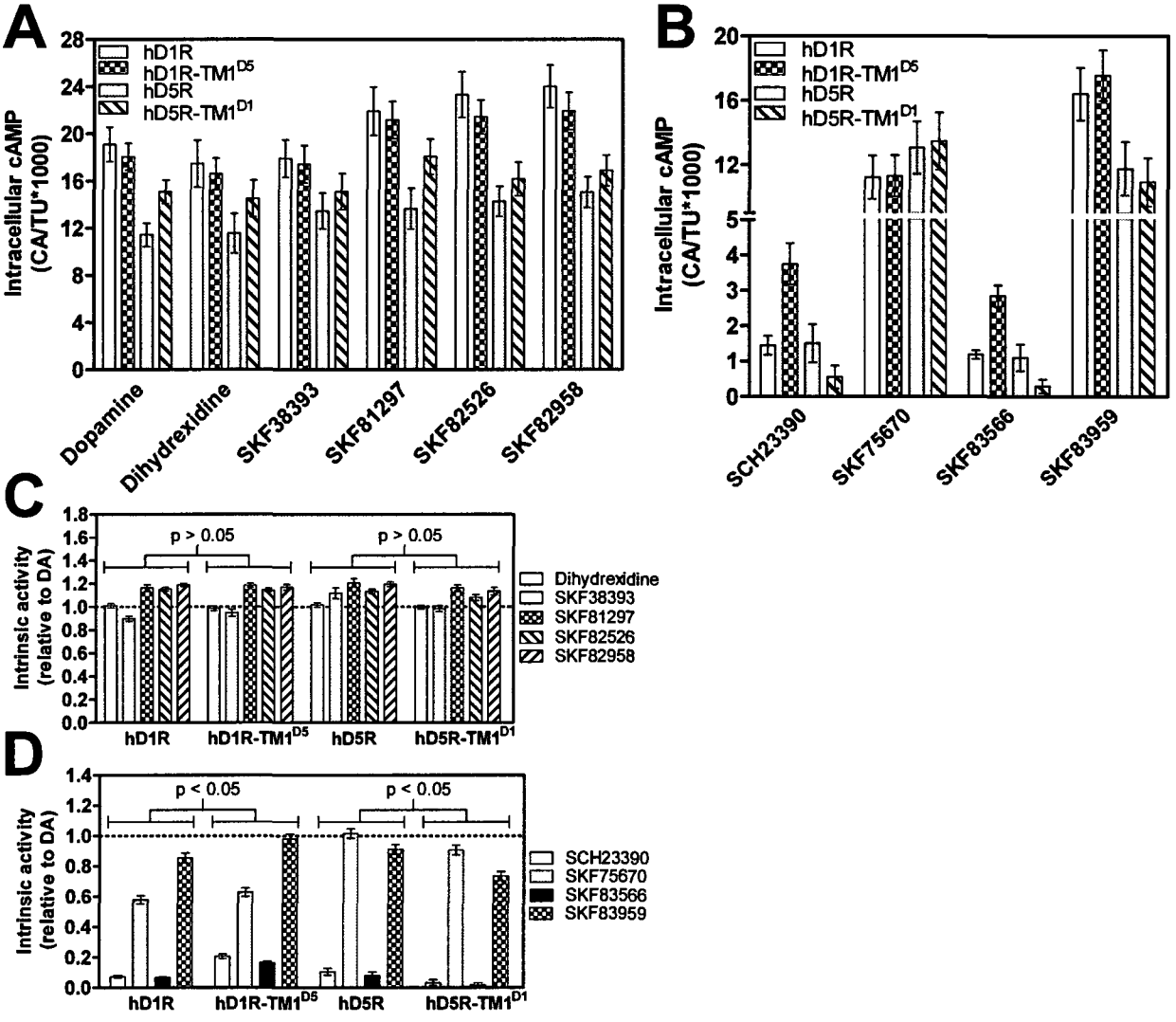


while behaving as a full agonist in cells containing similar levels of hD5R (Fig. 9*B*). The E_{max} values elicited by SKF75670 in cells expressing TM1 chimeras were not changed relative to cells expressing their respective parent wild type receptors (Fig. 9*B*). Interestingly, SCH23390 and SKF83566, two partial agonists at hD1R and hD5R, both exhibited drastic increase and decrease in their sub-maximal responses generated in cells expressing hD1R-TM1^{D5} and hD5R-TM1^{D1}, respectively (Fig. 9*B*). The distinct changes observed in E_{max} values of synthetic agonists prompted us to explore the role of TM1 of hD1-like receptors in controlling their intrinsic activities relative to DA.

Intrinsic activity of synthetic benzazepine agonists is regulated by TM1 of D1-like receptors- Dihydrexidine is a full agonist at hD1R and hD5R with intrinsic activities indistinguishable from DA (Fig. 9*C*). Meanwhile, three benzazepines (SKF81297, SKF82526 and SKF82958) displayed greater intrinsic activities relative to DA in cells expressing hD1R and hD5R (Fig. 9*C*). Our data show that the TM1 swap between D1-like receptors had no impact on the intrinsic activity of these compounds as assessed with hD1R-TM1^{D5} and hD5R-TM1^{D1} (Fig. 9*C*). SKF38393 and SKF75670 are partial agonists in hD1R-expressing cells while they behave as full agonists in cells harboring hD5R (Fig. 9*C*). Interestingly, intrinsic activities of SKF38393 and SKF75670 were increased and reduced in cells expressing hD1R-TM1^{D5} and hD5R-TM1^{D1}, respectively (Fig. 9*C*). These findings suggest that TM1 of D1-like receptors may impart the degree of intrinsic activity to partial agonists. This hypothesis was tested using three benzazepines (SKF83959, SKF83566 and SCH23390) behaving as partial agonists at both wild type receptors. The intrinsic activities of these benzazepines displayed a drastic augmentation in cells expressing hD1R-TM1^{D5} relative to cells harboring hD1R (Fig. 9*C*). In an

opposite manner, the intrinsic activities of these drugs were severely reduced in cells expressing hD5R-TM1^{D1} (Fig. 9*C*). Overall, we observe that the intrinsic activity pattern for these three drugs in cells expressing wild type receptors (i.e. hD5R > hD1R) is switched in cells harboring similar levels of hD1R-TM1^{D5} and hD5R-TM1^{D1} (Fig. 9*C*).

Figure 9. Activity of dopaminergic agonists on HEK293 cells expressing wild type or TM1-swapped D1-like receptors. HEK293 cells were transfected with wild type or chimerical receptor using the following amount of DNA per dish: 0.025 μg of hD1R, hD1R-TM1^{D5} and hD5R and 0.025-0.038 μg of hD5R-TM1^{D1}. The receptor expression in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) are as follows: 2.0 ± 0.3 (hD1R), 1.9 ± 0.3 (hD1R-TM1^{D5}), 2.0 ± 0.3 (hD5R), 2.0 ± 0.2 (hD5R-TM1^{D1}). Bars represent the arithmetic mean ± S.E. of four to ten experiments done in triplicate determinations after subtracting the constitutive activity of the receptor. *A.* Net intracellular [^{3}H]-cAMP production obtained with full agonists. *B.* Net intracellular [^{3}H]-cAMP production obtained with partial agonists. *C.* Intrinsic activity of full agonists relative to DA on cells expressing wild type and TM1-swapped receptors. The activity of full agonists in cells expressing wild type receptor is not changed in cells expressing TM1-swapped receptors (two-way ANOVA, $p>0.05$). *D.* Intrinsic activity of partial agonists relative to DA in cells expressing wild type and TM1-swapped receptors. The activity of partial agonists in cells expressing wild type receptor is altered in cells expressing TM1-swapped receptors (two-way ANOVA, $p<0.05$).



DISCUSSION

The aforementioned results strongly suggest that DA interaction with and activation of hD1R and hD5R are differentially modulated by NT and TM1 sequences. Moreover, whether acting as agonists or inverse agonists, binding of synthetic drugs to D1-like receptors is predominantly dependent on TM1. Our findings also reveal that TM1 plays an essential role in regulating the intrinsic activity and efficacy of synthetic agonists. Thus, NT and TM1 shape distinct conformational interplays separately regulating binding properties and coupling efficiency of D1-like receptors.

NT and TM1 of D1-like receptors have no impact on constitutive activity– Studies using constitutively active mutant (CAM) adrenergic receptors led to the formulation of the "allosteric ternary complex model" (or two-state model) whereby GPCRs are in an equilibrium between inactive R and active R* states (19,20). The two closely related D1-like subtypes were the first reported GPCRs naturally displaying a distinct propensity for adopting more readily either R (hD1R) or R* (hD5R) as seen with wild type and CAM adrenergic receptors, respectively (15). Several distinct GPCR structural determinants have been implicated in the regulation of constitutive activation (54). Notably, while being important for ligand binding function, intramolecular interactions between TM1, TM7 and H8 regulate the formation of GPCR active and inactive states (41,46-49,55). Interestingly, CT and helix 8 (H8) regions of D1-like receptors has been implicated in the regulation of their constitutive activation status (23-26). Recently, a mutation of Thr37 in TM1 of hD1R (also conserved in hD5R) was shown to reduce its constitutive activity (56). However, the drastically lower mutant receptor levels observed in the latter study

likely explained this effect (56). In contrast, we showed herein that the exchange of NT and/or TM1 does not alter the degree of constitutive activity of hD1R and hD5R at similar receptor levels. Furthermore, we noted that maximal inhibition of the basal activity of wild type and chimerical receptors by inverse agonists was essentially unchanged. Our findings are somewhat supported by a recent crystallographic study showing that TM1 conformation of the photointermediate opsin (a catalytically active ligand-free form of rhodopsin) is unchanged when compared with that of 11-*cis*-retinal-bound rhodopsin (inactive form) (28). Therefore, our data suggest that amino acid variations in NT and TM1 of D1-like receptors cannot account for the striking differences between constitutive activity of hD1R and hD5R.

Context-dependence of NT and TM1 pairing in ligand binding properties– Our data strongly suggest that DA affinity for hD1R and hD5R is differentially determined by the contextual combination of NT and TM1. Indeed, there was no detectable change in DA affinity for the chimerical hD1R harboring NT^{D5} and $TM1^{D5}$ whereas hD1R-NT^{D5} and hD1R-$TM1^{D5}$ chimeras displayed a similar increased DA affinity. Potentially, our data imply that both NT^{D1} and $TM1^{D1}$ sequences are required for hampering DA accessibility to orthosteric binding sites of hD1R. Interestingly, the presence of both NT^{D5} and $TM1^{D5}$ sequences can serve as surrogate determinants in controlling lower DA affinity at hD1R. In contrast, occurrence of both NT^{D1} and $TM1^{D1}$ sequences in chimerical D5R cannot act as substitutes of native NT and TM1 regions of wild type hD5R. In fact, increased DA affinity for hD5R-$NTTM1^{D1}$ chimera is only recapitulated with hD5R-$TM1^{D1}$ suggesting that accessibility to DA binding residues of wild type hD5R is modulated by TM1 independently of contextual NT sequences. Likewise, our studies point to a sole role of

TM1 in regulating accessibility of synthetic ligands to binding sites of wild-type D1-like receptors. The distinct requirement of NT and TM1 sequences in ligand binding of D1-like receptors is potentially explained by different interaction sites for DA and synthetic drugs.

Our results also indicate that the $TM1^{D5}$ impact on lowering ligand affinity at hD1R-$TM1^{D5}$ was lesser than the overall $TM1^{D1}$ effect mediating increased ligand affinity at hD5R-$TM1^{D1}$. Nevertheless, these asymmetrical affinity changes translated into drastically distinct patterns for ligand selectivity as assessed with wild type and chimerical D1-like receptors. We infer that a different TM1 packing within the membrane-spanning helix bundle of hD1R and hD5R potentially underlies subtype-specific accessibility and alignment of key binding residues for ligand affinity and selectivity. Indeed, studies have demonstrated that different TM1 packing and orientation inside of TM bundle of GPCRs can regulate the formation of a more open tertiary structure facilitating accessibility of diffusible ligands to different binding sites. (4,8,10,44,57,58). Furthermore, the crystal structure of opsin unraveled two different openings located between TM5 and TM6 and between TM1 and TM7 (28). It was proposed that the opening between TM1 and TM7 is the exit path for all-*trans*-retinal. Possibly, the exchange of TM1 between hD1R and hD5R may modify the closure of the exit path in chimeras leading to changes in ligand affinities. Studies have also identified a cholesterol binding site between TM1, TM2, TM3 and TM4 of the human β2AR (59). Interestingly, hD1R harbors a strict cholesterol consensus motif (CCM) while hD5R contains a less restrictive CMM (59). Thus, the TM1 exchange may reshape the cholesterol binding site regulating functional properties of hD1R and hD5R. Recently,

the involvement of TM1 in the formation of a GPCR dimerization interface has been highlighted (60-64). Intriguingly, evidence suggests that NT may modulate how TM1 contributes to GPCR dimerization (36,65). However, the functional relevance of NT and TM1 in dimerization of D1-like receptors and other GPCRs remains to be fully appreciated.

Context-dependence of NT and TM1 pairing in DA-induced activation– We also demonstrate that DA efficacy is dependent on the NT and TM1 pairs harbored by D1-like receptors. However, the context-dependence regulation of DA efficacy by these two regions does not match with the effects of NT and TM1 pairing on DA affinity. Indeed, exchange of the entire NT-TM1 segment between D1-like receptors leads to increased DA efficacy in cells expressing hD5R-NTTM1^{D1} (NT^{D1} + $TM1^{D1}$) and hD1R-NTTM1^{D5} (NT^{D5} + $TM1^{D5}$), which display an increase and no change in DA affinity, respectively. Likewise, receptors containing NT^{D5} and $TM1^{D1}$ sequences have higher coupling efficiency following DA binding whereas those harboring NT^{D1} and $TM1^{D5}$ sequences do not display increased E_{max}. It thus seems that the type of NT-TM1 segment connected to the TM2-TM7 backbone of D1-like receptors dictates DA-mediated maximal responses. This complex regulation of DA efficacy mediated by NT and TM1 is not easily understood. In fact, NT regions were disordered or not visible in crystallized adrenergic and adenosine receptors (5-8). However, the crystal structure of rhodopsin shows that the NT domain makes contact with all extracellular loops (4,66). Potentially, our data are explained by a role of TM1 in facilitating contacts between NT and extracellular faces of D1-like receptors. Additionally, studies elegantly demonstrated a structural interplay between TM1 and H8 (4-8,46-50). Notably, agonists decrease the proximity between the

N-terminal portion of H8 and cytoplasmic end of TM1 of M3 muscarinic receptors (49). Previous data from our lab suggest that the extracellular loop 3 (EL3), CT and H8 of D1-like receptors control DA efficacy (23-26). Thus, the present findings possibly hint at a link between NT and EL3 in modulating TM1 and H8 proximity. Alternatively, agonist efficacy can be regulated by GPCR desensitization and internalization (67). Interestingly, studies have reported that polymorphisms in NT altered regulation of GPCR responsiveness (30,35). It remains to be established whether unique desensitization and internalization properties underlie DA efficacy in cells expressing chimerical D1-like receptors.

Discrepancies between D1-like receptor properties and "allosteric ternary complex model"– The "allosteric ternary complex model" proposes that differences in drug properties are linked to their relative affinity and preference for binding to and stabilizing R and R* (19,20). Briefly, agonist affinity and intrinsic activity is increased while inverse agonist affinity is decreased at more constitutively active receptors or R* relative to GPCRs in R state (19,20). These distinguishing features between R and R* are mostly recapitulated with wild type hD1R and hD5R (15). However, results reported herein using chimeras and synthetic ligands cannot be explained by the "allosteric ternary complex model". First, the notion of decreased inverse agonist affinity at R* is not supported by our binding data with hD5R-TM1^{D1} and hD1R-TM1^{D5}, which virtually display no selectivity for inverse agonists while these chimeras retain the high and low constitutive activity of their respective parent wild type receptors. Second, our study also reveals that benzazepines (SKF38393, SKF75670, SKF83566 SKF83959 and SCH23390, see Fig. S1) behaving as partial agonists at hD1R (low constitutive activity) have

generally a greater intrinsic activity at hD5R (high constitutive activity) whereas they generally exhibit lower affinity for hD5R relative to hD1R. Third, we demonstrate that the phenotypic intrinsic activity of partial agonists at wild type receptors was switched following the exchange of TM1. Notably, SCH23390 and SKF83566 become stronger partial agonists at hD1R-TM1^{D5} whereas they completely loose their ability to activate hD5R-TM1^{D1}. Therefore, TM1 shapes structural interplays controlling whether a ligand is partial agonist or neutral (antagonist) at D1-like receptors. Opposite effects on intrinsic activities and affinities of partial agonists mediated by TM1 swap cannot thus be interpreted in the context of "allosteric ternary complex model". We believe that our data may be best reasoned by a GPCR multistate model (68-70), whereby partial and full agonists bind to intermediary (R') and R* states with distinct properties culminating in the stabilization of different R* states. In support of this view, the methyl group on the nitrogen of the azepine ring may underlie the lower affinity of these drugs for hD5R since full benzazepine agonists lack this chemical hallmark (Fig. S1). Additionally, halogens replacing the *meta*-hydroxyl group on the catechol ring of SCH23390 and SKF83566 may be responsible for low intrinsic activity at hD1R and hD5R as these hydroxyls are thought to bind to canonical Ser residues of TM5 and trigger receptor activation. In fact, SKF75670 and SKF83959 harboring both hydroxyl groups have a greater intrinsic activity than SCH23390 and SKF83566 at hD1R and hD5R. Thus, results obtained with structurally related benzazepine ligands imply the existence of specific binding sites for partial agonists and stabilization properties of R*.

Conclusions – Overall, we have highlighted for the first time the structural importance of NT and TM1 of D1-like dopaminergic receptors in defining their subtype-specific ligand

affinity and ligand-induced AC activation. We believe that our findings will have a general relevance to other GPCRs. Given that compromised D1-like receptors have been implicated in several pathophysiological conditions, our work may provide structural insight into the development of novel D1-like subtype-selective drugs with higher therapeutic potential.

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FOOTNOTES

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Table S1. Construction of chimerical receptors using a polymerase chain reaction-based overlapping approach. Two rounds of PCR were necessary to construct each chimerical receptor. The first round generated two products, A and B, amplified from the identified template using primer pair P1-P2 and P3-P4, respectively. Based on their overlapping region (15-18 nucleotides), products A and B were subjected to a second round of PCR using primer pair P5-P6.

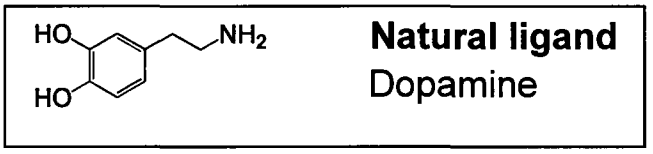
Construct	Oligonucleotide primers (5'-3')	Template (1st round)
hD1R-NTTM1^{D5}	**P1**: CCATGGTGATGCGGTTTT	hD1R-NT^{D5} in pCMV5
	P2: GTTCCCCAGGAGCGTCCAGATGATGAGCAGCGT CAGCAAACAGGC	
	P3: ACGCTCCTGGGGAACGTGCTGGTCTGTGCTGCC ATTGTCAGGTTCCGACACCTGCGG	hD1R in pCMV5
	P4: AGTTCAAGATGAAGA	
	P5: GGAATTCAAGCTTGCCGCCACCATGCTGCCGCC AGGCAGC	
	P6: CAGAGGTTGAGGATGGAT	
hD1R-NT^{D5}	**P1**: CCATGGTGATGCGGTTTT	hD5R in pCMV5
	P2: CGACAGGAAACAGGCAGTGACCACCTGTGAGG GCCCCAG	
	P3: CTGGGGCCCTCACAGGTGGTCACTGCCTGTTTC CTGTCG	hD1R in pCMV5
	P4: GTCTCAGCCAGGGAAGTG	
	P5: GGAATTCAAGCTTGCCGCCACCATGCTGCCGCC AGGCAGC	
	P6: CAGAGGTTGAGGATGGAT	
hD1R-TM1^{D5}	**P1**: CCATGGTGATGCGGTTTT	hD1R in pCMV5
	P2: GTTCCCCAGGAGCGTCCAGATGATGAGCAGCGT CAGCAAACAGGCAGTGAGGATACG	
	P3: ACGCTCCTGGGGAACGTGCTGGTCTGTGCTGCC ATTGTCAGGTTCCGACACCTGCGG	hD1R in pCMV5
	P4: GTCTCAGCCAGGGAAGTG	
	P5: GGAATTCACGCGTGCCGCCACCATGAGACTCTG AACACC	
	P6: CAGAGGTTGAGGATGGAT	

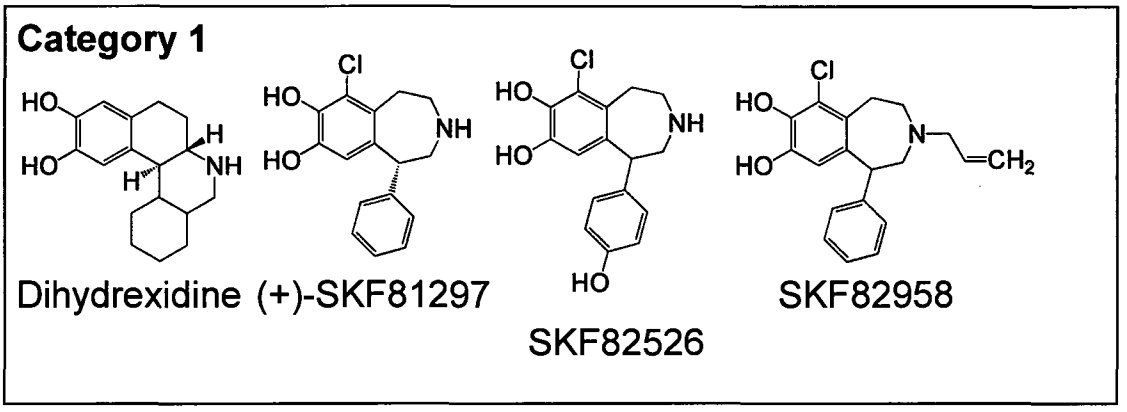
Table S1. Continued.

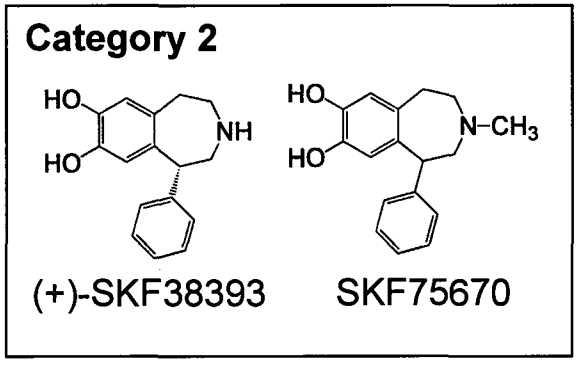
Construct	Oligonucleotide primers (5'-3')	Template (1st round)
hD5R-NTTM1^{D1}	**P1**: CCATGGTGATGCGGTTTT	hD5R-NT^{D1} in pCMV5
	P2: GTTGCCCAGCAGGGTGGAGAGGATGAGTAGGG ACAGGAAGCAGGC	
	P3: ACCCTGCTGGGCAACACGCTGGTGTGCGCAGCC GTCATCCGGAGCCGCCACCTGCGC	hD5R in pCMV5
	P4: AGTTAAGGATGAAGA	
	P5: GGAATTCACGCGTGCCGCCACCATGAGGACTCT GAACACC	
	P6: TCACGATCATGATGGCAA	
hD5R-NT^{D1}	**P1**: CCATGGTGATGCGGTTTT	hD1R in pCMV5
	P2: GGTCAGCAGGCAGGCGGTGAGGATACGAACAG AGAA	
	P3: TTCTCTGTTCGTATCCTCACCGCCTGCCTGCTGA CC	hD5R in pCMV5
	P4: AGTTAAGGATGAAGA	
	P5: GGAATTCACGCGTGCCGCCACCATGAGGACTCT GAACACC	
	P6: TCACGATCATGATGGCAA	
hD5R-TM1^{D1}	**P1**: CCATGGTGATGCGGTTTT	hD5R in pCMV5
	P2: GTTGCCCAGCAGGGTGGAGAGGATGAGTAGGG ACAGGAAGCAGGCGGTGACCACCTG	
	P3: ACCCTGCTGGGCAACACGCTGGTGTGCGCAGCC GTCATCCGGAGCCGCCACCTGCGC	hD5R in pCMV5
	P4: AGTTAAGGATGAAGA	
	P5: GGAATTCAGATCTGCCGCCACCATGCTGCCGCC AGGCAGC	
	P6: TCACGATCATGATGGCAA	

Figure S1. Chemical structures of dopamine and benzazepine ligands. Agonists are categorized according to their instrinsic activity measured in HEK293 cells compared with that of the natural ligand, dopamine (DA). Ligands of category 1 have an intrinsic activity ≥ 1 (compared with DA) at both hD1R and hD5R. Category 2 contains ligands with sub-maximal activity (≤1) at hD1R while displaying full activity (≥ 1) at hD5R. Ligands of category 3 are characterized by their sub-maximal activity (≤ 1) at both hD1R and hD5R..

Intrinsic activity: Category 1 → ≥ 1 (hD1R); ≥ 1 (hD5R)
Category 2 → < 1 (hD1R); ≥ 1 (hD5R)
Category 3 → < 1 (hD1R); < 1 (hD5R)







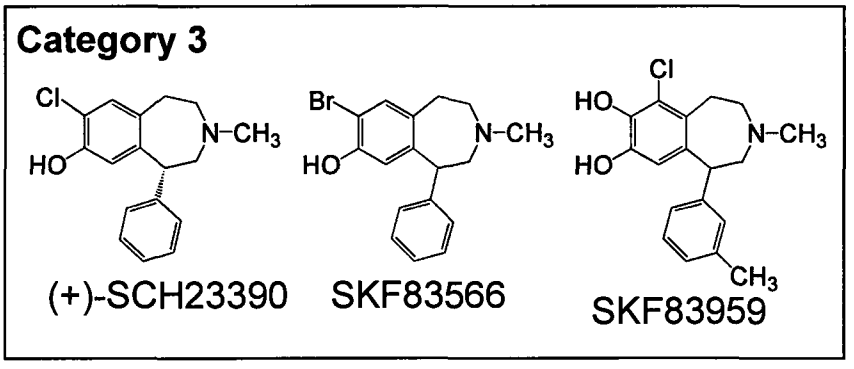
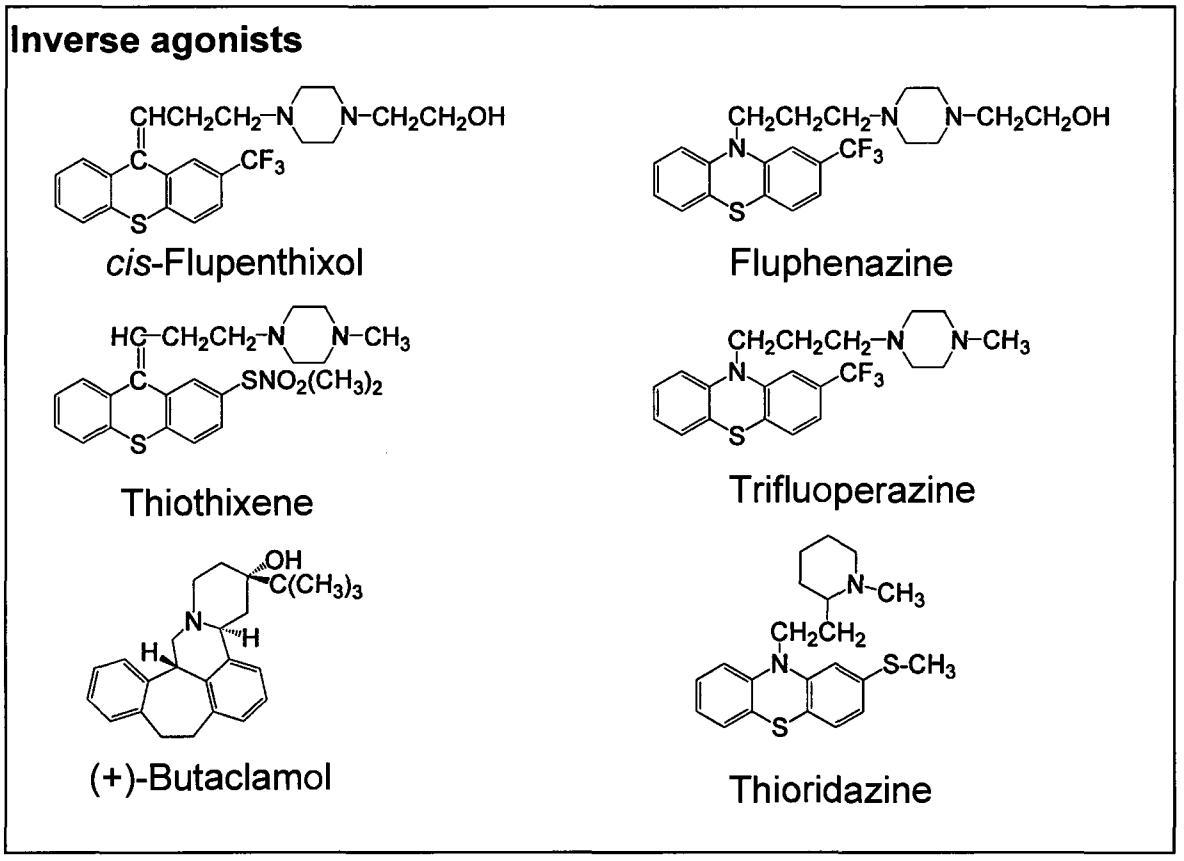


Figure S2. Chemical structures of antipsychotic drugs. Antipsychotic drugs possess an inverse agonist activity in HEK293 cells expressing both hD1R and hD5R.



Chapter 3. Manuscript #2

Middle segment residues of transmembrane domain 1 of human D1 and D5 dopaminergic receptors modulate ligand binding and G protein coupling functionalities

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Keywords: Dopamine, G protein-coupled receptors, Ligand binding, Transmembrane region 1

Abbreviations: B_{max}, maximal binding capacity, CT, cytoplasmic tail, DA, dopamine, E_{max}, maximal efficacy, GPCR, G protein coupled receptor, HEK293, human embryonic kidney 293, TM, transmembrane domain

ABSTRACT

Previously, we showed that drug properties at human D1-like dopaminergic receptors (hD1R and hD5R) are controlled by their transmembrane region 1 (TM1). Here, we constructed chimeras in which divergent residues located in the extracellular end, middle segment (mid) and intracellular end of TM1 were separately swapped between hD1R and hD5R. Our data demonstrate that permutation of variant residues located in the extracellular or intracellular ends does not impact ligand affinity. However, hD5R-TM1mid^{D1} and hD1R-TM1mid^{D5}, chimeras generated by the exchange of the two variant middle segment residues of TM1, bind to ligands with an increased and decreased affinity, respectively. These affinity changes culminated in a loss of selectivity for inverse agonists and partial agonists while increasing selectivity of full agonists at hD5R-TM1mid^{D1} relative to hD1R-TM1mid^{D5}. Importantly, these findings were reminiscent of those obtained with full TM1 chimeras. We then assessed the functional role of variant residues of TM1mid (Ser36$^{1.45}$ and Thr42$^{1.51}$ in hD1R; Trp53$^{1.45}$ and Val59$^{1.51}$ in hD5R). Studies with these single point mutants indicate that changes in ligand properties are mainly promoted by residue 1.45 and somewhat required a cooperative function of residue 1.51. Moreover, our data suggest that increase in DA efficacy detected with hD5R-TM1^{D1} relative to hD5R was recapitulated with hD5R-TM1mid^{D1} and also following the swap of residue 1.45 of hD5R. Potentially, these results hint toward a role of residues 1.45 and 1.51 in reorienting TM1 upon receptor binding and activation as they face opposite side in TM1. In conclusion, residues 1.45 and 1.51 of TM1 of D1-like subtypes play an important role in receptor binding and activation. Notably, this could not be predicted from currently available crystal structures of G protein-coupled receptors.

INTRODUCTION

The dopaminergic system regulates many functions in the human body such as locomotion, neuroendocrine activity, cognition, positive reinforcement as well as cardiovascular and renal functions (Missale et al. 1998). Dopamine (DA), a catecholamine, exerts its signaling properties by binding to seven membrane-spanning proteins also named G protein-coupled receptors (GPCRs). Two families of DA receptors are distinguished by their pharmacological and intracellular signaling properties: D1-like receptors (D1R and D5R) are coupled to $G\alpha_s/\alpha_{olf}$ proteins and increase the intracellular cAMP production by adenylyl cyclase (AC) whereas D2-like receptors ($D2_SR/D2_LR$, D3R and D4R) inhibit AC through stimulation of $G\alpha_i/\alpha_o$ proteins (Missale et al. 1998). Currently available drugs, however, do not substantially discriminate between D1R and D5R, which becomes an issue since development D1-like subtype-specific ligands could have beneficial physiological effects (Giorgioni et al. 2008; Zhang et al. 2009). Indeed, modulation of the activity of D1-like receptors has potential clinical applications in restoring normal working memory function of schizophrenics and aged individuals (Williams and Castner 2006; George et al. 2007; Mu et al. 2007; Nagai et al. 2009), neuroprotection and alleviation of symptoms in Parkinson's disease (Mailman et al. 2001; Lewis et al. 2006) as well as treatment of essential hypertension (Jose et al. 2003).

The 80% sequence identity between transmembrane (TM) domains of D1R and D5R has been an obstacle to the discovery of D1-like subtype-selective ligands (Jarvie and Caron 1993). Nevertheless, discrepancy in the binding characteristics of D1-like receptors provides an opportunity to assess the impact of divergent residues in the TM regions. For instance, full agonists generally have higher affinity for D5R whereas partial

agonists, antagonists and inverse agonists have lower affinity for D5R compared with D1R (Sunahara et al. 1991; Tiberi et al. 1991; Tiberi and Caron 1994; Demchyshyn et al. 2000; D'Aoust and Tiberi 2009). Mutagenesis studies have revealed that the third intracellular loop (IL3) and cytoplasmic tail (CT) modulate DA binding affinity and selectivity for D1R and D5R (Charpentier et al. 1996; Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al. 2003; Tumova et al. 2004). Importantly, none of these cytoplasmic regions appeared to change the affinity of antagonists and inverse agonists. In addition, mutations of TM residues that play a role in agonist and antagonist binding are found in both D1R and D5R, thus do not contribute to distinguish D1-like receptors (Pollock et al. 1992; Tomic et al. 1993; Cravchik and Gejman 1999). We recently reported that the exchange of TM1 between D1-like receptors caused an increase in ligand affinity for hD5R-TM1^{D1} chimera compared with hD5R whereas ligands bind to hD1R-TM1^{D5} chimera with a decreased affinity relative to hD1R (D'Aoust and Tiberi 2009). While studies on other GPCRs suggested that the TM1 helix plays a crucial structural role in the receptor conformation (Suryanarayana et al. 1992; Liu et al. 1995; Hwa et al. 1997; Shi et al. 2001; Hamdan et al. 2002; Bosch et al. 2003; Han et al. 2005), our work suggested that TM1 of D1-like receptors confers ligand affinity and selectivity.

Functionally, cells expressing D5R exhibit higher constitutive activity than cells expressing D1R, which can be inhibited by antipsychotics (Tiberi and Caron 1994; Cai et al. 1999; Martin et al. 2001; D'Aoust and Tiberi 2009). Furthermore, although DA is ~10 times more potent at D5R than D1R, the efficacy of DA and other full agonists is higher in HEK293 cells expressing D1R relative to those harboring D5R (Tiberi and Caron 1994; Charpentier et al. 1996; Iwasiow et al. 1999; Jackson et al. 2000; Tumova et al.

2003; Tumova et al. 2004; D'Aoust and Tiberi 2009). Several studies suggested that intracellular domains control signaling properties of GPCRs (Hall et al. 1999; Bockaert et al. 2003). Accordingly, our lab and others demonstrated the importance of IL3 and CT but also of extracellular domains in mediating the signaling properties of D1-like receptors (Charpentier et al. 1996; Iwasiow et al. 1999; Demchyshyn et al. 2000; Jackson et al. 2000; Tumova et al. 2003; Tumova et al. 2004; D'Aoust and Tiberi 2009). Additionally, we recently obtained evidence that TM1 can modulate the agonist properties at D1-like receptors (D'Aoust and Tiberi 2009). Indeed, the intrinsic activity of prototypical D1-like benzazepine partial agonists is modulated by TM1 sequences. Furthermore, cells transfected with hD5R-TM1^{D1} chimera generate greater DA-mediated maximal activity than cells expressing wild type hD5R. Yet, the DA-mediated activity of the hD1R-TM1^{D5} chimera was not altered suggesting that the TM1 of D1-like receptors differentially regulate signal transduction.

Divergent residues in the TM1 of D1-like receptors are spread out along the whole helical domain. Therefore, residues located near the cytoplasmic end of TM1 could offset residues near the extracellular end of TM1 and vice versa. In the present study, we explore the functional importance of the seven-residue difference in TM1 of D1-like subtypes in regulating ligand binding and G protein coupling properties using hD1R and hD5R harboring triple, double and single point mutations of TM1 divergent residues. Notably, we identify two residues located in the middle segment of the TM1 that are crucial for ligand binding and DA-dependent stimulation of AC activity.

MATERIALS AND METHODS

Materials

(+)-*N*-[methyl-^{3}H]SCH23390 (84-88 Ci/mmol) and [^{14}C]cAMP (250-275 mCi/mmol) were acquired from GE Healthcare. [2,8-^{3}H]adenine (24-27 Ci/mmole) was purchased from PerkinElmer. Dopamine (DA) hydrochloride, (+)-butaclamol hydrochloride, *cis*-flupenthixol dihydrochloride, fluphenazine dihydrochloride, trifluoperazine dihydrochloride, thioridazine hydrochloride, thiothixene hydrochloride, SKF82958 (chloro-APB) hydrobromide, SKF83566 hydrochloride and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich. SKF82526 (fenoldopam) hydrochloride and SKF83959 hydrobromide were from Tocris. Taq DNA polymerase, gentamicin, minimal essential media (MEM), phosphate buffered saline (PBS) and fetal bovine serum (FBS) were acquired from Invitrogen. MCL-204 hydrobromide was a gift from Dr. Neumeyer (McLean Hospital, Belmont, MA).

Expression constructs

We used a site-directed polymerase chain reaction-based overlap extension approach to construct triple, double and single point mutant receptors: hD1R-TM1up^{D5}, hD1R-TM1mid^{D5}, hD1R-TM1down^{D5}, hD5R-TM1up^{D1}, hD5R-TM1mid^{D1}, hD5R-TM1down^{D1}, hD1R-S36W, hD1R-T42V, hD5R-W53S, hD5R-V59T (Fig. 1). PCR primer sequences obtained from Sigma Genosys are described in *Supplementary information* (Table S1). Wild type human D1 (hD1R) and D5 receptor (hD5R) DNA sequences cloned in the pCMV5 expression vector were used as templates. Two PCR products, A and B, were amplified separately using primer pairs P1-P2 and P3-P4, respectively. An overlapping region (15-18 nucleotides) between A and B allowed the

amplification of the final PCR product using primers P5-P6. PCR reactions were carried out with Taq DNA polymerase in an Eppendorf thermal Mastercycler using the following conditions: 1 cycle (94°C for 3 min, 46-55°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 46-55°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 the following conditions: 1 cycle (94°C for 3 min, 46-55°C for 1 min, 72°C for 10 min), 25 cycles (94°C for 45 s, 46-55°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). Linearized pCMV5 (*EcoR*I-*Bgl*II) containing hD1R was used to construct hD1R-TM1up^{D5}, hD1R-TM1mid^{D5}, hD1R-TM1down^{D5} and linearized pCMV5 (*EcoR*I-*Bsm*I) containing hD5R was used to construct hD5R-TM1up^{D1}, hD5R-TM1mid^{D1}, hD5R-TM1down^{D1}, hD5R-W53S and hD5R-V59T. Point mutants hD1R-S36W and hD1R-T42V were first subcloned in linearized (*EcoR*I-*Bgl*II) pBlueScript II SK+ (Stratagene) containing hD1R coding sequence. Automated fluorescent DNA sequencing (Applied Biosystems 3730 DNA Analyzer) performed at the StemCore Laboratories of the Ottawa Genomics Innovation Centre (Ottawa, ON) confirmed the integrity of the coding sequences.

Amino acid nomenclature

Residues are numbered according to their position in the primary sequence of hD1R or hD5R using the Weinstein-Ballesteros nomenclature (Ballesteros and Weinstein 1995; Visiers et al. 2002). In the latter nomenclature, the first digit corresponds to the TM domain (from 1 to 7) which is followed by a number designating the residue position in the TM relative to the most conserved residue in that helix of related GPCRs, which is

assigned the number 50 (Fig. S1). Relative to this residue, residues closer to the N terminus have decreasing numbers and residues closer to the C terminus have increasing numbers.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Tissue Culture Collection, CRL-1573) were cultured at 37°C with 5% CO_2 atmosphere in MEM with Earle's salts supplemented with 10% v/v heat-inactivated FBS and 20 μg/mL gentamicin. Cells were seeded in 100-mm dishes (2.5 x 10^6 cells/dish) and transiently transfected by a modified calcium-phosphate method described previouly using a total amount of 5 μg of receptor DNA/dish (Tumova et al. 2004). Empty pCMV5 vector was used to adjust DNA amounts when less than 5 μg of receptor DNA was needed for transfections. All experiments were performed with cells from 41 to 52 passages.

Membrane preparation

After 18-24h incubation with the DNA-calcium-phosphate precipitate, HEK293 cells were washed with PBS, trypsinized, reseeded in 150-mm dishes and grown for an additional 48 h. Then, HEK293 cells were washed with cold PBS, scraped in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA, pH 8.0), and centrifuged at 40,000 x *g* for 20 min at 4°C. Pellets were washed in lysis buffer using a Brinkmann Polytron (17,000 rpm for 15 s) and centrifuged at 4°C (40,000 x *g* for 20 min). The washing procedure was repeated once. The final pellets were resuspended in lysis buffer and crude membranes were frozen in liquid nitrogen and stored at -80°C until used. Prior to freezing, an aliquot of different receptor membrane preparations were utilized for saturation studies as described in the next section.

Radioligand binding

Fresh or frozen membranes thawed on ice were mixed in a resuspension buffer (62.5 mM Tris-HCl, pH 7.4; 1.25 mM EDTA, pH 8.0) using a Brinkmann Polytron (17,000 rpm for 15 s). Radioligand saturation and competition assays were performed 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) with 100 µL of membranes in a total volume of 500 µL using *N*-[methyl-^{3}H]-SCH23390 as radioligand. Saturation studies were done using concentrations of *N*-[methyl-^{3}H]-SCH23390 ranging from 0.02 to 8 nM. Non-specific binding was delineated using 10 µM of *cis*-flupenthixol. For competition studies, frozen membranes were thawed on ice, mixed in resuspension buffer and incubated with a constant concentration of *N*-[methyl-^{3}H]-SCH23390 (0.5-1 nM) and increasing concentrations of cold competing ligand. Competition studies using DA were done in the presence of 0.1 mM ascorbic acid (final in assays). Binding assays were incubated for 90 min at room temperature and terminated using rapid filtration through glass fiber filters (GF/C, Whatman). The filters were washed three times with 5 mL of cold washing buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl) and bound radioactivity was determined by liquid scintillation counting (Beckman Counter, LS6500). Protein concentration was determined using the Bio-Rad assay kit with bovine serum albumin (BSA) as standard. To determine the equilibrium dissociation constant (K_d, nM), the inhibitory dissociation constant (K_i, nM) and receptor number (R, pmol/mg of membrane proteins) values, binding isotherms were analyzed using the non-linear curve-fitting program GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Whole-cell cAMP assay

G protein-mediated activation of AC by wild type and mutant receptors was assessed using a whole cell cAMP assay as described previously (Johnson and Salomon 1991; D'Aoust and Tiberi 2009). Following an overnight incubation with the DNA-calcium phosphate precipitate, HEK293 cells were reseeded in 6- or 12-well dishes. The following day, medium was changed for fresh MEM containing 5% v/v FBS, gentamicin (20 μg/mL) and [^{3}H]-adenine (1-2 μCi/mL) and HEK293 cells were metabolically labeled overnight at 37°C and 5% CO_2. The next day, the labeling medium was removed and HEK293 cells were incubated in 20 mM HEPES-buffered MEM containing IBMX (1 mM) in the presence or absence of drugs for 30 min at 37°C. DA was tested in the presence of 0.1 mM ascorbic acid (final in assays). After the incubation period, the medium was aspirated and each well filled with 1 mL of lysis solution (2.5% (v/v) perchloric acid, 0.1 mM cAMP and [^{14}C]cAMP (5 μCi, 8000-12,000 dpm)) for 30 min at 4°C. The lysates were then transferred to tubes containing 0.1 mL of a neutralizing solution (4.2 M KOH), mixed by vortex and subjected to a low-speed centrifugation (1500 rpm, 10 min) at 4°C to eliminate salt precipitates. [^{3}H]cAMP was determined from supernatants purified by sequential chromatography using Dowex and alumina columns as described before (Johnson and Salomon 1991). The amount of [^{3}H]cAMP (CA) over the total amount of intracellular [^{3}H]-adenine (TU) was calculated to determine the relative AC activity (expressed as CA/TU X 1000). Dose-response curves to DA were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02. Receptor numbers (B_{max} values) were determined using a saturating concentration (7 nM) of *N*-[methyl-^{3}H]-SCH23390.

Statistics

Equilibrium and inhibitory dissociation binding constants (K_d and K_i) are expressed using the geometric mean with the 95% lower and upper confidence interval. All other data are reported as arithmetic means ± standard error unless stated otherwise. Dose-response curves to DA were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02 using constrained or unconstrained parameters (bottom or constitutive activity, top or maximal stimulation, log of EC_{50} or log of effective concentration that elicits 50% of maximal stimulation, slope factor). This analysis was performed to establish whether differences observed between best-fitted values were statistically different, i.e., whether constraining a specific curve parameter to a shared or fixed value worsens the goodness of fit. Student's t test and one-way ANOVA with Newman-Keuls multiple comparison test and two-way ANOVA with Bonferroni posttests were used to compare two or multiple groups, respectively. The statistical tests were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) with a critical level of probability set at $\alpha = 0.05$.

RESULTS

The middle segment of TM1 of D1-like receptors modulates ligand binding properties

Previously, we showed that structurally unrelated ligands with various intrinsic efficacies (agonist, inverse agonist) bound to hD5R-TM1^{D1} chimera with increased affinity while interacting with hD1R-TM1^{D5} with decreased affinity relative to their

respective parent wild type receptors (D'Aoust and Tiberi 2009). To delineate residues of TM1 of human D1-like receptors involved in ligand binding, we constructed chimeras in which three segments of the TM1 were individually swapped between human D1R and D5R as depicted in figure 1. Saturation studies using *N*-[methyl-^{3}H]-SCH23390 show that mutant receptors retain their high affinity for this D1-like prototypical drug suggesting that the conformation of the ligand binding pocket is correctly folded (Table I). On the one hand, permuting residues near the extracellular (up) or intracellular (down) end of TM1 does not change the equilibrium dissociation constant value (K_d) of *N*-[methyl-^{3}H]-SCH23390 compared with parent wild type receptors (Table I). On the other hand, exchanging residues in the middle segment of TM1 results in an increased affinity of *N*-[methyl-^{3}H]-SCH23390 for hD5R-TM1mid^{D1} and a decreased affinity for hD1R-TM1mid^{D5} (Table I).

Additionally, swapping the whole TM1 domain slightly increases the maximal level of membrane-bound receptor expression (B_{max}) of hD1R-TM1^{D5} in HEK293 cells while the expression of hD5R-TM1^{D1} is decreased relative to parent wild type receptor (Table I). Interestingly, permuting only the middle segment of TM1 causes a more drastic increase and decrease of the B_{max} for hD1R-TM1mid^{D5} and hD5R-TM1mid^{D1}, respectively (Table I). While mutation in the extracellular or intracellular end of TM1 of hD1R does not affect B_{max}, hD5R-TM1up^{D1} and hD5R-TM1down^{D1} chimeras exhibit higher expression than wild type hD5R (Table I). This represents the first evidence of a counterbalance mechanism between residues of the TM1 in the regulation of D1-like receptor expression.

Figure 1. Primary sequence of the first transmembrane domain (TM1) of human D1-like receptors and schematic representation of wild type and mutant receptors. *Left panel*, topology of the TM1 of wild type human D1R. *Right panel*, topology of the TM1 of wild type human D5R. Identical residues between D1-like receptors are shown in black. The three delimited segments are identified in red (TM1up), blue (TM1mid) and green (TM1down).

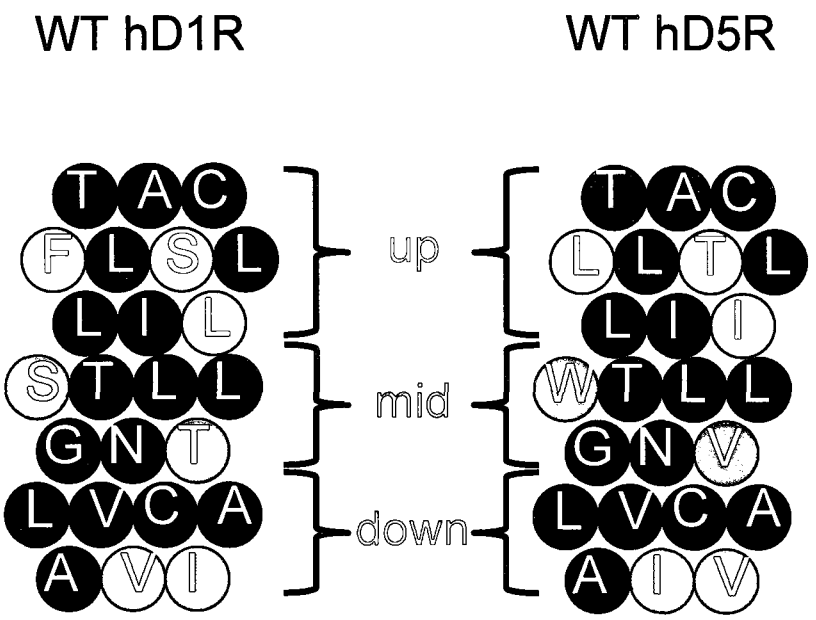


Table I. Dissociation constants (K_d) and maximal binding capacity (B_{max}) values of *N*-[methyl-^{3}H]-SCH23390 for wild-type and multiple point mutant D1-like receptors. K_d are expressed as geometric means and fold over parent wild type receptor from six experiments done in duplicate determinations with the lower and upper 95% of confidence interval in brackets. B_{max} are expressed as arithmetic means and fold over parent wild type receptor from six experiments done in duplicate determinations with the lower and upper 95% of confidence interval in brackets. *, $p<0.05$ when compared with hD1R. #, $p<0.05$ when compared with hD5R.

Receptor	K_d (nM)	Fold over parent wt	B_{max} (pmol/mg prot.)	Fold over parent wt
hD1R	**0.29** (0.24-0.37)	**1**	**9.2** (5.7-12.7)	**1**
hD1R-TM1^{D5}	**0.41** (0.33-0.52)	**1.48** (0.88-2.08)	**10.1** (6.3-14.0)	**1.12** (0.90-1.34)
hD1R-TM1up^{D5}	**0.30** (0.22-0.39)	**1.04** (0.76-1.32)	**9.5** (6.4-12.5)	**1.09** (0.81-1.36)
hD1R-TM1mid^{D5}	**0.39** (0.32-0.48)	**1.36*** (1.02-1.69)	**13.2** (9.3-17.2)	**1.49*** (1.30-1.68)
hD1R-TM1down^{D5}	**0.30** (0.22-0.42)	**1.04** (0.83-1.25)	**8.2** (6.1-10.2)	**0.94** (0.76-1.12)
hD5R	**0.54** (0.41-0.70)	**1**	**9.7** (7.2-12.1)	**1**
hD5R-TM1^{D1}	**0.26** (0.18-0.36)	**0.50#** (0.36-0.63)	**6.2** (4.8-7.6)	**0.66#** (0.46-0.85)
hD5R-TM1up^{D1}	**0.50** (0.42-0.60)	**0.96** (0.74-1.17)	**12.1** (8.8-15.3)	**1.26#** (1.02-1.49)
hD5R-TM1mid^{D1}	**0.26** (0.17-0.38)	**0.50#** (0.32-0.68)	**4.4** (3.3-5.5)	**0.46#** (0.38-0.54)
hD5R-TM1down^{D1}	**0.57** (0.51-0.63)	**1.09** (0.79-1.39)	**14.1** (11.2-16.9)	**1.48#** (1.21-1.75)

The roles of the three segments of TM1 were further investigated using several ligands in competition studies (DA, benzazepines and heterocyclic antipsychotics; Fig. S2). Our results strongly support the hypothesis that the middle segment of TM1 is a crucial structural determinant of ligand binding. Indeed, ligand affinities for hD1R-TM1mid^{D5} and hD5R-TM1mid^{D1} are highly reminiscent of the values obtained with hD1R-TM1^{D5} and hD5R-TM1^{D1}, respectively (Table II and III). Thus, ligands have increased affinities for hD5R-TM1mid^{D1} and decreased affinities for hD1R-TM1mid^{D5}. Swapping the extracellular or intracellular end of the TM1 does not generally affect the affinity of ligands for D1-like mutant receptors (Table II and III). Interestingly, the full agonists tested (DA, SKF82526, SKF82958) tend toward having a lower affinity for hD5R-TM1down^{D1} relative to hD5R (Table II). A graphical representation plotting the percent change in ligand affinity for chimeras relative to parent wild type receptor depicts a generalized affinity decrease for hD1R-TM1^{D5} and hD1R-TM1mid^{D5} except for DA, which binds these chimeras with increased affinity (Table II, Fig. 2*A*). Conversely, all ligands independently of their intrinsic efficacies (agonist, inverse agonist) display higher affinity for hD5R-TM1^{D1} and hD5R-TM1mid^{D1} relative to hD5R (Fig. 2*B*). Overall, our data show that the middle segment of TM1 is responsible for the effects observed with the full exchange of TM1 sequences. Consequently, we investigated the individual role of the two residues forming the middle segment of TM1.

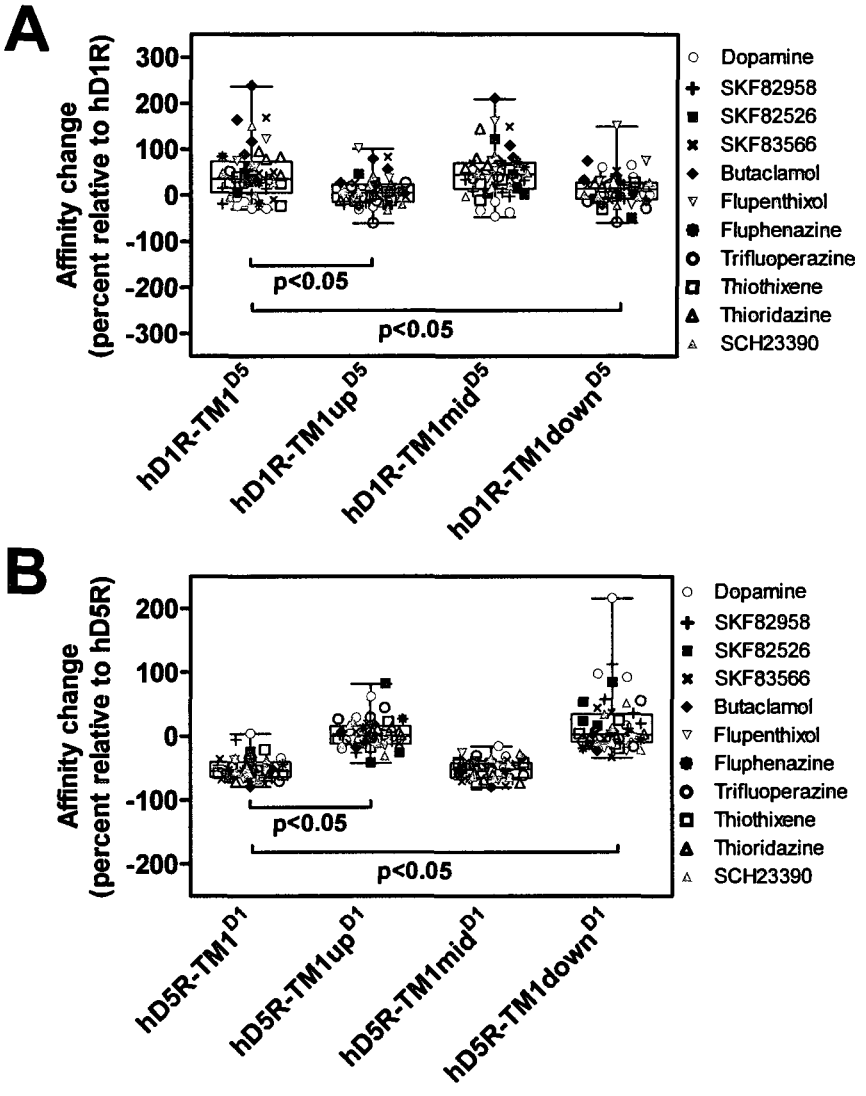
Table II. Inhibitory dissociation constants (K_i) values of dopamine and benzazepine ligands for wild type and multiple point mutant D1-like receptors. K_i are expressed as geometric means and fold over parent wild type receptor from four to five experiments done in duplicate determinations with the lower and upper 95% confidence interval in brackets. *, $p<0.05$ when compared with hD1R; #, $p<0.05$ when compared with hD5R.

Receptor	Dopamine		SKF82526		SKF82958		SKF83566	
	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over
hD1R	**3195** (2178-4688)	**1**	**31.1** (21.4-45.1)	**1**	**21.1** (16.0-27.8)	**1**	**0.96** (0.45-2.05)	**1**
hD1R-TM1^{D5}	**2400** (1615-3568)	**0.75*** (0.64-0.87)	**41.9** (32.3-54.5)	**1.36*** (1.10-1.62)	**22.8** (14.2-36.6)	**1.10** (0.83-1.36)	**1.45** (1.14-1.83)	**1.63** (0.44-2.82)
hD1R-TM1up^{D5}	**2577** (1661-3999)	**0.82** (0.58-1.05)	**34.0** (25.1-46.0)	**1.12** (0.80-1.43)	**19.7** (13.3-29.1)	**0.94** (0.74-1.14)	**1.19** (0.83-1.70)	**1.28** (0.67-1.89)
hD1R-TM1mid^{D5}	**2090** (1166-3747)	**0.67*** (0.45-0.88)	**40.7** (32.8-50.5)	**1.37** (0.74-1.99)	**24.0** (16.7-34.4)	**1.15** (0.94-1.35)	**1.66** (0.99-2.77)	**1.78*** (1.03-2.53)
hD1R-TM1down^{D5}	**4403** (2250-8617)	**1.40** (0.94-1.87)	**28.7** (15.2-54.3)	**0.97** (0.58-1.35)	**24.5** (16.4-36.7)	**1.18** (0.96-1.39)	**1.15** (0.76-1.75)	**1.23** (0.81-1.66)
hD5R	**252** (117-541)	**1**	**14.8** (11.9-18.5)	**1**	**8.5** (6.6-10.8)	**1**	**1.78** (1.11-2.86)	**1**
hD5R-TM1^{D1}	**173** (80-375)	**0.76** (0.15-1.37)	**6.9** (4.9-9.9)	**0.49#** (0.27-0.71)	**4.4** (2.9-6.8)	**0.57#** (0.15-0.98)	**0.72** (0.56-0.92)	**0.43#** (0.14-0.72)
hD5R-TM1up^{D1}	**280** (153-515)	**1.16** (0.57-1.75)	**14.0** (8.6-22.7)	**1.02** (0.42-1.61)	**7.8** (6.9-8.9)	**0.93** (0.78-1.08)	**1.78** (1.28-2.47)	**1.01** (0.77-1.24)
hD5R-TM1mid^{D1}	**140** (92-214)	**0.59#** (0.25-0.92)	**7.7** (5.9-10.2)	**0.53#** (0.42-0.64)	**3.6** (2.6-5.0)	**0.44#** (0.28-0.59)	**0.69** (0.47-1.02)	**0.43#** (0.11-0.75)
hD5R-TM1down^{D1}	**486** (269-878)	**2.05** (0.74-3.37)	**18.9** (14.6-24.6)	**1.32** (0.86-1.79)	**12.1** (8.4-17.4)	**1.47** (0.97-1.97)	**1.87** (1.46-2.41)	**1.10** (0.52-1.68)

Table III. Inhibitory dissociation constants (K_i) values of antipsychotics for wild type and multiple point mutant D1-like receptors. K_i are expressed as geometric means from four experiments done in duplicate determinations with the lower and upper 95% confidence interval in brackets. [&], $p<0.05$ when compared with hD1R; *, $p<0.05$ when compared with hD5R.

Receptor	(+)-Butaclamol		Thioridazine		*cis*-Flupenthixol		Fluphenazine		Trifluoperazine		Thiothixene	
	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over	Ki (nM)	Fold over
hD1R	**2.0** (1.1-3.7)	**1**	**40.1** (26.6-60.4)	**1**	**3.4** (1.2-9.9)	**1**	**9.4** (5.0-17.7)	**1**	**21.0** (6.8-64.1)	**1**	**52.4** (41.0-66.9)	**1**
hD1R-TM1^{D5}	**4.9** (3.3-7.3)	**2.51*** (1.47-3.55)	**69.2** (43.1-111)	**1.74*** (1.38-2.09)	**5.7** (1.6-20.6)	**1.71*** (1.12-2.31)	**11.8** (5.2-26.8)	**1.30** (0.64-1.96)	**20.8** (16.1-26.8)	**1.12** (0.33-1.91)	**59.5** (36.0-98.3)	**1.17** (0.72-1.61)
hD1R-TM1up^{D5}	**2.6** (1.3-4.9)	**1.34** (0.64-2.04)	**43.5** (24.9-75.9)	**1.10** (0.80-1.39)	**4.0** (1.1-15.0)	**1.26** (0.38-2.14)	**10.1** (5.6-18.2)	**1.08** (0.96-1.20)	**17.6** (11.4-27.1)	**0.92** (0.30-1.55)	**49.4** (33.9-72.0)	**0.95** (0.80-1.10)
hD1R-TM1mid^{D5}	**4.2** (3.3-5.4)	**2.18*** (1.18-3.17)	**73.4** (43.0-125)	**1.86*** (1.24-2.48)	**5.9** (2.1-16.1)	**1.80** (0.92-2.67)	**14.3** (9.4-21.9)	**1.54*** (1.17-1.91)	**22.2** (14.6-33.7)	**1.15** (0.46-1.83)	**63.8** (38.7-105)	**1.25** (0.81-1.68)
hD1R-TM1down^{D5}	**2.2** (1.4-3.7)	**1.18** (0.48-1.87)	**46.2** (34.5-62.0)	**1.16*** (1.01-1.30)	**4.8** (3.3-7.2)	**1.57** (0.41-2.73)	**10.3** (5.1-20.7)	**1.10** (0.91-1.29)	**15.4** (7.0-34.1)	**0.79** (0.26-1.33)	**50.4** (29.9-85.0)	**0.99** (0.60-1.37)
hD5R	**27.3** (18.9-39.6)	**1**	**144** (113-183)	**1**	**9.0** (3.5-23.1)	**1**	**23.8** (18.0-31.5)	**1**	**27.7** (23.8-32.2)	**1**	**289** (195-429)	**1**
hD5R-TM1^{D1}	**10.1** (6.7-15.4)	**0.39#** (0.20-0.58)	**52.9** (33.2-84.6)	**0.38#** (0.23-0.52)	**4.1** (1.1-14.8)	**0.46#** (0.27-0.65)	**10.9** (6.9-17.2)	**0.46#** (0.35-0.58)	**14.1** (7.0-28.4)	**0.53#** (0.25-0.81)	**141** (119-168)	**0.51#** (0.22-0.80)
hD5R-TM1up^{D1}	**27.6** (19.1-39.8)	**1.02** (0.78-1.26)	**155** (121-198)	**1.08** (0.99-1.16)	**8.4** (3.3-21.7)	**0.94** (0.81-1.08)	**24.5** (16.8-35.8)	**1.04** (0.76-1.32)	**34.7** (26.3-45.8)	**1.26#** (1.00-1.52)	**286** (207-397)	**1.00** (0.73-1.27)
hD5R-TM1mid^{D1}	**12.1** (6.2-23.8)	**0.49#** (0.17-0.80)	**48.9** (31.5-75.8)	**0.35#** (0.23-0.46)	**4.3** (1.1-17.4)	**0.50#** (0.21-0.79)	**10.2** (3.3-32.0)	**0.44#** (0.18-0.70)	**14.7** (6.1-35.1)	**0.54#** (0.15-0.92)	**131** (78.3-220)	**0.48#** (0.22-0.74)
hD5R-TM1down^{D1}	**26.9** (20.3-35.7)	**0.99** (0.75-1.23)	**143** (113-181)	**1.00** (0.95-1.304)	**7.7** (2.6-22.2)	**0.86** (0.70-1.02)	**22.5** (15.1-33.5)	**0.95** (0.78-1.12)	**28.6** (18.3-44.5)	**1.06** (0.54-1.59)	**281** (252-314)	**0.99** (0.67-1.30)

Figure 2. Affinity change (in %) of dopaminergic ligands for multiple point mutant receptor relative to parent wild type receptor. For each drug tested, percent change in affinity relative to wild type receptor was calculated from binding affinity values reported in Tables I, II and III. Each box represents the 25th and 75th percentiles and whiskers show the minimum and maximum values of dopaminergic ligand affinity changes; $p<0.05$, one-way ANOVA followed by Newman-Keuls. No differences are observed between TM1 chimeras and TM1mid chimeras. All values except those of hD1R-TM1up^{D5} and hD5R-TM1up^{D1} are different from 0; Student't t test, $p<0.05$. (DA, red circle; SKF82526, blue square; (+)-Butaclamol, purple diamond; cis-Flupenthixol, green inverted triangle, *N*-[methyl-^{3}H]-SCH23390, grey triangle; SKF82958, +; SKF83566, x; Fluphenazine, *; Trifluoperazine, open circle; Thiothixene, open square; Thioridazine, open triangle. (A) Box-and-whisker plot of mutant D1R relative to wild type hD1R. (B) Box-and-whisker plot of mutant D5R relative to wild type hD5R.



Identification of a TM1 residue controlling ligand binding of D1-like receptors

We individually swapped the residues of the middle segment of TM1 between D1-like receptors. Ser36$^{1.45}$ and Thr42$^{1.51}$ in TM1 of hD1R were mutated to Trp and Val, respectively and vice versa in hD5R (Trp53Ser$^{1.45}$ and Val59Thr$^{1.51}$) to create four point mutant chimeras: hD1R-S36W, hD1R-T42V, hD5R-W53S and hD5R-V59T. Saturation studies with *N*-[methyl-^{3}H]-SCH23390 demonstrate that point mutant receptors retain their high affinity for this ligand (Table IV). The maximal levels of membrane-bound functional receptor expression (B_{max}) of single point mutant receptors are reported in Table IV. Permuting only residue 1.45 leads to a ~50% increase and decrease in hD1R-S36W and hD5R-W53S receptor levels, which is reminiscent of findinds obtained with hD1R-TM1mid^{D5} and hD5R-TM1mid^{D1} (Table IV). B_{max} values of hD1R-T42V and hD5R-V59T are similar to those of wild type receptors (Table IV). Additionally, *N*-[methyl-^{3}H]-SCH23390 has decreased affinity for hD1R-S36W whereas its affinity for hD1R-T42V is similar to hD1R. Conversely, *N*-[methyl-^{3}H]-SCH23390 displays an increased affinity for hD5R-W53S, a result similar to that obtained with swapping the middle segment of the TM1. Meanwhile, a slight increase in affinity is also observed with hD5R-V59T, suggesting that both residues play a role in ligand binding.

A possible cooperation of amino acids in the middle segment of TM1 in ligand binding

Subsequently, the binding properties of point mutant chimeras were studied using six compounds of various chemical structures. Interestingly, all single point mutant

Table IV. Dissociation constants (K_d) and maximal binding capacity (B_{max}) values of *N*-[methyl-^{3}H]-SCH23390 for wild type and single point mutant D1-like receptors. K_d are expressed as geometric means and fold over parent wild type receptor from four experiments done in duplicate determinations with the lower and upper 95% of confidence interval in brackets. B_{max} are expressed as arithmetic means and fold over parent wild type receptor from four experiments done in duplicate determinations with the lower and upper 95% of confidence interval in brackets. *, $p<0.05$ when compared with hD1R. #, $p<0.05$ when compared with hD5R.

Receptor	K_d (nM)	Fold over parent wt	B_{max} (pmol/mg prot.)	Fold over parent wt
hD1R	**0.35** (0.27-0.45)	**1**	**12.0** (8.3-15.7)	**1**
hD1R-TM1mid^{D5}	**0.49** (0.39-0.62)	**1.42** (0.80-2.06)	**17.2** (14.1-20.3)	**1.47** (0.95-1.99)
hD1R-S36W	**0.39** (0.31-0.49)	**1.11** (0.99-1.24)	**17.2** (15.8-18.7)	**1.47*** (1.04-1.90)
hD1R-T42V	**0.34** (0.30-0.38)	**0.98** (0.73-1.22)	**11.4** (10.3-12.4)	**0.97** (0.71-1.22)
hD5R	**0.46** (0.33-0.63)	**1**	**9.9** (7.1-12.7)	**1**
hD5R-TM1mid^{D1}	**0.20** (0.16-0.26)	**0.45**$^{\#}$ (0.38-0.52)	**4.5** (2.4-6.6)	**0.45**$^{\#}$ (0.30-0.60)
hD5R-W53S	**0.25** (0.19-0.34)	**0.55**$^{\#}$ (0.49-0.61)	**4.6** (3.1-6.1)	**0.47**$^{\#}$ (0.32-0.62)
hD5R-V59T	**0.40** (0.27-0.58)	**0.88** (0.69-1.08)	**9.9** (7.9-12.0)	**1.04** (0.58-1.49)

chimeras gain affinity for DA relative to parent wild type receptors, suggesting that the two variant amino acids of the middle segment shape the orthosteric binding site of the natural ligand (Table V). As observed with *N*-[methyl-^{3}H]-SCH23390, all ligands tested display an increased affinity for both hD5R-W53S and hD5R-V59T although the increase does not always reach a level comparable to hD5R-TM1mid^{D1}, suggesting an additive effect or cooperation of both residues (Table V). Indeed, DA, SKF82526, SKF83959, thioridazine and butaclamol have increased affinity for both hD5R point mutant receptors relative to hD5R but this increase is smaller than that observed with hD5R-TM1mid^{D1} (Table V). Similarly, the reduced affinities of SKF83959, thioridazine and (+)-butaclamol for hD1R-TM1mid^{D5} are not fully recapitulated with either hD1R-S36W or hD1R-T42V (Table V). These results strongly suggest that both residues in the middle segment of TM1 are cooperating to influence ligand affinity.

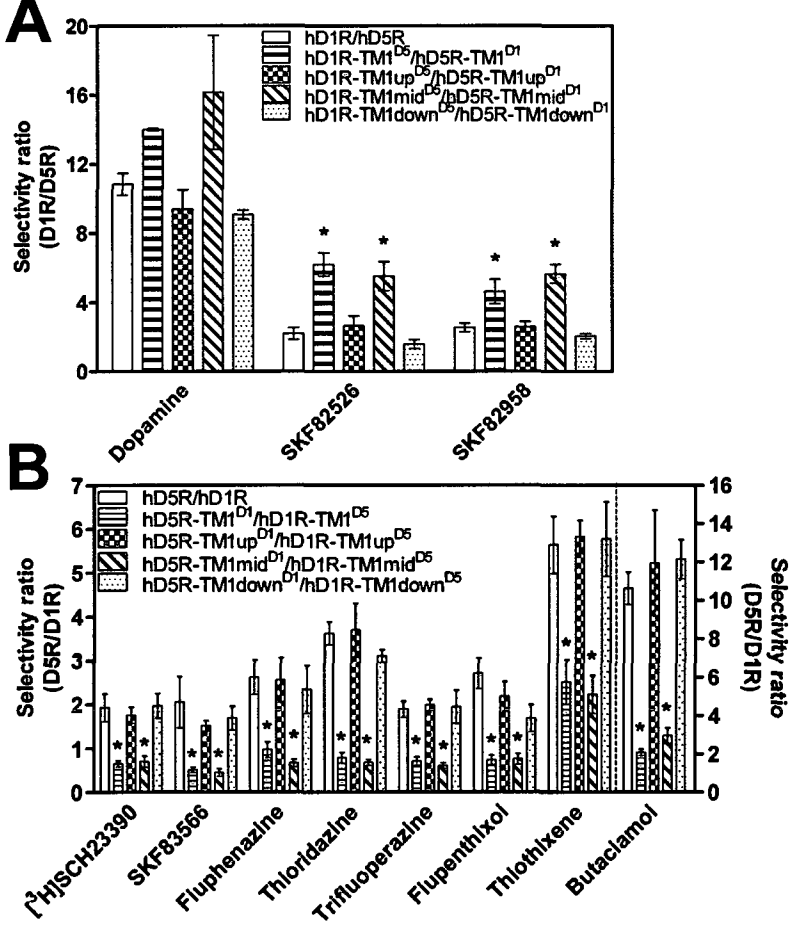
Middle segment residues of TM1 control ligand selectivity

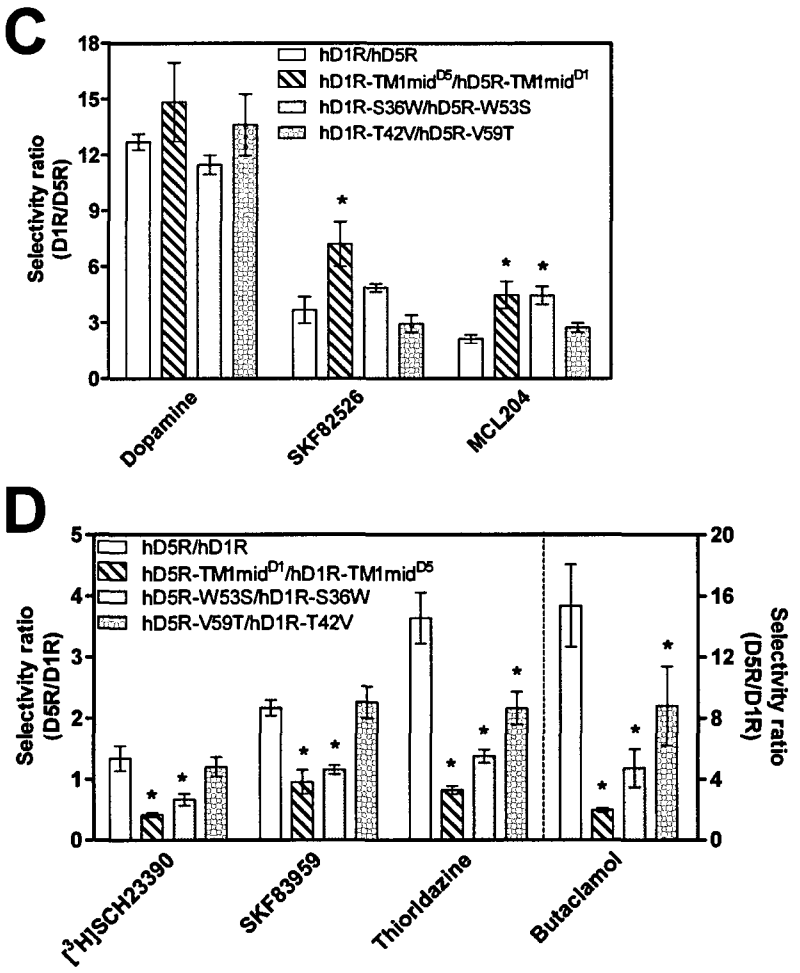
The variations in ligand affinity described earlier lead to changes in the D1-like selectivity profile of ligands. DA exhibits a ~10-fold selectivity ratio for hD5R over hD1R, comparable with previous studies (Sunahara et al. 1991; Tiberi and Caron 1994; Iwasiow et al. 1999; Demchyshyn et al. 2000; Jackson et al. 2000; Tumova et al. 2003; D'Aoust and Tiberi 2009). Exchanging the whole TM1 domain or the middle segment of TM1 slightly increases the selectivity of DA for hD5R, although the effect is not detectable statistically (Fig. 3*A*). In addition, selectivity ratios of all synthetic ligands are changed for D1-like chimeras harboring a swapped TM1 domain compared with wild type receptors (Fig. 3*A* and *B*). In fact, selectivity ratios of ligands with higher affinity for hD5R are increased after swapping the TM1 domain while drugs with higher affinity for

Table V. Inhibitory dissociation constants (K_i) values of dopaminergic ligands for wild type and single point mutant D1-like receptors. K_i are expressed as geometric means and fold over parent wild type receptor from four experiments done in duplicate determinations with the lower and upper 95% confidence interval in brackets. *, $p<0.05$ when compared with hD1R; #, $p<0.05$ when compared with hD5R.

Receptor	Dopamine		SKF82526		SKF83959		MCL-204		(+)-Butaclamol		Thioridazine	
	K_i (nM)	Fold over	K_i (nM)	Fold over	K_i (nM)	Fold over	K_i (nM)	Fold over	K_i (nM)	Fold over	K_i (nM)	Fold over
hD1R	**3422** (3091-3789)	**1**	**44.5** (27.5-72.1)	**1**	**0.87** (0.29-2.66)	**1**	**8.7** (7.2-10.5)	**1**	**1.5** (0.8-3.0)	**1**	**40.5** (20.1-81.3)	**1**
hD1R-TM1mid^{D5}	**2362** (1549-3600)	**0.70*** (0.47-0.93)	**42.7** (26.5-68.8)	**0.97** (0.71-1.23)	**1.00** (0.33-2.98)	**1.20** (0.52-1.88)	**9.7** (5.5-17.0)	**1.15** (0.61-1.69)	**4.0** (2.5-6.4)	**2.82** (0.89-4.75)	**68.5** (38.5-122)	**1.72*** (1.12-2.32)
hD1R-S36W	**2276** (1581-3276)	**0.68*** (0.45-0.91)	**39.2** (31.7-48.4)	**0.93** (0.40-1.45)	**0.83** (0.32-2.18)	**0.97** (0.66-1.28)	**8.6** (5.4-13.7)	**1.00** (0.71-1.29)	**2.5** (1.3-4.8)	**1.85** (0-3.78)	**53.4** (24.6-116)	**1.35** (0.82-1.88)
hD1R-T42V	**2739** (2053-3655)	**0.82** (0.51-1.13)	**33.2** (20.6-53.5)	**0.81** (0.20-1.42)	**0.72** (0.27-1.91)	**0.84** (0.57-1.10)	**10.1** (6.3-16.1)	**1.18** (0.78-1.57)	**2.0** (1.1-3.6)	**1.49** (0.20-2.77)	**53.8** (25.1-115)	**1.37** (0.73-2.01)
hD5R	**270** (227-320)	**1**	**13.1** (6.4-27.0)	**1**	**1.88** (0.72-4.87)	**1**	**4.2** (2.6-6.8)	**1**	**22.3** (15.5-32.2)	**1**	**144** (85.3-244)	**1**
hD5R-TM1mid^{D1}	**164** (122-221)	**0.61#** (0.48-0.74)	**6.2** (4.8-8.0)	**0.49#** (0.26-0.72)	**0.89** (0.34-2.34)	**0.49#** (0.29-0.69)	**2.3** (1.3-3.9)	**0.55#** (0.39-0.71)	**7.9** (5.1-12.3)	**0.36#** (0.25-0.48)	**55.7** (28.6-108)	**0.39#** (0.26-0.52)
hD5R-W53S	**199** (148-268)	**0.74#** (0.59-0.90)	**8.0** (6.1-10.6)	**0.68** (0.05-1.31)	**0.96** (0.37-2.47)	**0.52#** (0.38-0.65)	**2.0** (1.6-2.4)	**0.48#** (0.33-0.62)	**10.5** (7.4-14.9)	**0.48#** (0.31-0.64)	**72.8** (41.3-128)	**0.51#** (0.45-0.56)
hD5R-V59T	**206** (180-235)	**0.77#** (0.58-0.96)	**11.6** (8.9-15.2)	**1.00** (0.06-1.93)	**1.59** (0.73-3.49)	**0.85** (0.70-1.00)	**3.7** (3.0-4.6)	**0.91** (0.61-1.20)	**15.9** (12.2-20.6)	**0.73#** (0.46-0.99)	**113** (76.4-168)	**0.79#** (0.68-0.89)

Figure 3. Selectivity ratios of dopaminergic ligands for wild type and mutant forms of D1-like receptors. For each drug tested, selectivity ratio was calculated from binding affinity values reported in Tables I, II, III, IV and V. Bars represent the arithmetic mean ± S.E. of four to six experiments done in duplicate determinations. (A) Selectivity ratios of DA, SKF82958 and SKF82526 were calculated for each experiment by dividing the affinity for wild type or mutant hD1R by the affinity for their respective hD5R counterpart. *, $p<0.05$ when compared with wild type hD1R/hD5R selectivity ratio. (B) Selectivity ratios of *N*-[methyl-^{3}H]-SCH23390, SKF83566, fluphenazine, thioridazine, trifluoperazine, *cis*-flupenthixol, thiothixene and (+)-butaclamol were calculated for each experiment by dividing the affinity for wild type or mutant hD5R by the affinity for their respective hD1R counterpart. Ratios of butaclamol are plotted on the right Y axis. *, $p<0.05$ when compared with wild type hD5R/hD1R selectivity ratio. (C) Selectivity ratios of DA, SKF82526 and MCL-204 were calculated for each experiment by dividing the affinity for wild type or mutant hD1R by the affinity for their respective hD5R counterpart. *, $p<0.05$ when compared with wild type hD1R/hD5R selectivity ratio. (D) Selectivity ratios of *N*-[methyl-^{3}H]-SCH23390, SKF83959, thioridazine, and (+)-butaclamol were calculated for each experiment by dividing the affinity for wild type or mutant hD5R by the affinity for their respective hD1R counterpart. Ratios of butaclamol are plotted on the right Y axis. *, $p<0.05$ when compared with wild type hD5R/hD1R selectivity ratio.




C
hD1R/hD5R
hD1R-TM1mid^{D5}/hD5R-TM1mid^{D1}
hD1R-S36W/hD5R-W53S
hD1R-T42V/hD5R-V59T
Selectivity ratio (D1R/D5R)
18
15
12
9
6
3
0
Dopamine
SKF82526
MCL204
D
hD5R/hD1R
hD5R-TM1mid^{D1}/hD1R-TM1mid^{D5}
hD5R-W53S/hD1R-S36W
hD5R-V59T/hD1R-T42V
Selectivity ratio (D5R/D1R)
Selectivity ratio (D5R/D1R)
5
4
3
2
1
0
20
16
12
8
4
0
[^{3}H]SCH23390
SKF83959
Thioridazine
Butaclamol

hD1R exhibit reduced selectivity ratios with TM1-swapped receptors (Fig. 3*A* and *B*). Exchanging the extracellular or intracellular end of the TM1 does not modify selectivity ratios compared with wild type receptors (Fig. 3*A* and *B*). Importantly, permuting the middle segment of the TM1 causes identical selectivity ratio changes as swapping the whole TM1 domain for all ligands tested (Fig. 3*A* and *B*). Thus, these results strongly suggest that the middle segment of TM1 controls ligand selectivity for D1-like subtypes.

Furthermore, plotting selectivity ratios of ligands for D1-like point mutant receptors unveils a cooperative mechanism between both residues of the middle segment of TM1 (Fig. 3*C* and *D*). Indeed, while swapping residue 1.45 between D1-like receptors lowers the selectivity ratio of SCH23390, SKF83959, thioridazine and butaclamol, it does not systematically reach the level attained with TM1mid chimeras (Fig. 3*D*). Interestingly, this incomplete reduction of the selectivity ratio is somewhat completed by the swap of residue 1.51, which decreases the selectivity ratios of three compounds ([^{3}H]-SCH23390, thioridazine, butaclamol) (Fig. 3*D*). The slight increase in DA selectivity obtained with TM1mid chimeras may be explained by the exchange of residue 1.51, as DA is slightly more selective towards hD5R-V59T than hD1R-T42V (Fig. 3*C*). However, the increase in selectivity of SKF82526 for hD5R-TM1mid^{D1} relative to hD1R-TM1mid^{D5} is not recapitulated with either single point mutation, suggesting that both residues of the TM1 middle segment participate to confer a higher selectivity for hD5R chimeras (Fig. 3*C*). In striking contrast, the increase in selectivity of another benzazepine, MCL-204, obtained with TM1mid chimeras is solely mediated by residue 1.45, suggesting that both middle segment residues control ligand selectivity (Fig. 3*C*). Interestingly, MCL-204 was initially reported as a ligand displaying a 109-fold selectivity

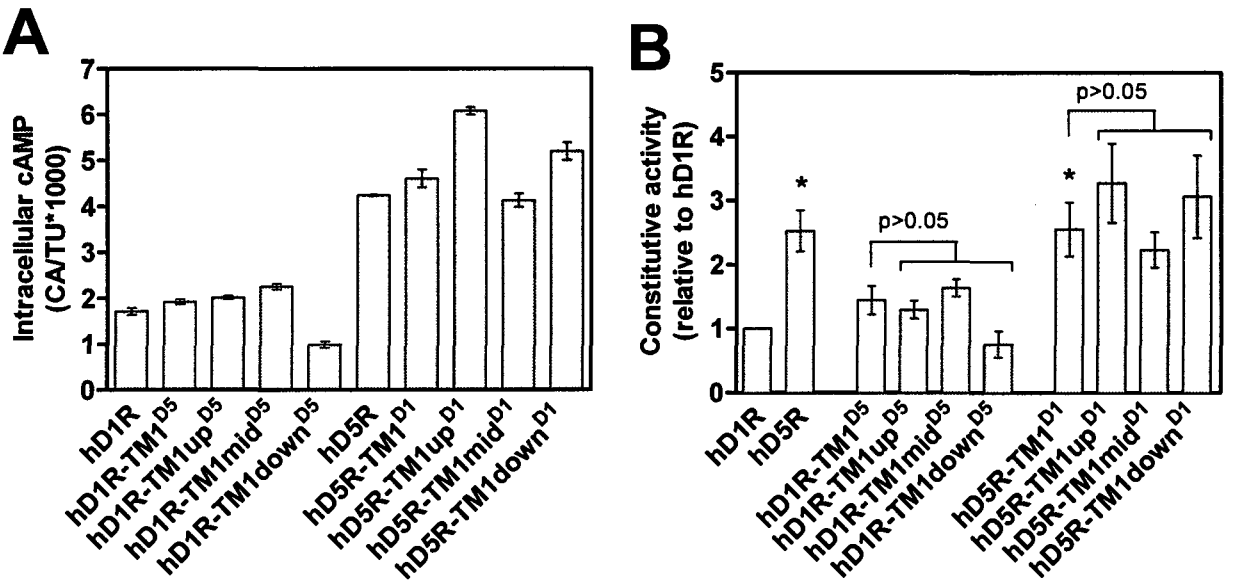
for D1R over D5R (Neumeyer et al. 2003). However, our results show instead a 2-fold selectivity for hD5R over hD1R (Fig. 3*C*). This difference in ligand binding values may be explained by the use, in the initial study, of rat striatal tissue for D1R and transfected HEK293 cells for hD5R (Neumeyer et al. 2003), whereas our results are obtained with HEK293 membrane preparations for both human receptors.

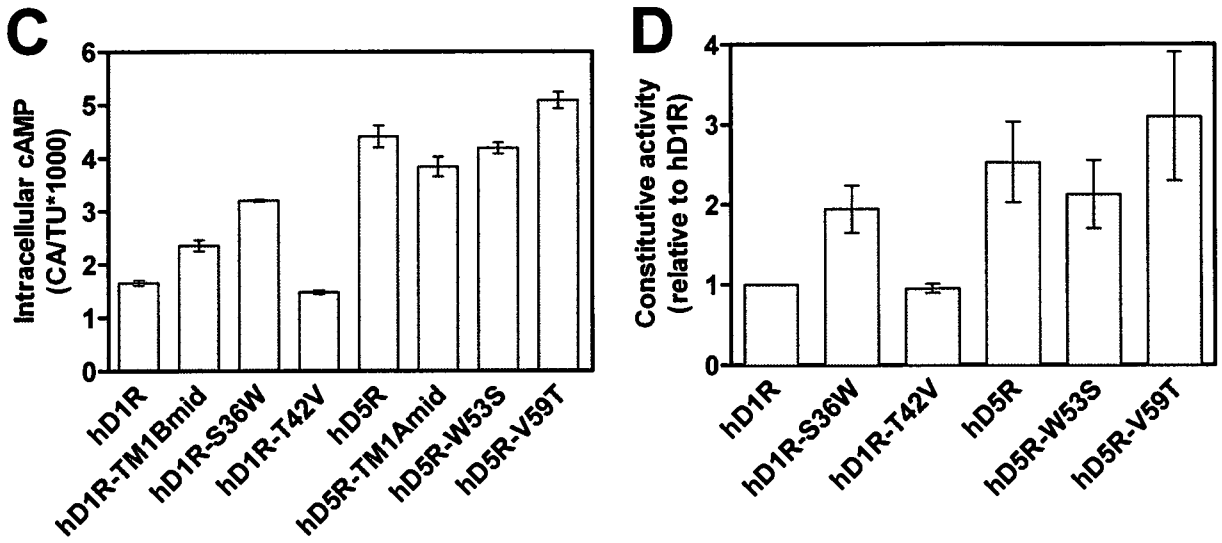
Agonist-independent activity of D1-like receptors is not controlled by TM1

Recently, we showed that cells expressing D1-like chimerical receptors with a swapped TM1 domain stimulated the AC in the absence of an agonist to a similar extent than parent wild type receptor (D'Aoust and Tiberi 2009). However, hD1R-TM1^{D5} chimera had a slight tendency to have a higher constitutive activity indicating a possible counteraction of residues located throughout the helical domain. The slightly higher constitutive activity of hD1R-TM1^{D5} is also seen in this study. However, the constitutive activities of hD1R-TM1up^{D5}, hD1R-TM1mid^{D5} and hD1R-TM1down^{D5} are comparable to the full chimera, suggesting that the TM1 of hD1R does not participate in the ligand-independent activity of the receptor (Fig. 4*B*). The constitutive activities of hD5R and hD5R-TM1^{D1} are ~2.5 times higher than hD1R (Fig. 4*B*). Like it was observed for hD1R mutants, ligand-independent activities of hD5R-TM1up^{D1}, hD5R-TM1mid^{D1} and hD5R-TM1down^{D1} are reminiscent to that of hD5R-TM1^{D1} (Fig. 4*B*). Differences in receptor expression explain the variation in constitutive activity of chimeras. Therefore, swapping individual segments of TM1 do not drastically alter the constitutive activity of D1-like receptors.

Moreover, the constitutive activities of single point mutant receptor are not different than their respective wild type receptor (Fig. 4*D*). Although the activity of

Figure 4. Agonist-independent activity of wild type and mutant forms of D1-like receptors expressed in HEK293 cells. (A) Representative example of the constitutive activity of wild type and mutant D1-like receptors using raw data. (B) Constitutive activity of each mutant hD1R or hD5R and wild type hD1R was plotted as relative to hD1R constitutive activity. *, $p<0.05$ when compared with hD1R (value of 1). Receptor expression is expressed as the arithmetic mean ± S.E. (pmol/mg membrane proteins) and is as follows: 13.6 ± 0.8 (hD1R); 12.2 ± 0.4 (hD1R-TM1^{D5}); 14.6 ± 1.2 (hD1R-TM1up^{D5}); 17.8 ± 1.3 (hD1R-TM1mid^{D5}); 10.9 ± 1.1 (hD1R-TM1down^{D5}); 9.8 ± 0.8 (hD5R); 6.0 ± 0.2 (hD5R-TM1^{D1}); 9.8 ± 0.6 (hD5R-TM1up^{D1}); 4.5 ± 0.6 (hD5R-TM1mid^{D1}); 13.0 ± 1.4 (hD5R-TM1down^{D1}). Each bar represents the arithmetic mean ± S.E. of four to seven experiments done in triplicate determinations and expressed as relative to the constitutive activity of hD1R. (C) Representative example of the constitutive activity of wild type and single point mutant D1-like receptors using raw data. (D) Constitutive activity of each point mutant hD1R or hD5R was plotted as relative to hD1R constitutive activity. *, $p<0.05$ when compared with hD1R (value of 1). Receptor expression is expressed as the arithmetic mean ± S.E. (pmol/mg membrane proteins) and is as follows: 14.4 ± 4.1 (hD1R); 18.1 ± 2.8 (hD1R-S36W); 14.1 ± 3.8 (hD1R-T42V); 10.6 ± 2.7 (hD5R); 5.4 ± 1.0 (hD5R-W53S); 9.8 ± 2.1 (hD5R-V59T). Each bar represents the arithmetic mean ± S.E. of three experiments done in triplicate determinations and expressed as relative to the constitutive activity of hD1R.



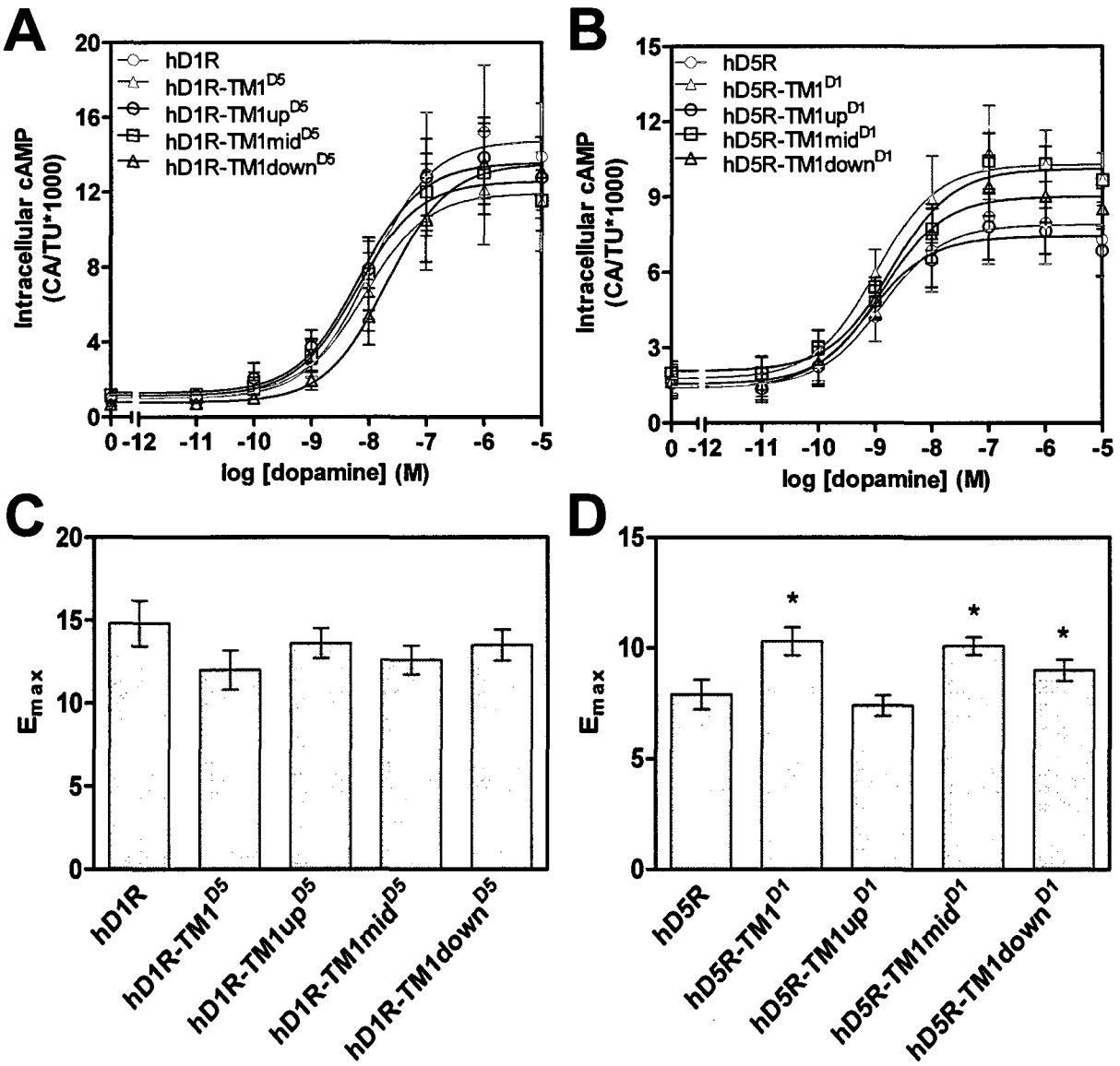

C
Intracellular cAMP
(CA/TU*1000)
0
1
2
3
4
5
6
hD1R
hD1R-TM1Bmid
hD1R-S36W
hD1R-T42V
hD5R
hD5R-TM1Amid
hD5R-W53S
hD5R-V59T
D
Constitutive activity
(relative to hD1R)
0
1
2
3
4
hD1R
hD1R-S36W
hD1R-T42V
hD5R
hD5R-W53S
hD5R-V59T

hD1R-S36W may appear increased relative to hD1R, it is likely explained by the drastically higher receptor expression of this mutant receptor (see legend of Fig. 4*D*). Overall, TM1 residues do not play an important role in conferring D1-like subtype-specific agonist-independent properties.

Efficacy of dopamine in HEK293 expressing D1-like receptors depends on a critical segment of TM1

Dose-response curves to DA were performed on HEK293 cells expressing similar low levels of mutant and wild type D1-like receptors to assess the role of the different segments of TM1 on the efficacy (E_{max}) and potency (EC_{50}) of DA. Maximal stimulation of AC elicited by DA is greater in cells expressing hD1R than hD5R. Swapping the whole TM1 leads to a higher E_{max} in cells expressing hD5R-TM1^{D1} than hD5R while no effect is observed for hD1R-TM1^{D5} chimera (Fig. 5). Exchanging any segment of TM1 in hD1R with its hD5R counterpart does not alter DA-mediated maximal activity compared with wild type hD1R (Fig. 5*A* and *C*). On the other hand, a higher E_{max} is obtained with hD5R-TM1mid^{D1}, reminiscent of hD5R-TM1^{D1} while hD5R-TM1down^{D1} also displays a greater E_{max} value compared with hD5R (Fig. 5*B* and *D*). As observed in previous studies, DA possesses a ~10-fold higher EC_{50} in cells expressing wild type hD5R than hD1R (see legend of Fig. 5). Differences in the EC_{50} values of mutant receptors compared with parent wild type receptors were not detectable statistically, although the EC_{50} values of hD1R-TM1down^{D5} and hD5R-TM1mid^{D1} chimeras have a propensity to be lower (see legend of Fig. 5).

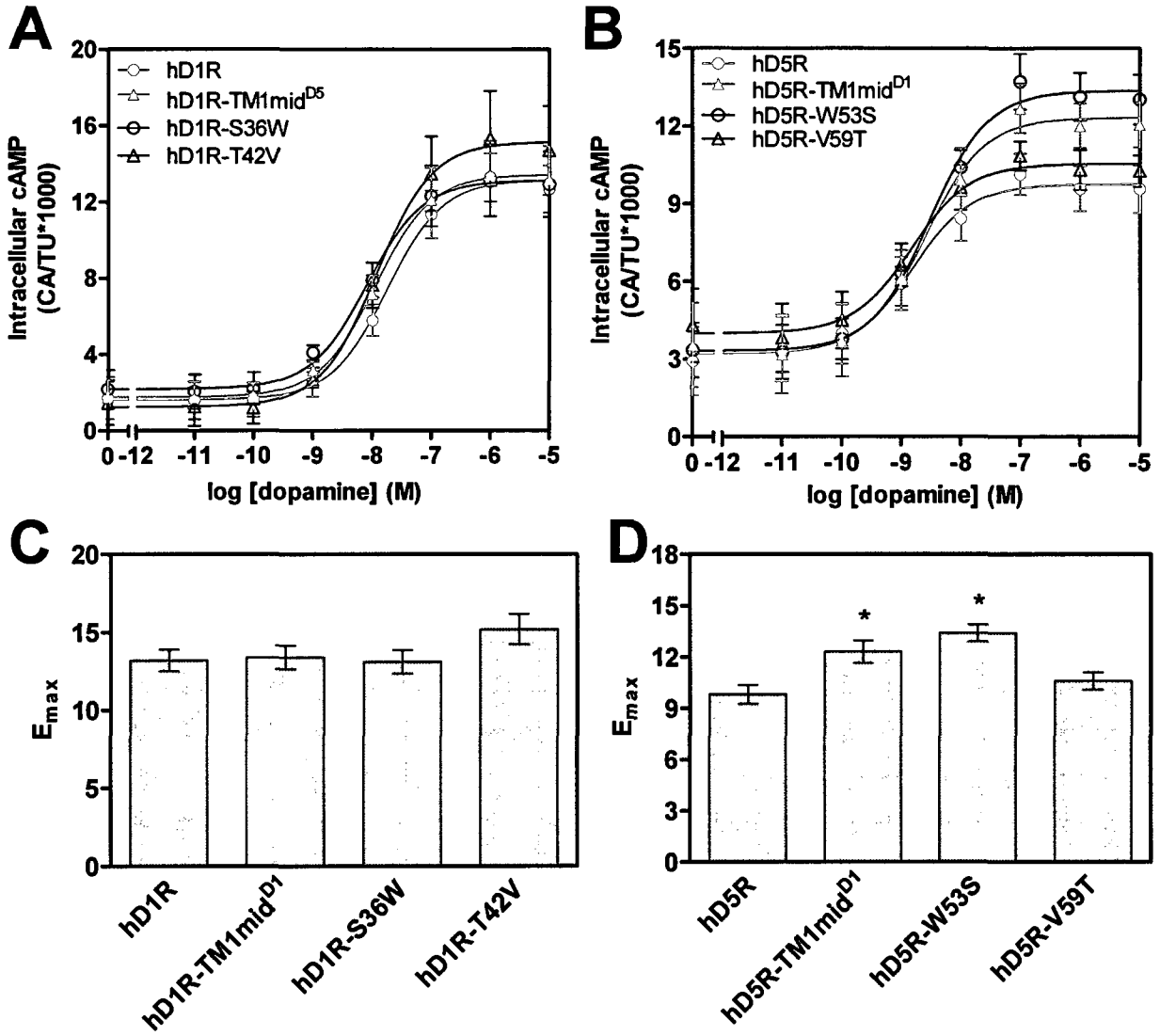
Figure 5. DA-mediated activation of adenylyl cyclase by wild type and multiple point mutant D1-like receptors expressed in HEK293 cells. HEK293 cells were transfected with wild type or mutant receptor using the following amount of DNA per dish: 0.02-0.03 μg of hD1R, 0.02-0.03 μg of hD1R-TM1^{D5}, 0.02 μg of hD1R-TM1up^{D5}, 0.02-0.03 μg of hD1R-TM1mid^{D5}, 0.02-0.03 μg of hD1R-TM1down^{D5}, 0.02 μg of hD5R, 0.03 μg of hD5R-TM1^{D1}, 0.02 μg of hD5R-TM1up^{D1}, 0.03-0.04 μg of hD5R-TM1mid^{D1}, 0.02 μg of hD5R-TM1down^{D1}. Receptor expression in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) is as follows: 1.9 ± 0.9 (hD1R), 1.4 ± 0.7 (hD1R-TM1^{D5}), 1.9 ± 0.7 (hD1R-TM1up^{D5}), 1.7 ± 0.9 (hD1R-TM1mid^{D5}), 1.6 ± 0.8 (hD1R-TM1down^{D5}), 1.4 ± 0.4 (hD5R), 1.7 ± 0.6 (hD5R-TM1^{D1}), 1.4 ± 0.5 (hD5R-TM1up^{D1}), 1.3 ± 0.2 (hD5R-TM1mid^{D1}). 1.8 ± 0.4 (hD5R-TM1down^{D1}). Each point represents the arithmetic mean ± S.E. of three to four experiments done in triplicate determinations and expressed as [^{3}H]-cAMP formed (CA) over the total amount of [^{3}H]-adenine uptake (TU) X 1000. DA dose-response curves were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02 using constrained or unconstrained parameters. The EC_{50} (concentration that elicits 50% of the maximal response) corresponds to the best-fitted value with the 95% lower and upper confidence interval in parenthesis (expressed in nM): 10.7 (2.2-52.2) for hD1R, 8.7 (1.5-51.6) for hD1R-TM1^{D5}, 6.9 (2.1-23.4) for hD1R-TM1up^{D5}, 6.4 (1.8-22.9) for hD1R-TM1mid^{D5}, 20.8 (6.8-64.5) for hD1R-TM1down^{D5}, 1.31 (0.18-9.4) for hD5R, 1.05 (0.25-4.4) for hD5R-TM1^{D1}, 0.80 (0.17-3.7) for hD5R-TM1up^{D1}, 1.91 (0.77-4.7) for hD5R-TM1mid^{D1}, 1.37 (0.40-4.6) for hD5R-TM1down^{D1}. (A) DA dose-response curves for mutant and wild type hD1R. (B) DA dose-response curves for mutant and wild type hD5R.


A
Intracellular cAMP (CA/TU*1000)
hD1R
hD1R-TM1D5
hD1R-TM1upD5
hD1R-TM1midD5
hD1R-TM1downD5
log [dopamine] (M)
B
Intracellular cAMP (CA/TU*1000)
hD5R
hD5R-TM1D1
hD5R-TM1upD1
hD5R-TM1midD1
hD5R-TM1downD1
log [dopamine] (M)
C
Emax
hD1R
hD1R-TM1D5
hD1R-TM1upD5
hD1R-TM1midD5
hD1R-TM1downD5
D
Emax
hD5R
hD5R-TM1D1
hD5R-TM1upD1
hD5R-TM1midD1
hD5R-TM1downD1

A single residue in TM1 of hD5R modulates dopamine efficacy

Since the most prominent effect on DA efficacy was obtained with hD5R-TM1mid^{D1}, dose-response curves to DA were performed with single point mutant receptors in order to pinpoint which residue of the middle segment of TM1 controls the efficacy of DA in cells expressing hD5R-TM1mid^{D1}. The E_{max} and EC_{50} values of single point mutant hD1Rs are reminiscent of wild type hD1R (Fig. 6, *A* and *C*). Importantly, an increased DA-mediated activation of AC relative to hD5R is observed with hD5R-W53S, a value comparable to hD5R-TM1mid^{D1}, while hD5R-V59T responds to DA like hD5R (Fig. 6*B* and *D*). Moreover, the EC_{50} value of hD5R-W53S is ~2 times higher than hD5R as observed with hD5R-TM1mid^{D1} (see legend of Fig. 6).

Figure 6. DA-mediated activation of adenylyl cyclase by wild type and mutant D1-like receptors expressed in HEK293 cells. HEK293 cells were transfected with wild type or mutant receptor using the following amount of DNA per dish: 0.02 μg of hD1R, 0.012-0.03 μg of hD1R-TM1mid^{D5}, 0.016-0.024 μg of hD1R-S36W, 0.02 μg of hD1R-T42V, 0.02 μg of hD5R, 0.036-0.05 μg of hD5R-TM1mid^{D1}, 0.03-0.05 μg of hD5R-W53S, 0.02 μg of hD5R-V59T. Receptor expression in pmol/mg of membrane proteins (expressed as arithmetic mean ± S.E.) is as follows: 1.6 ± 0.3 (hD1R), 1.7 ± 0.4 (hD1R-TM1mid^{D5}), 1.8 ± 0.3 (hD1R-S36W), 1.7 ± 0.5 (hD1R-T42V), 1.6 ± 0.3 (hD5R), 1.3 ± 0.2 (hD5R-TM1mid^{D1}), 1.4 ± 0.1 (hD5R-W53S) and 1.8 ± 0.1 (hD5R-V59T). Each point represents the arithmetic mean ± S.E. of four to five experiments done in triplicate determinations and expressed as [^{3}H]-cAMP formed (CA) over the total amount of [^{3}H]-adenine uptake (TU) X 1000. DA dose-response curves were simultaneously analyzed by a four-parameter logistic equation using GraphPad Prism version 5.02 using constrained or unconstrained parameters. The EC_{50} (concentration that elicits 50% of the maximal response) corresponds to the best-fitted value with the 95% lower and upper confidence interval in parenthesis (expressed in nM): 17.6 (7.5-41.1) for hD1R, 11.2 (4.3-29.0) for hD1R-TM1mid^{D5}, 8.1 (2.9-22.7) for hD1R-S36W, 11.7 (4.2-32.5) for hD1R-T42V, 1.53 (0.35-6.7) for hD5R, 2.65 (0.73-9.6) for hD5R-TM1mid^{D1}, 2.99 (1.28-7.0) for hD5R-W53S, 1.49 (0.37-6.0) for hD5R-V59T. (A) DA dose-response curves for wild type and mutant hD1R. (B) DA dose-response curves for wild type and mutant hD5R.


A
hD1R
hD1R-TM1mid^{D5}
hD1R-S36W
hD1R-T42V
Intracellular cAMP (CA/TU*1000)
log [dopamine] (M)
B
hD5R
hD5R-TM1mid^{D1}
hD5R-W53S
hD5R-V59T
Intracellular cAMP (CA/TU*1000)
log [dopamine] (M)
C
E_{max}
hD1R
hD1R-TM1mid^{D1}
hD1R-S36W
hD1R-T42V
D
E_{max}
*
*
hD5R
hD5R-TM1mid^{D1}
hD5R-W53S
hD5R-V59T

Discussion

Growing evidence suggests that the TM1 domain is an important structural component modulating conformational and functional properties of GPCRs (Suryanarayana et al. 1992; Gether et al. 1993; Sakamoto et al. 1993; Liu et al. 1995; Hwa et al. 1997; Shi et al. 2001; Hamdan et al. 2002; Bosch et al. 2003; Han et al. 2005; Klammt et al. 2007; Li et al. 2007; Assil-Kishawi et al. 2008). The TM1 helix has also received attention for its role in GPCR dimerization, which can modify functional properties of GPCRs (Overton and Blumer 2002; Klammt et al. 2007; Casciari et al. 2008; Mancia et al. 2008). Accordingly, we recently showed that a hD5R chimera harboring the TM1 of hD1R (hD5R-TM1^{D1}) and its hD1R counterpart (hD1R-TM1^{D5}) had altered ligand binding, receptor expression and ligand-dependent activation of AC properties (D'Aoust and Tiberi 2009).

Clearly, our study demonstrates that residues located in the middle segment of the TM1 mediate those changes in D1-like receptor functionalities. Indeed, the altered B_{max} values of D1-like receptor chimeras having only a permutated TM1 middle segment suggest that this segment of the TM1 helix distinctly regulates membrane-bound receptor expression of hD1R and hD5R. Furthermore, our results suggest that the middle segment controls the orientation and interactions of TM1 and do so by exerting dominant actions that shadowed those mediated by the extracellular and intracellular ends of TM1. Interestingly, a cholesterol-binding site was found in a TM interface encompassing the TM1 to TM4 region, which may regulate the structure and stability of GPCRs (Hanson et al. 2008). Consequently, residues in TM1 and more specifically in the middle segment may modulate cholesterol binding and thus impart changes to functional properties and

conformation of docking site. Since D1R and D5R are predicted to have different cholesterol consensus motifs (Hanson et al. 2008), it is highly possible that $Trp53^{1.45}$ in hD5R makes hydrophobic interactions with a cholesterol molecule whereas $Ser36^{1.45}$ of hD1R is not involved cholesterol binding. Our results with point mutant receptors are in agreement with this hypothesis because mutating residue 1.45 in hD5R (hD5R-W53S, hD5R-TM1mid^{D1}, hD5R-TM1^{D1}) resulted in a decreased B_{max} (an index of GPCR stability), while replacement of serine for tryptophan in hD1R increased the B_{max} values of hD1R-TM1^{D5}, hD1R-TM1mid^{D5} and hD1R-S36W.

The middle segment of TM1 also controls ligand binding as altered ligand affinity and selectivity values obtained with hD1R-TM1mid^{D5} and hD5R-TM1mid^{D1} are reminiscent of what was observed when swapping the whole TM1 domain. While slight changes in agonist affinity for hD5R-TM1down^{D1} can be observed, our results suggest that the intracellular or extracellular end of TM1 do not generally modulate drug affinity. Interestingly, swapping individually the two residues of the middle segment of the TM1 helix could not identically reproduce the binding results obtained with the TM1mid mutant receptors. Thus, the two residues in the middle segment of TM1 appear to cooperatively control ligand binding. The absence of a three-dimensional model for D1-like receptors causes a lack of important structural information about the localization, orientation and intermolecular interactions of TM1 residues. Relative to the most conserved residue of TM1 ($Asn^{1.50}$), crystallographic studies of rhodopsin, β1- and β2-adrenergic and adenosine A_{2A} receptors suggest that none of the seven divergent amino acids of the TM1 in D1-like receptors face the binding pocket crevice (Palczewski et al. 2000; Cherezov et al. 2007; Rasmussen et al. 2007; Jaakola et al. 2008; Warne et al.

2008). Likewise, although 6 residues in the TM1 of dopamine D2 receptor were identified as facing the binding site crevice by a substituted-cysteine-accessibility method (SCAM), only one of those residues near the TM1 intracellular end corresponds to one of the seven variant amino acids of TM1 of D1-like receptors (Shi et al. 2001). These residues are most likely implicated in intermolecular interactions with other residues in TM1 or in other TM domains such as TM2 or TM7, thereby shaping helical packing (Suryanarayana et al. 1992; Liu et al. 1995; Hamdan et al. 2002; Abdulaev 2003; Han et al. 2005; Li et al. 2007). Importantly, middle segment residues of hD1R, serine and threonine, are hydrophilic whereas those of hD5R, tryptophan and valine, are hydrophobic. Thus, the small size and hydrophilicity of $Ser36^{1.45}$ in hD1R compared with the bigger size, hydrophobicity and aromaticity of $Trp53^{1.45}$ in hD5R suggest that those residues have completely different intermolecular interactions. In comparison, crystallized receptors possess a leucine or alanine residue located at position 1.45. Consequently, these small hydrophobic residues might not be similarly oriented and most likely do not make identical interactions than serine or tryptophan in D1-like receptors. Also, $Thr42^{1.51}$ in hD1R has an identical core structure than $Val59^{1.51}$ in hD5R but the instable hydroxyl group of threonine in the hydrophobic phospholipid bilayer must be solvated through hydrogen bonds. A small rotation of the TM1 helix would permit the interaction of residues 1.45 and 1.51 with other TM domains leading to conformational changes of the binding pocket or docking site. In agreement with our hypothesis, the multistate binding model of GPCR proposes that the binding of a functional group of a ligand leads to conformational rearrangements that could unveil new intermolecular interactions (Kobilka 2004). Therefore, the conformational remodeling of TM bundle

upon ligand binding would be affected by the TM1 of D1-like receptors. Interestingly, divergent residues of the middle segment of TM1 of D1-like receptors face opposite sides of the helix. Thus, a helical rotation bringing one residue closer to an adjacent helical domain would move away the other residue from its adjacent domain. Since we observed that mutation of residue 1.45 induces a more robust effect on ligand binding, membrane-bound receptor expression and DA efficacy than mutation of residue 1.51, our results suggest that residue 1.45 is the cornerstone of the TM1 helix. Although the role played by residue 1.51 seems less crucial, its specific intermolecular interactions fine-tunes ligand binding and DA efficacy. Notably, residue 1.51 immediately follows the conserved $Asn^{1.50}$ residue of GPCRs which is thought to make hydrogen bonds with the conserved $Asp^{2.50}$ residue in TM2 of GPCR (Liu et al. 2004). Thus, changing the spatial orientation and hydrophilicity of residue 1.51 may influence residue 1.50 and affect ligand-dependent functional properties of D1-like receptors.

Indeed, although swapping different segments of the TM1 results in slight changes of agonist-independent activity, it is fair to say that the TM1 does not contribute to the distinct constitutive activity of D1-like receptors. However, our present results indicate that residue 1.45 located in the middle segment of TM1 of hD5R plays a crucial role controlling the extent of the receptor activation following DA stimulation. The greater response elicited by DA stimulation of hD5R-W53S relative to hD5R suggests that residue 1.45 affects the overall movement of TM regions following receptor activation. In fact, it is believed that a region encompassing TM1-TM7-CT slightly moves upon photoactivation of rhodopsin (Altenbach et al. 2008). This core movement could be altered in hD5R chimeras (hD5R-W53S, hD5R-TM1mid^{D1}, hD5R-TM1^{D1}), thus

affecting DA-mediated signal transduction and ligand binding. Interestingly, the CT and more specifically the helix 8 region has been shown to control DA-mediated activation of D1-like receptors (Tumova et al. 2003; Tumova et al. 2004). Taken together, these studies suggest that the TM1 could indeed modulate the agonist-mediated movement of TM7 and CT in hD1R and hD5R. Furthermore, the slight increase in E_{max} of hD5R-TM1down^{D1} compared with hD5R could also reflect a change in the proximity between TM1, TM7 and CT. Interestingly, the crystal structure of ligand-free opsin receptor shows a lost interaction between Tyr306 of TM7 (NPxxY motif) and Phe313 of H8 compared with rhodopsin (Park et al. 2008). Biochemical studies of muscarinic receptors also showed that the distance between TM1 and H8 was increase following agonists exposure (Li et al. 2007). Thus, distinct TM1-TM7-H8 interhelical interactions between hD1R and hD5R could affect the activation-mediated movement of this GPCR region and lead to altered responses.

In conclusion, our study sheds light on the roles in ligand binding and efficacy played by critical residues in TM1 of D1-like receptors. We demonstrated that two residues located in the middle segment of the TM1 of D1-like receptors are crucial for subtype-specific ligand binding, selectivity and DA-mediated maximal stimulation of AC activity. Precisely, residue 1.45 probably acts as the cornerstone of TM1 helix, controlling the subtype-specific orientation or helical packing of the TM1 helix. In addition, residue 1.51 could make different intermolecular interactions in D1R and D5R, thus affecting the structure of the ligand binding pocket. A rotation of TM1 helix could result in an increased interaction with TM7, thereby affecting the movement of CT

following receptor activation. A crystal structure of D1-like receptors is critically needed to support these data and to promote the development of a subtype-specific drug.

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Table S1. Sequences of oligonucleotide primers used. P1 and P2 were used to amplify product A; P3 and P4 were used to amplify product B; P5 and P6 were used to amplify the overlap product of A and B. The pCMV5 vector containing hD1R DNA sequence was used as a template for the PCR of hD1R chimeras and pCMV5 vector containing hD5R was used for hD5R chimeras.

Construct	Oligonucleotide primers (5'-3')
	P2: GATGAGCAGCGTCAGTAGACAGGC
	P3: CTGACGCTGCTCATCATATCCACG
	P4: GTCTCAGCCAGGGAAGTG
	P5: CAAAATGTCGTAATAACCCCG
	P6: CAGAGGTTGAGGATGGAT
	P1: CCATGGTGATGCGGTTTT
hD1R-TM1mid^{D5}	P1: CCATGGTGATGCGGTTTT
	P2: GTTCCCCAGGAGCGTCCACAGGAT
	P3: ACGCTCCTGGGGAACGTGTTGGTC
	P4: GTCTCAGCCAGGGAAGTG
	P5: CAAAATGTCGTAATAACCCCG
	P6: CAGAGGTTGAGGATGGAT
hD1R-TM1down^{D5}	P1: CCATGGTGATGCGGTTTT
	P2: GACAATTGCAGCACAGACCAG
	P3: TGTGCTGCAATTGTCAGGTTC
	P4: GTCTCAGCCAGGGAAGTG
	P5: CAAAATGTCGTAATAACCCCG
	P6: CAGAGGTTGAGGATGGAT
hD1R-S36W	P1: CCATGGTGATGCGGTTTT
	P2: GTTCCCCAGGAGCGTCCACAGGAT
	P3: ACGCTCCTGGGGAACACGCTGGT
	P4: GTCTCAGCCAGGGAAGTG
	P5: CAAAATGTCGTAATAACCCCG
	P6: CAGAGGTTGAGGATGGAT
hD1R-T42V	P1: CCATGGTGATGCGGTTTT
	P2: TTCCCCAGGAGCGTGGACA
	P3: ACGCTCCTGGGGAACGTGTTGGTC
	P4: GTCTCAGCCAGGGAAGTG
	P5: CAAAATGTCGTAATAACCCCG
	P6: CAGAGGTTGAGGATGGAT

Table S1. Continued.

Construct	Oligonucleotide primers (5'-3')
hD5R-TM1up^{D1}	P1: CCATGGTGATGCGGTTTT
	P2: GATGAGTAGGGACAGAAAGCATGCGGT
	P3: CTGTCCCTACTCATCCTCTGGACC
	P4: AGTTAAGGATGAAGA
	P5: CAAAATGTCGTAATAACCCCG
	P6: TCACGATCATGATGGCAA
hD5R-TM1mid^{D1}	P1: CCATGGTGATGCGGTTTT
	P2: GTTGCCCAGCAGGGTGGAGATGAT
	P3: ACCCTGCTGGGCAACACGGTGGTG
	P4: AGTTAAGGATGAAGA
	P5: CAAAATGTCGTAATAACCCCG
	P6: TCACGATCATGATGGCAA
hD5R-TM1down^{D1}	P1: CCATGGTGATGCGGTTTT
	P2: GGCCGCGCACACCAGCACGTT
	P3: CTGGTGTGCGCGGCCGTCATACGG
	P4: AGTTAAGGATGAAGA
	P5: CAAAATGTCGTAATAACCCCG
	P6: TCACGATCATGATGGCAA
hD5R-W53S	P1: CCATGGTGATGCGGTTTT
	P2: GTTGCCCAGCAGGGTGGAGATGAT
	P3: ACCCTGCTGGGCAACGTG
	P4: AGTTAAGGATGAAGA
	P5: CAAAATGTCGTAATAACCCCG
	P6: TCACGATCATGATGGCAA
hD5R-V59T	P1: CCATGGTGATGCGGTTTT
	P2: GTTGCCCAGCAGGGTCCA
	P3: ACCCTGCTGGGCAACACGCTGGT
	P4: AGTTAAGGATGAAGA
	P5: CAAAATGTCGTAATAACCCCG
	P6: TCACGATCATGATGGCAA

Figure S1. Alignment of G protein-coupled receptor primary sequences. Primary sequences of hD1R and hD5R were aligned with those of crystallized GPCRs (human β2-adrenergic receptor, hb2AR; turkey β1-adrenergic receptor, tb1AR; human adenosine A_{2A} receptor, hA2AR; rhodopsin) using ClustalX (www.clustal.org). Bold residues are defined as forming transmembrane domain 1 (TM1). Residue 1.50 (Asn, N) is the most conserved residue among these related GPCRs. IL1, intracellular loop 1, EL1, extracellular loop 1.

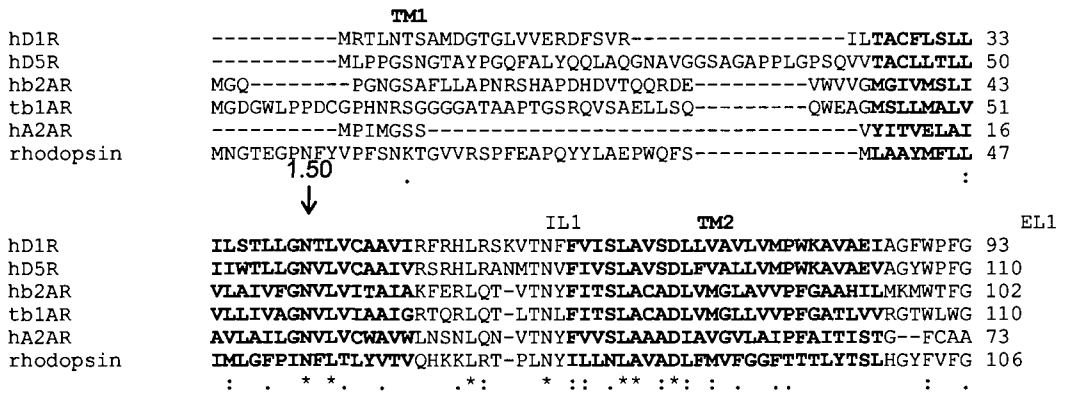
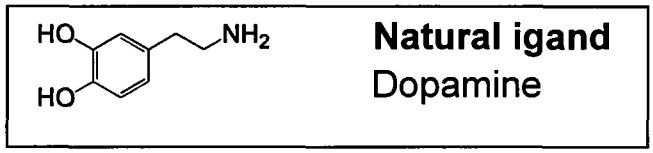
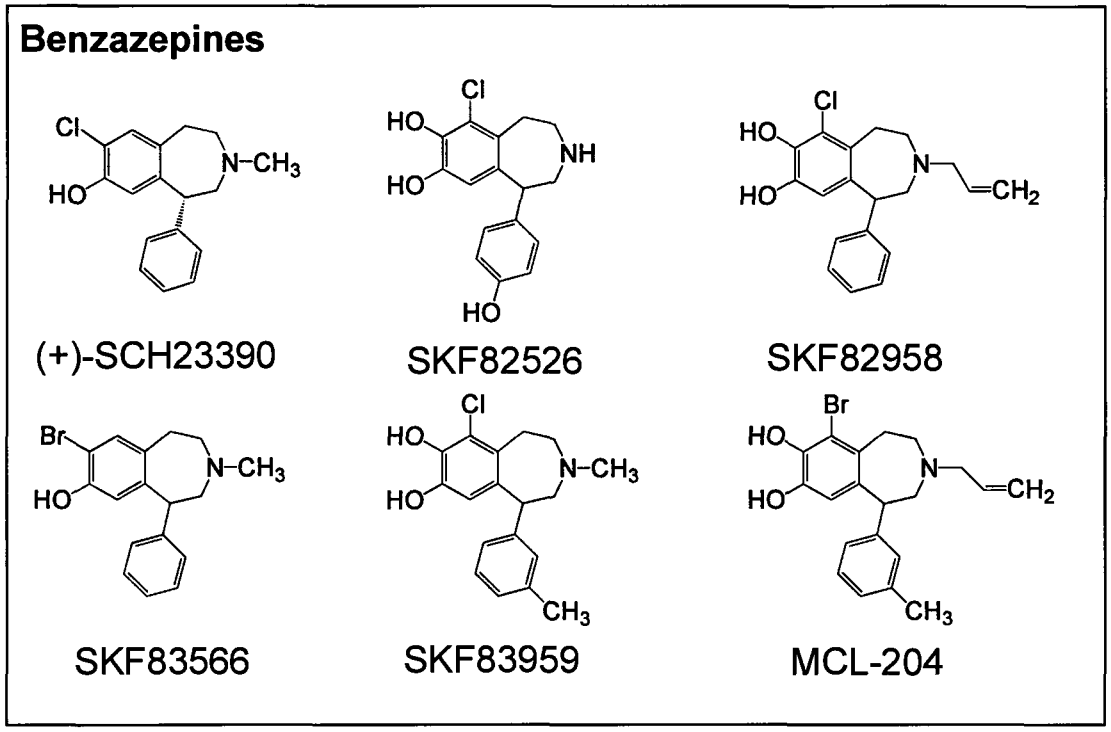
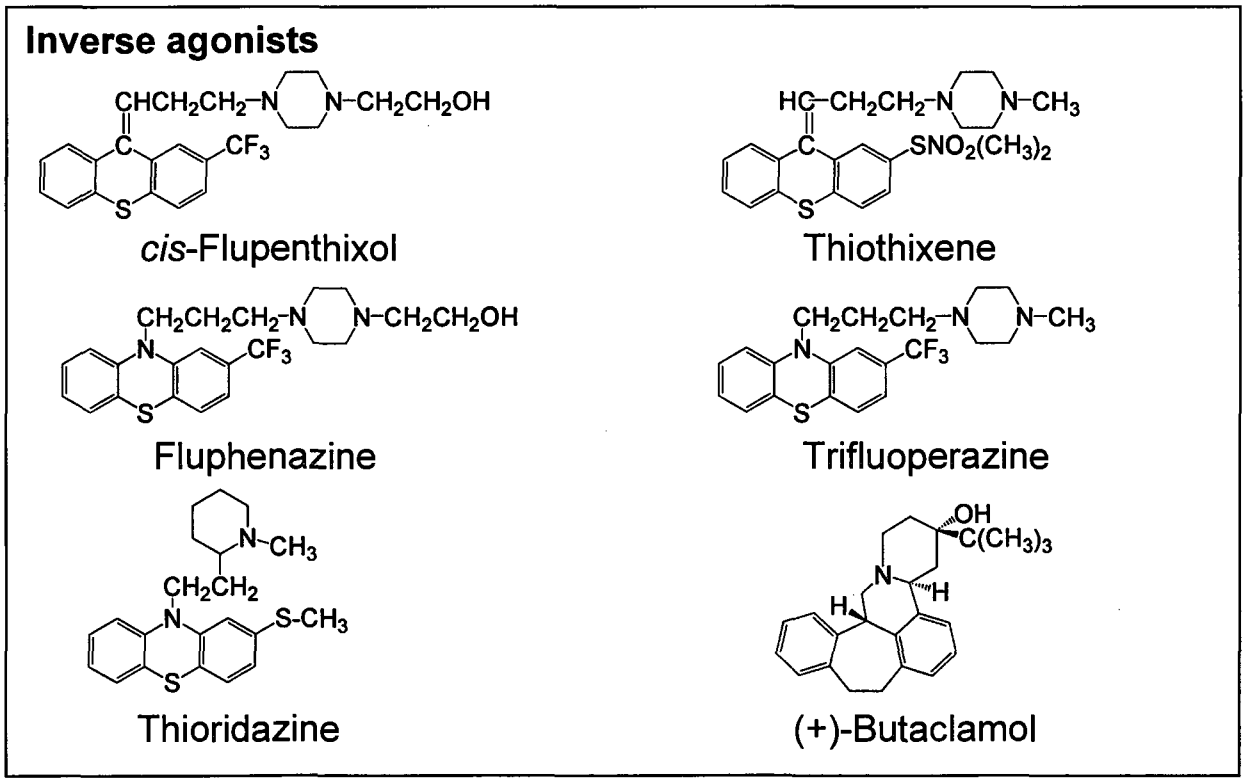


Figure S2. Chemical structures of dopaminergic ligands.







Chapter 4. General Discussion

Although D1-like dopaminergic receptors are well distributed in the human body and associated with many pathophysiological conditions like Parkinson's disease and schizophrenia, studies with D1R and D5R knockout mice have led to equivocal – and somewhat controversial – conclusions about their roles in locomotion, drug addiction and hypertension. Since no currently known ligands are able to discriminate D1-like receptors, a pharmacological characterization of the physiological roles of D1R and D5R is not possible. Structurally, D1-like receptors are highly identical, sharing over 60% identity in their primary sequences. Furthermore, while TM residues shaping the ligand binding pocket are 80% identical between D1R and D5R, D1-like receptors display distinct ligand binding and G protein coupling properties. Thus, to provide additional insight into their distinct functional properties that would help model a subtype-selective drug, I investigated the role of receptor structures with low degree of identity between D1-like receptors.

4.1 The amino terminal cassette of D1-like receptors

I assessed whether the amino terminal cassette of D1-like receptors, consisting of the amino terminal (NT) and first transmembrane (TM1) regions, shaped their subtype-specific functional properties. The low degree of identity between the amino terminal cassette of D1R and D5R hinted that this region could control the affinity and selectivity of dopaminergic ligands and contribute to the distinct G protein coupling properties. Therefore, using a chimerical approach, I swapped the amino terminal cassette between human D1R and D5R and probed the functional consequences of this permutation. An increased ligand affinity for the hD5R full chimera (hD5R-NTTM1^{D1}) and a decreased ligand affinity for the hD1R full chimera (hD1R-NTTM1^{D5}) were generally observed for

all synthetic ligands tested. This trend could be recapitulated with chimeras having only a swapped TM1 region (hD1R-TM1^{D5} and hD5R-TM1^{D1}). Computation of the selectivity ratios of dopaminergic ligands suggested that the TM1 controls synthetic ligand selectivity for D1-like receptors. Indeed, synthetic ligands with higher affinity for hD5R over hD1R displayed an increased selectivity for the hD5R-TM1^{D1} chimera versus hD1R-TM1^{D5} while synthetic drugs with higher affinity for hD1R almost completely lost their selectivity with TM1-swapped chimeras.

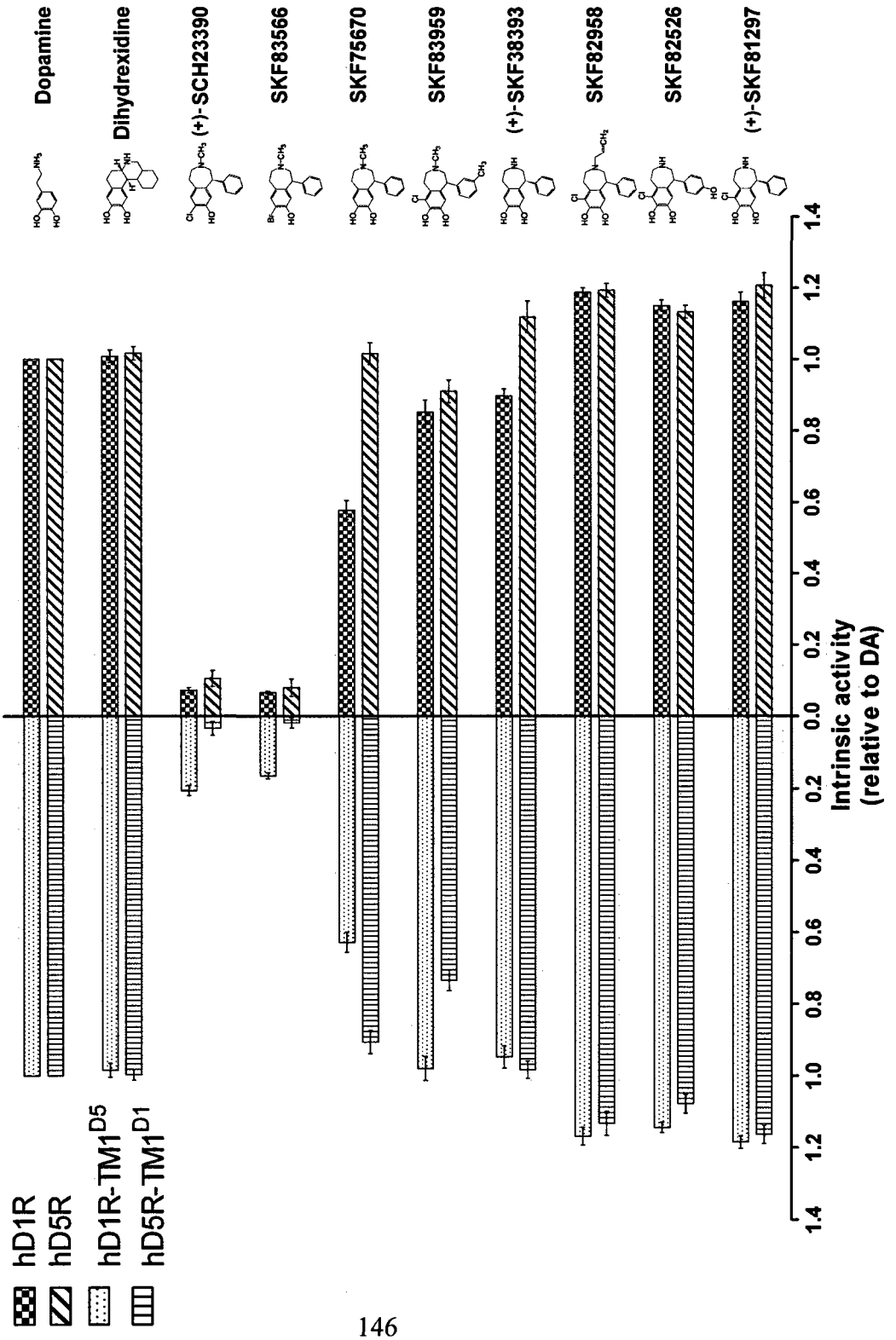
In addition, cells expressing hD1R-NTTM1^{D5} and hD5R-NTTM1^{D1} had greater DA-mediated activation of AC (E_{max}) than wild type hD1R and hD5R transfected cells, respectively. While cells expressing hD1R-TM1^{D5} responded to DA in a similar fashion than hD1R transfected cells, hD1R-NT^{D5} expressing cells had a greater E_{max} than hD1R, reminiscent of that observed with hD1R-NTTM1^{D5}. In stark contrast, cells expressing hD5R-TM1^{D1} but not hD5R-NT^{D1} transfected cells had an increased E_{max} suggesting that the amino terminal cassette of D1-like receptors regulates subtype-specific conformational rearrangements following DA stimulation leading to distinct G protein coupling properties. For instance, different helical packing and interactions in the extracellular domains could contribute to these effects. More importantly, TM1 sequences may underlie the drug properties of the classical D1-like benzazepine ligands.

4.2 Chemical structures and receptor activation: is there a link?

I also characterized the relationship between the chemical structures of benzazepine ligands and their efficacy at D1-like receptors (summarized in Fig. IX). SCH23390 is a classical D1-like benzazepine used in *in vitro* and *in vivo* studies. Although this compound displays a low agonistic activity in HEK293 cells expressing D1-like receptors

(Tiberi and Caron 1994; Sugamori et al. 1998) (shown in Chapter 2), it acts as an antagonist in primary cell cultures (Itoh et al. 1984; O'Boyle et al. 1989; Shah et al. 1995; Mottola et al. 1996). Structurally similar to SCH23390, SKF83566 also displayed a low agonistic activity in my study with hD1R or hD5R transfected cells. SCH23390 and SKF83566 possess an halogen atom instead of a hydroxyl group on the *meta* site of their catechol ring. Since both hydroxyl groups of the catechol ring are thought to bind serine residues in TM5 of bioamine receptors, replacing one hydroxyl by an halogen would likely change how drugs interact with binding pocket residues and promote a conformational remodeling that underlie the extent of E_{max}. Accordingly, replacing the halogen of SCH23390 and SKF83566 by a hydroxyl group should enhance the E_{max} of the compound. Indeed, SKF75670 had an increased E_{max}, although it did not behave as a full agonist in hD1R expressing cells. The partial agonistic activity of SKF75670 was also observed in rat striatal tissue, which express mostly D1R (Itoh et al. 1984; Andersen et al. 1987; O'Boyle et al. 1989; Arnt et al. 1992). Interestingly, these three compounds share a methyl group on the nitrogen heterocycle and removal of this group enhanced the activity of the benzazepine SKF38393 compared with SKF75670. Studies using rat striatal tissue also reported a similar difference in activity between SKF75670 and SKF38393 (Andersen et al. 1987; O'Boyle et al. 1989; Arnt et al. 1992). Therefore, the *meta*-hydroxyl group of the catechol ring and the nitrogen atom on the heterocycle of benzazepines are two crucial functional groups controlling the efficacy of synthetic agonists on D1-like receptors. Additionally, three compounds, SKF81297, SKF82526 and SKF82958, exhibit a higher efficacy than DA in D1-like receptor expressing cells. These drugs share a halogen atom on the *ortho* position of the catechol ring.

Figure IX. Relationship between the intrinsic activity of benzazepine ligands and their chemical structures. The intrinsic activities of dopaminergic ligands relative to dopamine calculated in Fig.9 *C* and *D* of Chapter 2 were plotted on the right side for wild type receptors and compared with those of TM1-swapped receptors (left side). Chemical structures of ligands are also shown.



Interestingly, SKF83959, which has this halogen on the *ortho* site but also a methyl on the nitrogen of the heterocycle, produces a higher E_{max} than SKF75670 in hD1R transfected cells. Potentially, the halogen on the *ortho* site makes new contacts with the receptor thereby increasing its activation.

The significant differences in SCH23390 efficacy observed between primary cell cultures and transfected HEK293 cells could be explained by the activation of other receptors in primary cultures like serotonin receptors (Ohlstein and Berkowitz 1985; Millan et al. 2001) or by unique intracellular signalling features (e.g. desensitization machinery, dimerization) in HEK293 cells versus striatal tissue. In addition, the efficacy of D1 agonists reported in various studies largely depends on the cellular system used, *i.e.* rat, monkey or human background and type of cells. For instance, SKF38393 and SKF81297 increased cAMP to higher levels in rat astrocytes than monkey or human astrocytes (Vermeulen et al. 1994) while no differences in cAMP levels were observed for SKF38393 in human or monkey striatum (Watts et al. 1993). Our experimental design, using HEK293 cells, provides a better way to compare the efficacy of ligands and to assess the relationship between the chemical structure and the efficacy of a ligand since no other receptors are expressed at levels that would affect ligand activity. Therefore, my data could become useful in medicinal chemistry for the rational design of drug controlling the activation of D1-like receptors.

According to the results obtained with chimeras, it appears that the TM1 domain of D1-like receptors also modulates sub-maximal agonistic efficacy. In fact, cells expressing hD5R-TM1^{D1} responded less to partial agonists (SCH23390, SKF83566, SKF75670 and SKF83959) while cells expressing hD1R-TM1^{D5} had increased activity following the

stimulation by those drugs. These results on the signalling properties of TM1-swapped chimeras suggest that the TM1 helix has opposite functions in controlling the sub-maximal activity of ligands in cells expressing D1-like receptors. As proposed for other GPCR, the existence of intermediate conformational states with specific functionalities could explain my results. Hypothetically, these intermediate states in D1-like receptors could be modulated by residues in the TM1 helix.

4.3 Cooperation of residues within the TM1 helix

Characterization of mutant receptors with multiple point mutations in the TM1 clearly established that the functional and structural significance of the TM1 of D1-like receptors was controlled by its middle segment. Ligand binding and DA-dependent activation of AC data with chimeras having only the middle segment of TM1 swapped between hD1R and hD5R (hD1R-TM1mid^{D5} and hD5R-TM1mid ^{D1}) were reminiscent of those obtained with hD1R-TM1^{D5} and hD5R-TM1^{D1}. Consequently, the two residues in the middle segment were individually swapped, thus generating four additional chimeras. Despite the fact that swapping a tryptophan for a serine residue in hD5R (hD5R-W53S) resulted in an increased DA-mediated E_{max} and an increased ligand affinity, swapping the other residue of the middle segment (hD5R-V59T) also caused an increased ligand affinity. This cooperation of both residues was even more obvious with hD1R chimeras since the decreased ligand affinity observed with hD1R-TM1mid^{D5} was not seen with either hD1R-S36W or hD1R-T42V. Interestingly, as depicted in figure X, corresponding residues in crystallized structures face opposite sides of the helix (blue and green, from β2AR). Therefore, those residues do not physically interact with each other and a rotation of the helix bringing a residue closer to another TM would move away the other residue

Figure X. Orientation of residues in the TM1 helix. The three-dimensional structure of helix 1 of human β2-adrenergic receptor-T4 lyzozyme fusion protein was taken from the Protein Data Bank (accession number: 2HR1). The residue corresponding to serine (hD1R) or tryptophan (hD5R) in β2AR-T4L is shown in blue, while the residue in green shows the position of threonine (hD1R) or valine (hD5R). The residues face opposite sides of the helix and do not interact with each other.

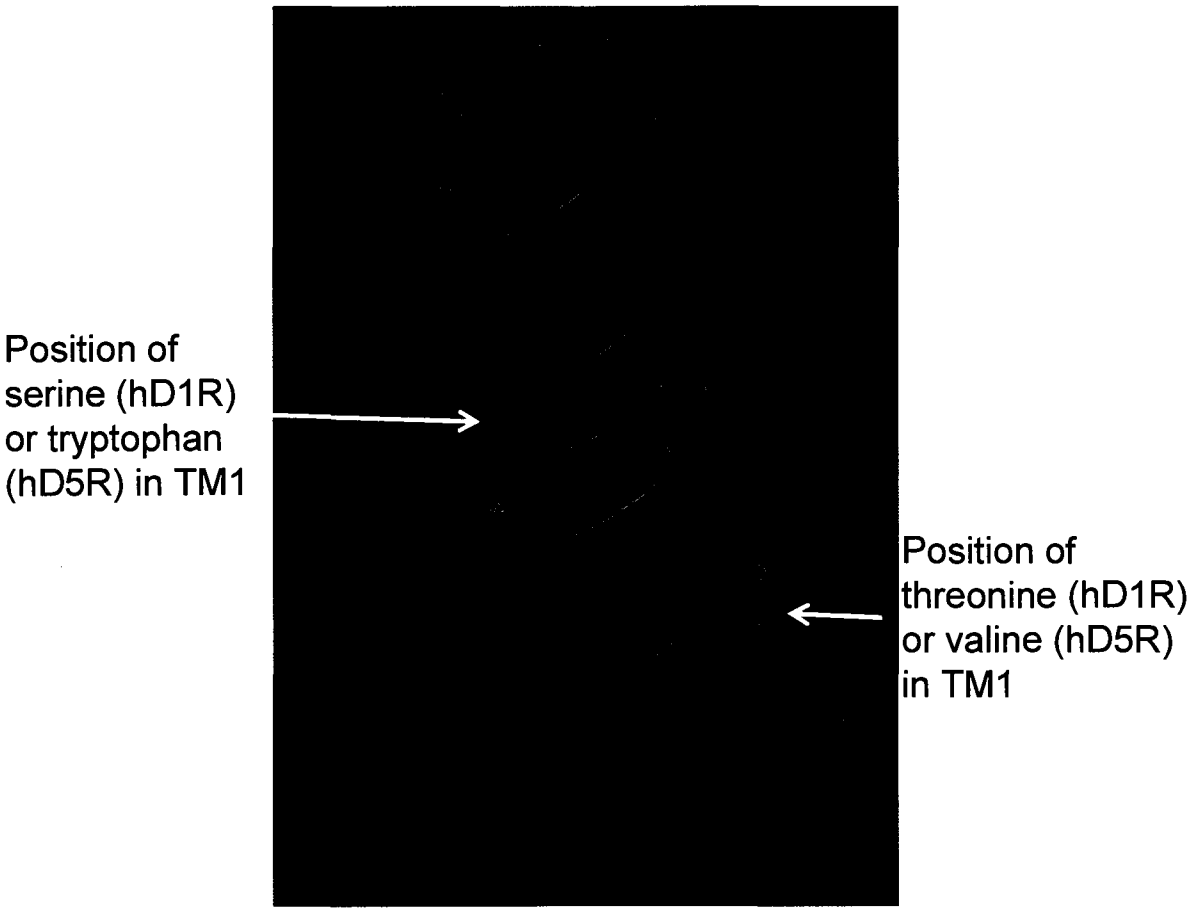
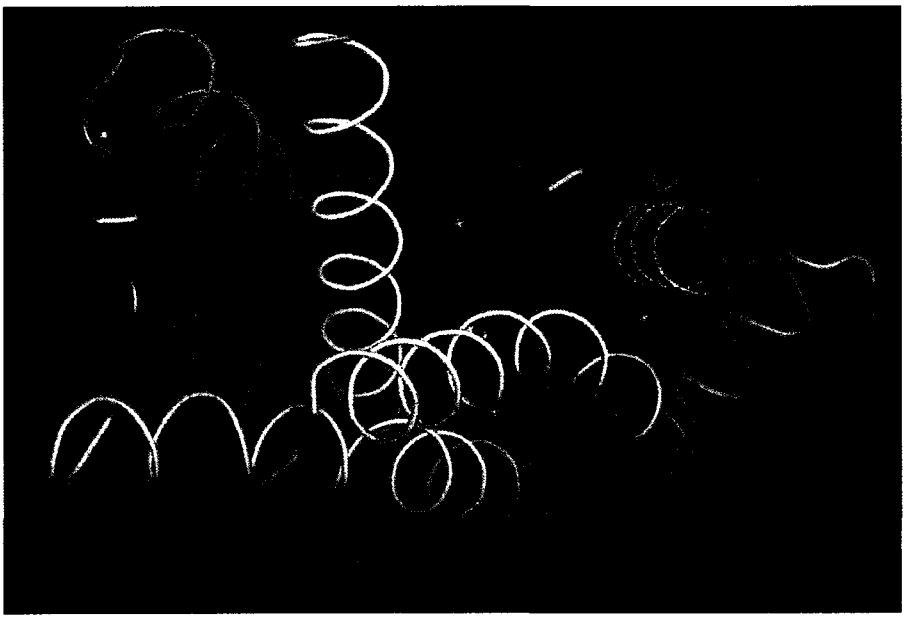
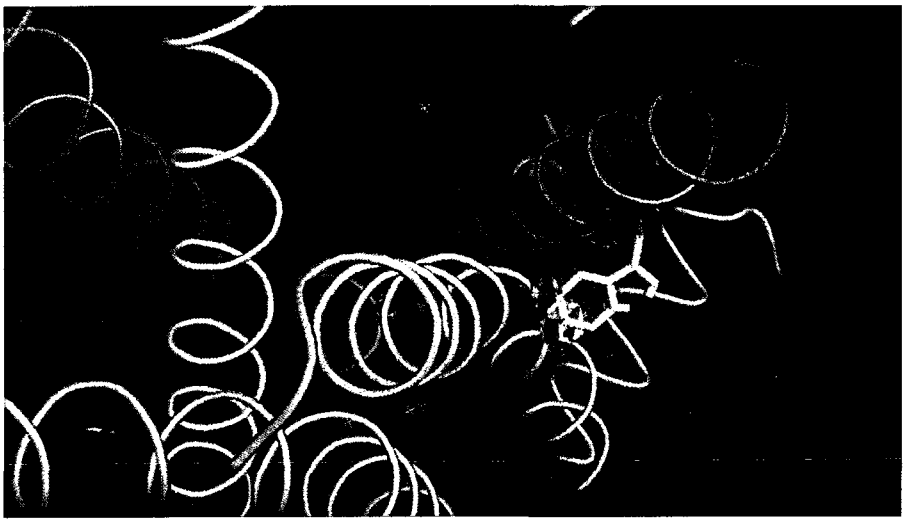


Figure XI. Possible interaction of TM1 middle segment residues of D1-like receptors with TM7. The three-dimensional structure of human β2-adrenergic receptor-T4 lyzozyme fusion protein was obtained from the Protein Data Bank (accession number: 2HR1). *A*, TM1 (red helix) middle segment residues (residue 1.45 in blue, 1.51 in green) do not interact with TM2 or TM7 in human β 2AR-T4L. *B*, Mutating alanine$^{1.45}$ (from β 2AR-T4L) to tryptophan (in hD5R) creates a possible connection between TM1 and TM7.

A.



B.



from its adjacent TM domain. Additionally, both residues of the middle segment do not appear to interact with adjacent TM domains in β2AR (Fig XI*A*). However, the unique residues in the TM1 middle segment of D1-like receptors could make contacts with adjacent TM domains, as shown by mutating the alanine$^{1.45}$ of β2AR with a tryptophan (as in hD5R) (Fig. XI*B*). Thus, a rotation or a different packing of the helix induced by the mutation of a residue will also influence the intermolecular interactions of the other residue. The hypothesis of a helical rotation finds supporting arguments from a multistep binding mechanism of agonists to various TM residues (next section). Conversely, crystallographic and biophysical studies suggest that TM1 helix does not move upon receptor activation. However, differences in residue 1.45 between D1-like receptors (Trp and Ser) and crystallized receptors (Ala and Leu) suggest that TM1 of D1R and D5R could undergo a distinct activation-mediated conformational rearrangement.

4.4 Sequential binding of ligands

Pharmacological studies led to the development of ligand binding and activation models. The most extensively studied one is the ternary complex model, stating that there was an intimate relationship between ligands, receptors and another component (later shown to be G proteins) in attributing ligand affinity and efficacy (De Lean et al. 1980). Then, the ternary complex model was extended to introduce the isomerization properties of GPCRs between the inactive and active states (Samama et al. 1993). Finally, a cubic ternary complex model was proposed to include all the possible coupling of the ligand/ receptor/G protein (Weiss et al. 1996). In light of our results, another model seems to be better suited. The mechanistic multistate binding model of GPCR activation was developed with the use of ligands with similar chemical structures. In an elegant study,

Liapakis *et al.* showed that essential functional groups (OH, CH_3) of ligands synergistically induced a maximal response in cells expressing β2AR (Liapakis et al. 2004). It was therefore inferred that the binding of a functional group caused a rearrangement of the binding pocket, creating an intermediate conformation of β2AR. This TM rearrangement allowed the binding of another functional group to a newly exposed residue, thus leading to the full activation of the receptor. Interestingly, additional intermediate conformational states of β2AR were proposed based on the movement of TM6 upon ligand stimulation (Swaminath et al. 2004). Therefore, a multistep binding process was defined as a mechanistic model of ligand binding (Kobilka 2004). This multistep binding model is supported by crystallographic studies showing that the alleged ligand binding pocket in β1AR and β2AR, as determined using synthetic molecules, is too wide to allow the simultaneous binding of the functional groups of epinephrine to TM3 and TM5 (Rosenbaum et al. 2007; Warne et al. 2008). Since both residues in the middle segment of TM1 of D1-like receptors appear to change ligand affinity, this multistep binding model could explain my results. Indeed, if the binding of a ligand depends on the orientation of a specific residue or a subset of residues in TM1, then the ligand-mediated reorientation of TM domains could unmask another residue of TM1 and consequently lead to a cooperation of TM1 residues.

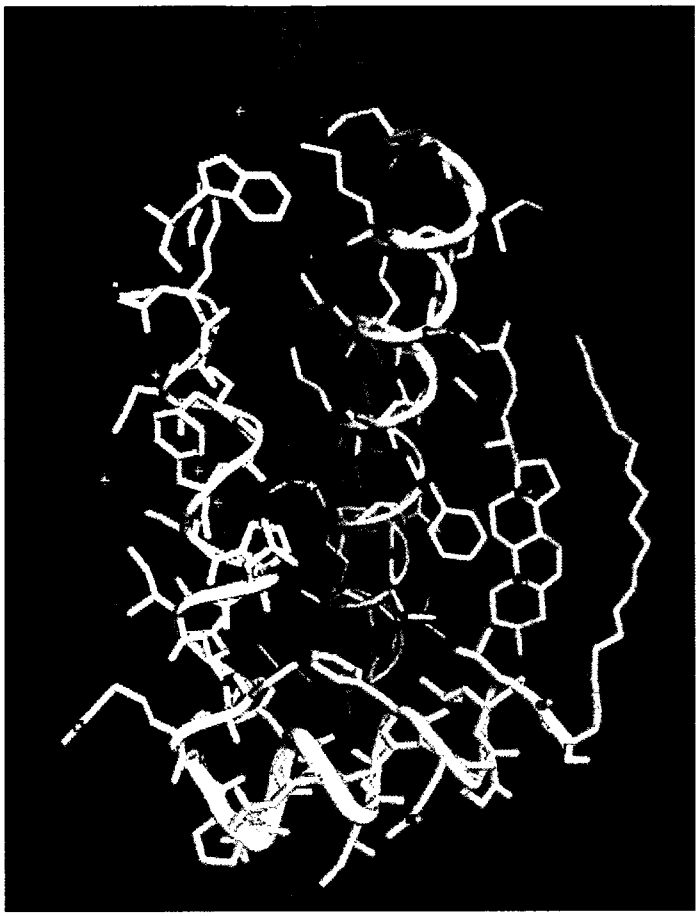
Another study also suggested that ligands with different structures and efficacies bind distinct residues of the binding pocket (Swaminath et al. 2005). Similar conclusions could be drawn from mutagenesis studies showing that the replacement of serine residues in TM5 of D1R caused more changes in the affinity of DA than SCH23390 (Pollock et al. 1992; Tomic et al. 1993). In my studies, the change in DA affinity observed with hD1R-

$TM1^{D5}$ is different than the changes observed with synthetic ligands, *i.e.* gain of affinity of DA versus loss of affinity of synthetic ligands. With respect to those differences, the binding of DA to D1R would require different conformational rearrangements than the binding of benzazepine ligands. Furthermore, differences in the chemical structure of benzazepine ligands would also require distinct conformational changes, which could lead to differences in desensitization and efficacy properties.

4.5 A possible cholesterol binding residue

Chimeras with mutated residues in the TM1 showed signs of altered membrane expression (an index of GPCR stability). The hD5R-NTTM1^{D1}, hD5R-TM1^{D1}, hD5R-TM1mid^{D1} and hD5R-W53S chimeras were all less expressed than hD5R while hD1R-TM1^{D5}, hD1R-TM1mid^{D5} and hD1R-S36W were more expressed than hD1R. The recent finding of cholesterol binding interface outside the ligand binding pocket crevice and formed by TM1, TM2, TM3 and TM4 suggest that cholesterol may influence receptor structure and stability (Hanson et al. 2008). Therefore, swapping the TM1 of hD1R and hD5R, which possess different cholesterol consensus motif, could influence the binding of cholesterol, thus affecting receptor expression at plasma membrane and stability. Importantly, the absence of Trp residue in TM1 of hD5R-W53S, hD5R-TM1mid^{D1} and hD5R-TM1^{D1} might result in a decrease proximity between TM1 and cholesterol because of a lost aromatic interaction, thus leading to decreased stability and receptor expression, as suggested by the reduced B_{max} values. Reciprocally, hD1R-S36W, hD1R-TM1mid^{D5} and hD1R-TM1^{D5}, which have acquired Trp in TM1, display an increased B_{max} value. These changes were obtained under conditions maximizing receptor expression in HEK293 cells. It remains to be determined, in more physiological conditions such as a

Figure XII. Proximity of cholesterol and cysteine-linked palmitoylation in GPCRs. Cholesterol molecules and cysteine-linked palmitoylation at the end of H8 (white) sit close to TM1 (red).



knock-in approach, whether tryptophan in TM1 does affect D1-like receptor expression.

Interestingly, cholesterol molecules are close to TM1 (in β2AR), which is also in close proximity with the cysteine-attached palmitate molecule in H8 (Fig. XII). Since H8 of D1-like receptors is crucial for regulation of DA binding conformation and DA-mediated stimulation of AC activity (Tumova et al. 2004), changes in the TM1-cholesterol interaction could influence the conformational rearrangement of H8 following activation, leading to altered responses as observed for hD5R chimeras.

4.6 Unsolved questions

This project clearly established that the amino terminal cassette of D1-like receptors contributes to their distinct ligand binding and G protein-coupling properties. In addition, divergent residues in the middle segment of TM1 of D1-like receptors control their subtype-specific ligand binding profile and DA-mediated AC activation. Nevertheless, some questions remain unresolved.

For instance, would the mutation of Ser36 in hD1R for alanine, a structurally similar residue without the hydroxyl group, have a similar impact as changing serine for tryptophan? In other words, is the size of the amino acid or its interactions with other amino acids (hydrogen bond, hydrophobic interaction) the important factor? Similarly, changing Trp53 of hD5R for phenylalanine or tyrosine would tell us the importance of size and aromaticity at this position.

In addition, what is the role of the middle segment of TM1 in mediating sub-maximal responses? Since the intrinsic activity of partial agonists (and inverse agonists) has not been probed for multiple point mutant chimeras, I cannot rule out the possibility that the intracellular or extracellular end of TM1 partake in the activation properties of

such ligands.

Another possible explanation for my results comes from receptor dimerization, which affects ligand binding, signalling properties and cell-surface expression. Since a proposed GPCR dimerization interface includes the TM1 region, swapping of the TM1 could affect dimerization of D1-like receptors, thus leading to altered functional properties. A TM1-dependent dimerization of D1-like receptors remains to be tested under our conditions.

Crystallographic studies of β-adrenergic receptors do not provide information about the interactions of the NT region with EL or TM domains since it was either truncated prior to crystallization or unresolved. Therefore, the role of the NT region in shaping the structural conformation of bioamine GPCR is not known. However, the effects observed in my studies with NT-swapped receptors indicate that the NT of D1-like receptors affects signal transduction and, to a lesser extent, ligand binding.

Finally, a recent study showed the position of water molecules within the transmembrane bundle of GPCRs based on available crystal structures (Angel et al. 2009). Interestingly, a water molecule sits next to TM1, making hydrogen bonds with $Asn^{1.50}$. As water molecules may be important for receptor functionality, it can be argued that swapping TM1 of D1-like receptors caused changes in water organization within the binding-pocket thus leading to altered affinity and efficacy as observed in my project. However, such effects remain to be elucidated.

4.7 Closing remarks

Overall, my research project brings new insights into the role of structural components (NT and TM1) of D1-like receptors that could be relevant for other GPCRs.

It also provides additional information about the relationship between the chemical structure of benzazepine ligands and their affinity and efficacy that could be used for drug design. Importantly, our data reveal that the TM1 shapes the selectivity of ligands, a fundamental issue for developing subtype-selective compounds for D1-like receptors. Since the regulation of the D1-like receptor family has a significant impact on human physiology, the development of a discriminating compound would lead to a greater understanding of the role of D1R and D5R and could result in better therapeutic treatments.

Chapter 5. References

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Chapter 6. Appendices

Schematic representations of the PCR approaches and cloning methods used to construct chimerical receptors are depicted in appendices A1, A2, A3 and A4. Two PCR products, A and B, are amplified using primer pair P1-P2 and P3-P4, respectively. A 15-18 nucleotide overlapping region between A and B allows the amplification of the final product with primers P5-P6. Appropriate restriction enzymes digest the PCR product and a vector containing either hD1R or hD5R. Linearized pCMV5 (*EcoR*I-*Bsm*I) containing hD5R was used to construct all hD5R chimeras (Fig. A1). Linearized pCMV5 (*EcoR*I-*Bgl*II) containing hD1R was used to construct hD1R-TM1up^{D5}, hD1R-TM1mid^{D5} and hD1R-TM1down^{D5} (Fig. A2). A three-piece ligation method using linearized pCMV5 (*EcoR*I-*Xba*I) containing hD1R and a hD1R fragment (*Bgl*II-*Xba*I) was employed to construct hD1R-TM1^{D5} (Fig. A3). Digested PCR product of hD1R-NTTM1^{D5}, hD1R-NT^{D5}, hD1R-S36W and hD1R-T42V was first subcloned into linearized pSK^{+} (*EcoR*I-*Bgl*II) containing hD1R. The full coding sequence of these hD1R chimeras was inserted into linearized pCMV5 (*EcoR*I-*Xba*I) (Fig. A4).

Figure A1. Schematic representation of the overlapping PCR-approach technique and cloning method used to construct all hD5R chimeras. Construction of hD5R-NTTM1D1, hD5R-NTD1, hD5R-TM1^{D1}, hD5R-TM1up^{D1}, hD5R-TM1mid^{D1}, hD5R-TM1down^{D1}, hD5R-W53S and hD5R-V59T.

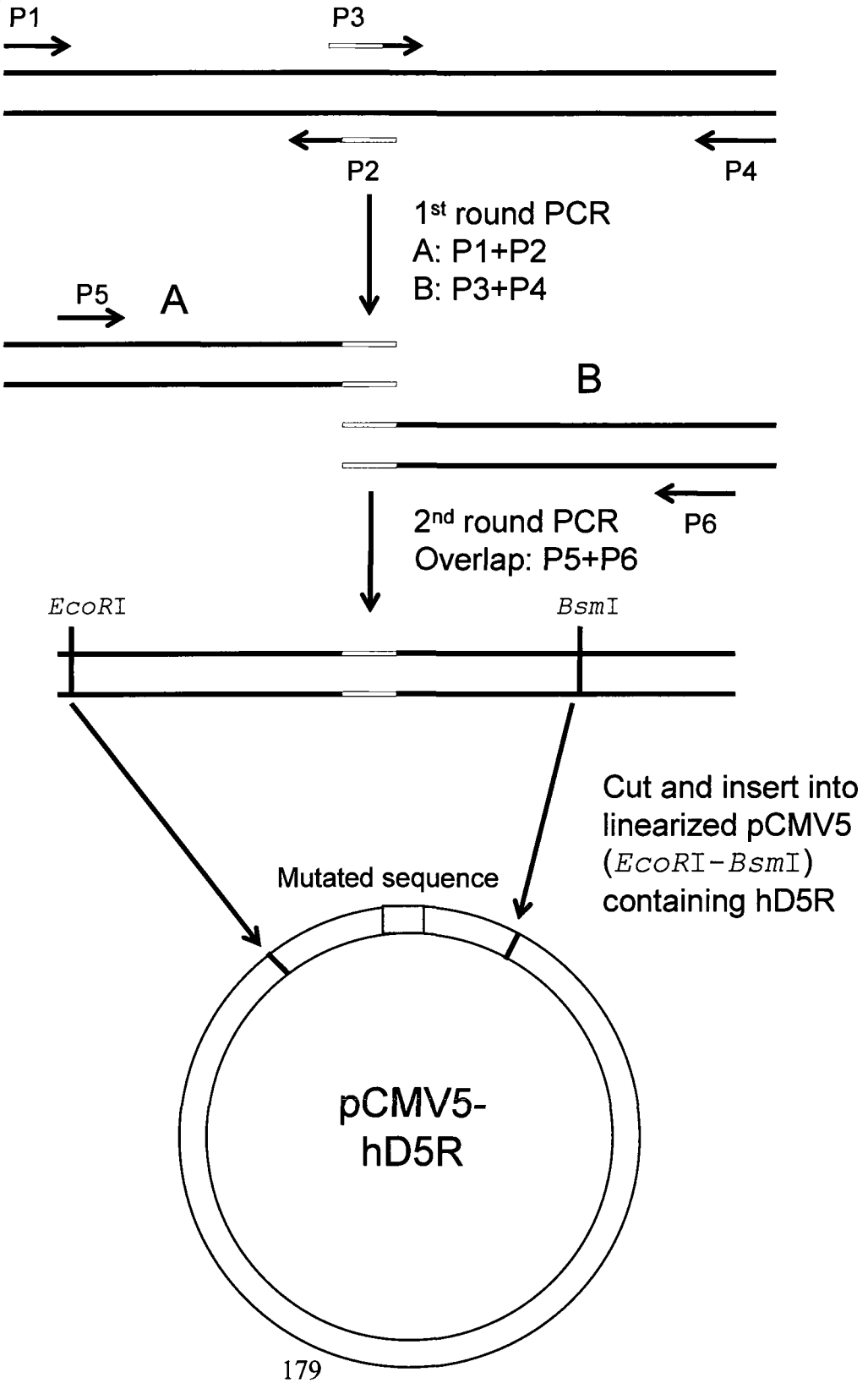

Figure A2. Schematic representation of the overlapping PCR-approach technique and cloning method used to construct multiple point mutant hD1R. Construction of hD1R-TM1up^{D5}, hD1R-TM1mid^{D5} and hD1R-TM1down^{D5}.

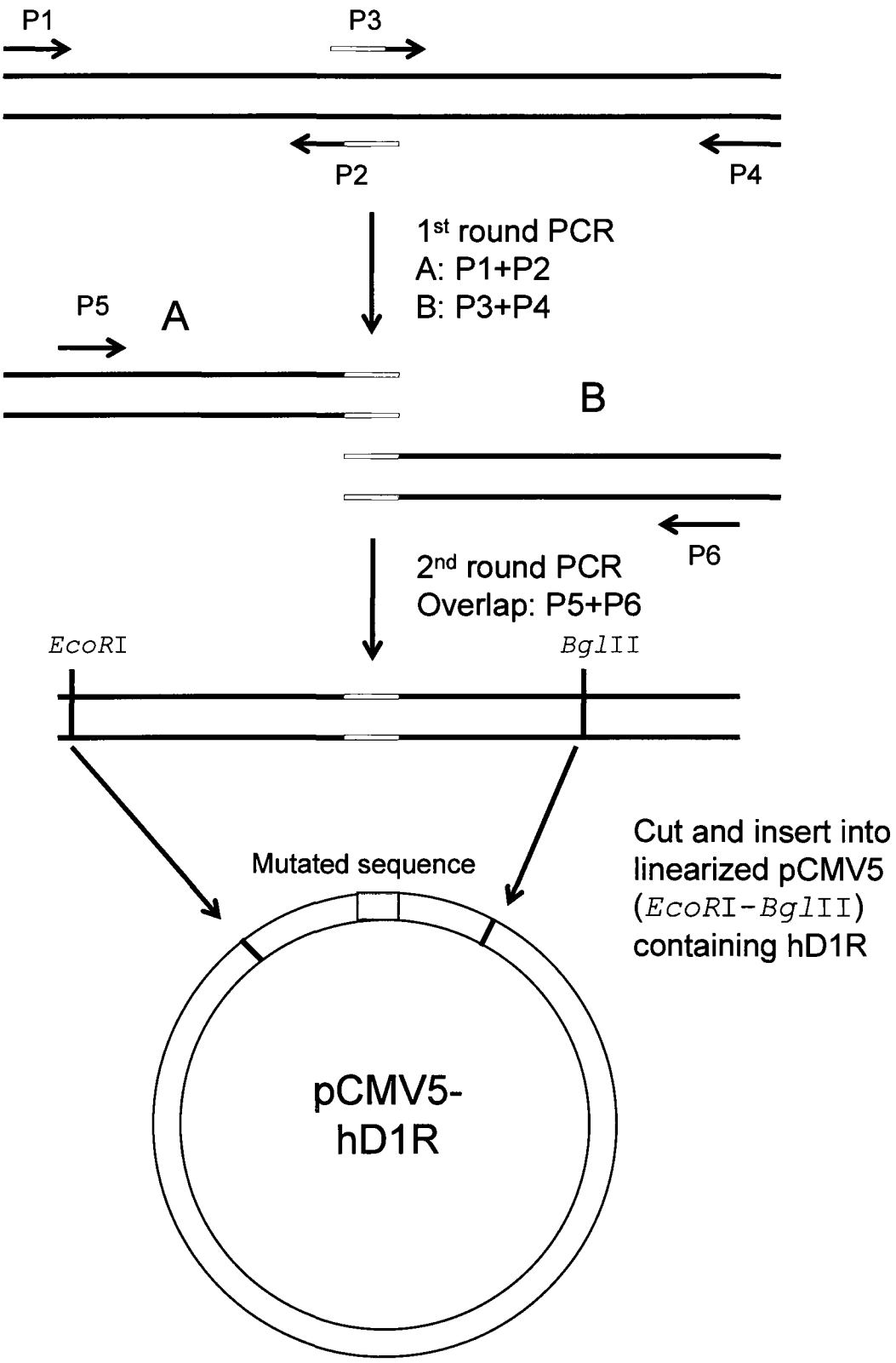

Figure A3. Schematic representation of the three-piece ligation method employed to construct a hD1R chimera. Construction of hD1R-TM1^{D5}.

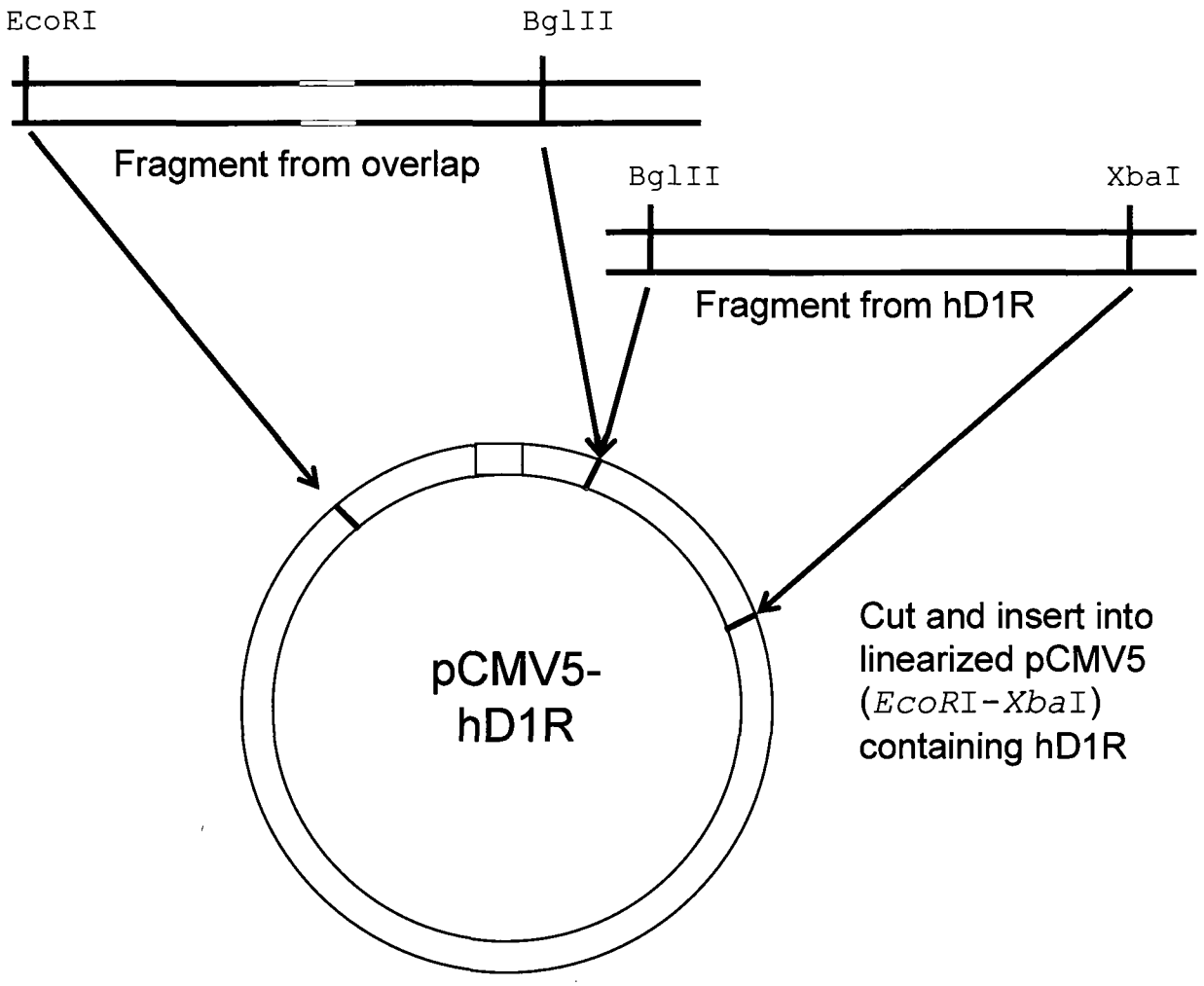

Figure A4. Schematic reprensentation of the subcloning and cloning methods emploued to construct hD1R chimeras. Construction of hD1R-NTTM1^{D5}, hD1R-NT^{D5}, hD1R-S36W and hD1R-T42V.