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Serine and Threonine Residues of the Cytoplasmic Tail of D1-Like Dopaminergic Receptors Differentially Modulate their Pharmacological and Regulatory Properties

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Serine and threonine residues of the cytoplasmic tail of D1-like dopaminergic receptors differentially modulate their pharmacological and regulatory properties

by

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Abstract

The molecular basis for distinct pharmacological and regulation properties of human D1-like dopaminergic receptors (D1R and D5R) remains elusive. To address this issue, the contribution of all serine (Ser) and threonine (Thr) of cytoplasmic tail (CT) of D1R and D5R was investigated using phosphodeficient (PDT) mutants. Results obtained using radioligand and whole cell cAMP assays indicate that Ser/Thr residues regulate in a subtype-specific manner the properties of D1R and D5R. PDT-D5R exhibited an increase in agonist binding affinity, constitutive activity and DA-mediated maximal stimulation. Meanwhile, only PDT-D1R exhibited a decrease in short-term desensitization compared to wild type receptor. In contrast, both mutants were resistant to agonist-induced down-regulation. Overall, the targeting Ser/Thr residues of CT may prove useful in the development of new dopaminergic drugs not acting at the transmembrane ligand binding pocket and thus potentially provide alternative therapeutic approaches for pathological conditions associated with compromised D1-like function such as Parkinson's.

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List of Abbreviations

AA: Ascorbic acid

A1R: Adenosine 1 receptor

AC: Adenylyl cyclase

ACh: Acetylcholine

AD: Alzheimer's disease

AIP1: ALG-2 interacting protein 1

AT1: Angiotensin 1 receptor

ATP: Adenosine triphosphate

β2AR: Beta-adrenergic 2 receptor

Bmax: Maximal binding capacity

BRET: Bioluminescence resonance energy transfer

cAMP: Cyclic adenosine monophosphate

CB1R: Cannabinoid one receptor

CMV: Cytomegalovirus

CRTH2: Chemoattractant receptor homologous molecule expressed on T helper type 2 cells

CT: C-terminal tail

D1R: Dopamine 1 receptor

D2R: Dopamine 2 receptor

D3R: Dopamine 3 receptor

D4R: Dopamine 4 receptor

D5R: Dopamine 5 receptor

DA: Dopamine

DAG: Diacylglycerol

DARPP-32: Dopamine activated protein of 32 kda

DHX: Dihydrexidine

DRIP78: Dopamine receptor interacting protein 78

EC50: Half-maximal effective concentration

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

FRET: Fluorescence resonance energy transfer

GABAR: Gamma-aminobutyric acid receptor

GAP: GTPase activating protein

GEF: Guanine nucleotide exchange factor

GDP: Guanosine diphosphate

GPCR: G protein-coupled receptor

GRK: GPCR kinase

GTP: Guanosine triphosphate

HEK: Human embryonic kidney

IBMX: 3-isobutyl-1-methylxanthine

IL3: Third intracellular loop

IP3: Inositol-3-phosphate

KO: Knockout

L-Dopa: 3,4-dihydroxy-L-phenylalanine

LTP: Long-term potentiation

MAPK: Mitogen-activated protein kinase

MEM: Minimal essential media

mGLUR5: Metabotropic glutamate 5 receptor

MOR: μ-opioid receptor

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

Nac: Nucleus accumbens

NMDAR: N-methyl-D-aspartate receptor

PAR2: Protease-activated receptor 2

PBS: Phosphate buffer saline

PD: Parkinson's disease

PDT: Phospho-deficient tail

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PSD-95: Postsynaptic density-95

SE: Standard error

Ser: Serine

Thr: Threonine

TMD: Transmembrane domain

TRL: Terminal receptor locus

V2: Vasopressin 2 receptor

Vmat: Vesicular monoamine transporter

WT: Wild type

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Introduction

1.1 Transmembrane receptors

Communication between distant cells is essential in a living organism to assure its survival. In that regard, transmembrane receptors are incredible evolutionary tools that cells have at their disposal. Transmembrane receptors bind to extracellular ligands and enable cells to communicate with each other even when separated by considerable distance. This gives an obvious advantage to cells that possess them compared to other cell to cell means of communication (e.g. gap junctions) since they need physical contact between cells to function, limiting the quickness of signal transmission. Transmembrane receptors also hold the distinct advantage of being able to activate multiple signaling pathways through the same receptor. Those signaling pathways are mediated by different classes of receptors such as ion channel linked receptors (found on most of the excitable cells), enzyme linked receptors like tyrosine phosphatase receptors, tyrosine or serine/threonine kinase receptors and G protein-coupled receptors (GPCRs). The latter are described in the following sections. The various activation paradigms that receptors can accomplish are possible because of two intrinsic properties. Firstly, receptors are able to bind to more than one ligand such as dopamine receptors which can bind dopamine (DA) as well as other chemical compounds (dihydrexidine and clozapine for example). Secondly, the intracellular portions of these receptors possess various domains that are modified when activated, enabling them to become docking site for other proteins. The different pathways activated by the receptors are also regulated by a myriad of proteins, leading to the possibility of a graded or sectored response if needed. Defects in regulating these numerous signaling pathways are deemed responsible for the onset of various diseases such as cancers (Bard et al., 2008; Hynes and MacDonald, 2009) or

neurological disorders (Mailman et al., 2001). It should come as no surprise that transmembrane receptors are one of the most targeted protein families in the pharmacological field.

1.2 GPCRs

GPCRs, also referred to as seven-transmembrane receptors, are proteins expressed in almost all living organisms, ranging from bacteria to human. It is also one of the largest gene family present in the human genome. In fact, around 1 to 2% of the whole genome is coding for GPCRs, resulting in about 850 different GPCRs. GPCRs have a broad spectrum of action in humans from enabling sight (Borochov-Neori and Montal, 1989) to regulating blood pressure level (Missale et al., 1998) or controlling neuronal pathways (Missale et al., 1998). Most of these receptors have now been functionally characterized and a majority have a ligand assigned to them. However, some of them are still without a proper ligand and are considered orphans (Chung et al., 2008).

1.2.1 Structure of GPCRs

Even though a large number of GPCRs exist, most of them share a common structural similarity (illustrated in Fig. 1). An early hypothesis was that all GPCRs possess an extracellular N-terminal tail, seven transmembrane domains each connected by intracellular and extracellular loops and a cytoplasmic C-terminal tail. This early claim was later confirmed when the first crystallography structure of a GPCR (rhodopsin) was published (Palczewski et al., 2000). Each structural component of GPCRs has a specific role, which will further be explained below.

Crystal structure of rhodopsin confirmed that GPCRs do indeed have 7 transmembrane domains (TMD). These domains, composed of alpha helices that are positioned in a anti-clockwise manner, serve as the main site for ligand docking (Park et al., 2008). The transmembrane regions also mediate the specific affinity of each receptor for their respective ligand. This is surprising considering that GPCRs share a relatively high degree of TMDs sequence similarity between one another. This characteristic still holds true for the rhodopsin GPCR family even though its members bind to ligands that are quite distinct from each other (for example: dopamine compared to prostaglandin). Moreover, several studies have demonstrated that mutations of certain residues located in the transmembrane domains can change receptor binding affinity for agonists and inverse agonists (Park et al., 2008).

Another important site for ligand docking, at least for some GPCRs, is the N-terminal domain. Receptors with a longer N-terminal chain use it to stabilize large ligands, such as hormones, to the TMD binding pocket. In this case, the N-terminal acts as a sort of lid, blocking the escape of the ligand from the TMDs. Specific residues located in the N-terminal chain can also serve as sites for post-translational modifications (Kristiansen, 2004), such as glycosylation. N-terminal chain asparagine residues in a majority of GPCRs can be glycosylated, resulting in a higher stability of the receptor conformation thus facilitating its plasma membrane recruitment.

Extracellular loops are also important to maintain the structural integrity of the GPCRs. The general role of extracellular loops appears to join the different TMDs to one another.

Cysteine residues located in the second extracellular loop have been demonstrated to create a disulfide bridge with the TMDs in order to maintain a certain rigidity of the structure. Intracellular loops, on the other hand, appears to have a more functional role for GPCRs. Studies have demonstrated that the second and third intracellular loops are crucial elements for G protein coupling (Kristiansen, 2004). More specifically, the third intracellular loop (IL3) is an important structural component for G protein coupling, but also in the regulation of the receptor itself. In fact, phosphorylation of the IL3 is believed to be the most important step in the regulation of GPCRs by enabling its desensitization and subsequent G protein uncoupling. More details regarding the regulation of GPCRs will be given in section 1.5.

Lastly, the C-terminal tail (CT) also plays an important role in the regulation of the receptors (Kristiansen, 2004; Moore et al., 2007; Premont and Gainetdinov, 2007). As is the case for the IL3, the CT can also be phosphorylated. This process is believed to enable the recruitment of a protein called arrestin, generally leading to G protein uncoupling and internalization of the receptor. Receptor internalization, as G protein uncoupling, is a crucial step in the regulation of GPCRs. Moreover, the CT of GPCRs can be involved in the modulation of their functional characteristics as truncation of the CT leads to impairment in the signaling properties of some receptors (Chaar et al., 2001). More details on this particular region will be given in the last section of this introduction.

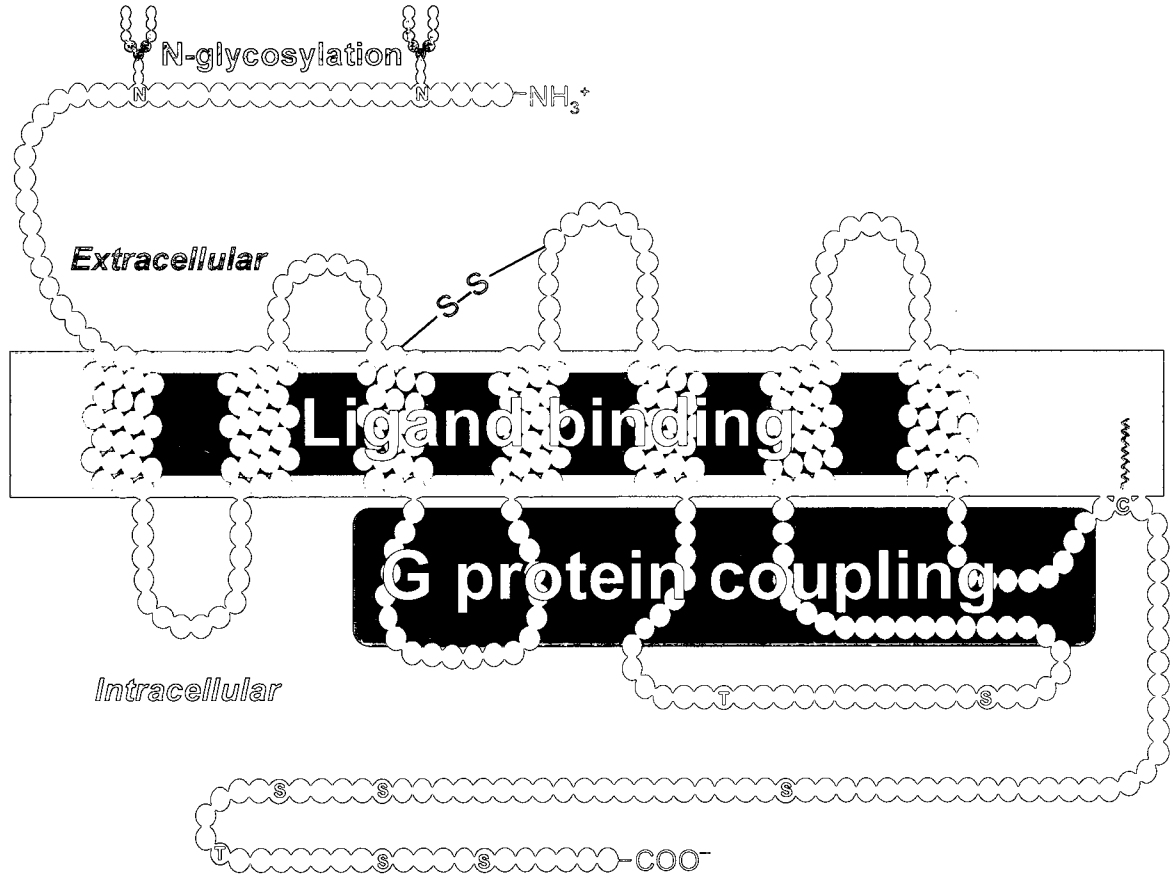


Figure 1: Structure of GPCRs with the main functional sites highlighted

1.2.2 Classification of GPCRs

Even though GPCRs all share structural homology, distinct differences between them still exist. In 1994, Kolakowski and colleagues designed the first classification system for GPCRs (Kolakowski, 1994). Their system comprises GPCRs that were discovered in humans and other living organisms. This classification is based on sequence and functional homologies between GPCRs. Receptors were divided into 5 distinct categories: Rhodopsin-like receptors (which are further sub-divided in 19 sub-families), secretin receptors, metabotropic glutamate/pheromone receptors, fungal mating pheromone receptors and the cyclic AMP receptors. However, Wang and al discovered in 1996 a new class of GPCRs that closely resembled the drosophila Frizzled receptors leading to the creation of a sixth class: the frizzled/smoothened receptors (Wang et al., 1996).

Rhodopsin-like receptors family is by far the largest class of GPCRs comprising 19 sub-families encompassing more than 250 receptors. Rhodopsin-like GPCRs are activated by various ligand ranging from hormones, biogenic amines to light. GPCRs are classified in that class by virtue of their primary sequence high homology, a trait of this GPCR family. Nonetheless, rhodopsin-like receptors still possess great binding specificity toward their respective ligand, evidenced by the wide range of ligands activating them.

Secretin receptors usually have a very long N-terminal that serves as a ligand docking site along with their TMD. This adaptation is useful since the long N-terminal can help stabilize the commonly big ligands binding to them (usually a hormone). Unlike

rhodopsin-like receptors, secretin receptors do not share a high primary sequence homology between members of the family.

Metabotropic glutamate receptors were classified in this family by their ability to bind to glutamate as well as their physiological activity. Members of this family are not to be mistaken for the ionic glutamate receptor, which are present at different regions of the brain and act independently of an association with a G protein. Metabotropic glutamate receptors transduce their signal mainly by coupling to the G protein subunit $G_{\alpha q}$ and activating the phospholipase C (PLC) pathway.

Other groups of GPCRs (fungal mating pheromone, cyclic AMP and Frizzled receptor) constitute a very small proportion of the remaining GPCRs. Some of them are not expressed in humans.

Another group studying the phylogenetic relations between the different families of GPCRs discovered certain deficiencies in the classification system of Kolakowski. Some phylogeny divergences were observed between receptors belonging to the same class, divergences that sometimes went up to 40% (Fredriksson et al., 2003; Perez, 2003). To alleviate this issue, a new classification theme was put forward and named GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin). It is important to note that this system applies only to GPCRs that have been discovered in human. The two classification systems are currently used by the scientific community.

1.2.3 G Protein

G proteins are the main mean of communication of GPCRs. This heterotrimeric protein is composed of an alpha subunit and a tightly bound beta/gamma subunit (Luttrell, 2008). G protein activation by GPCRs leads to the activation of downstream effectors and subsequent physiological effects. Given the large array of GPCRs and their different physiological roles, one could expect the amount of G proteins to be quite high. However, the opposite is true. In fact, only a handful of genes coding for subunits of G protein have been found (16 for alpha, 5 for beta and 12 for gamma).

G proteins are classified by their sequence similarity and their activity profile. G proteins are usually categorized only by their alpha subunit as it is considered their principal catalytic portion. There are four main G_{α} subunit families: $G_{\alpha s/olf}$, $G_{\alpha i/o}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$ (Luttrell, 2008). $G_{\alpha s}$ and $G_{\alpha i}$ are well known for their opposite action on the activation of adenylyl cyclase (AC), the enzyme generating the second messenger cyclic adenosine monophosphate (cAMP). GPCRs that couple to $G_{\alpha s}$ activate AC while those coupling to $G_{\alpha i}$ inhibit AC activity. $G_{\alpha q}$-coupled GPCRs activate the PLC pathway, leading to the creation of two important second messengers, inositol-3-phosphate (IP3) and diacylglycerol (DAG). $G_{\alpha 12/13}$-coupled GPCRs have now been demonstrated to be involved in the remodeling of cell cytoskeleton via the activation of Rho kinase.

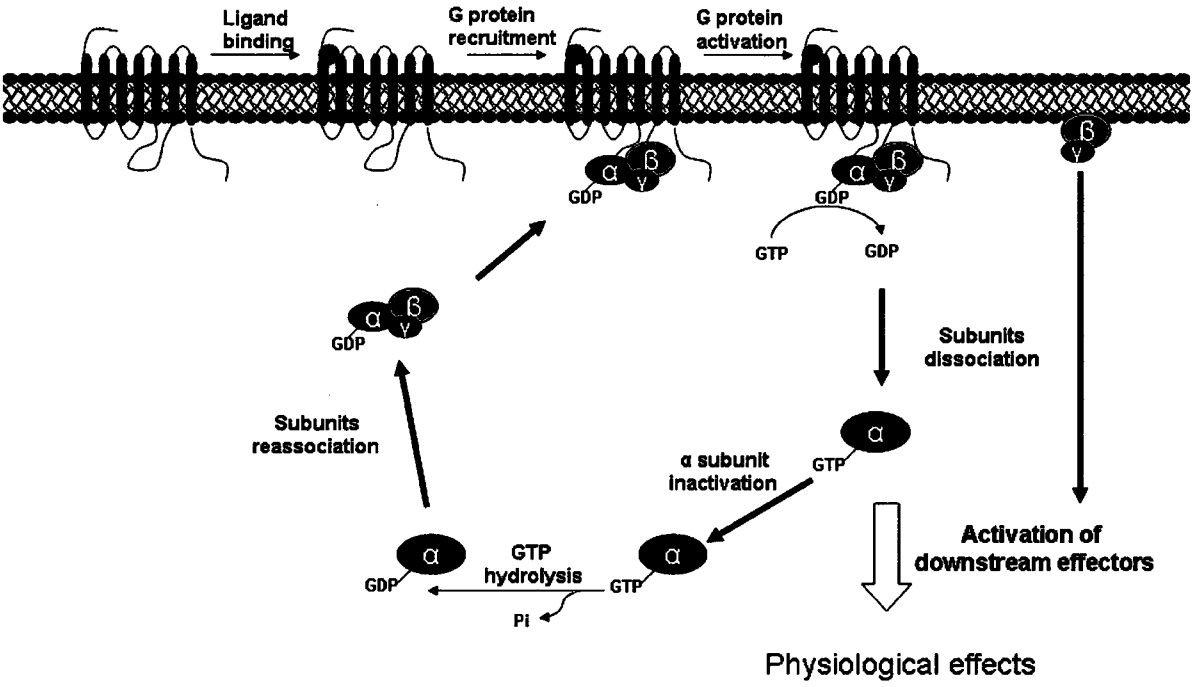


Figure 2: Activation cycle of G proteins

1.2.4 GPCR signaling

When first discovered, GPCRs were thought to exist in only two states: active and inactive. This theory led to the creation of the ternary model where the idea was that GPCRs would operate like a switch, alternating between an off and on state depending on ligand binding and G protein coupling to the receptor (Park et al., 2008). However, this model failed to explain the constitutive activity observed for some GPCRs (activity without the binding of a ligand). Hence, a new model (extended ternary complex model) was proposed where GPCRs would alternate between an inactive (R) and an active state (R*). The R* state of the receptor is the only conformation who can couple to G protein efficiently and is thus the one adopted by receptors showing constitutive activity. In this model, agonists have a higher affinity for the R* conformation of the receptor and shift the thermodynamic equilibrium towards the R* state while inverse agonists do the opposite (i.e. favor inactive state (R) formation). As for antagonists (neutral ligand), no conformation preference is shown. In that case, antagonists merely serve to block the ligand docking site for agonists or inverse agonists. The extended ternary complex model does not appear to be the final model for GPCRs activation as more evidence points towards an even more complex regulation (Park et al., 2008).

Physiological effects generated from GPCRs signaling is believed to be observed when a majority of GPCRs are present in the active state (R*). Stimulation by various stimuli such as light, smell, monoamine and chemokines leads to an increase in active state GPCRs and subsequent coupling to G proteins. In rare cases, some groups have observed signal transduction from GPCRs without the need for G protein coupling (Sun et al.,

2007). Generally, transduction of the signal occurs in sequential steps which are listed below and presented in figure 2 (Luttrell, 2008).

Firstly, ligand binding induces a conformational change of the receptor, allowing the recruitment of an inactive state of the heterotrimeric G protein. This point is not a consensus in the field as some groups believe that GPCRs are constitutively coupled to G proteins, regardless of its state (Park et al., 2008). In their inactive state, G proteins are bound to a guanosine diphosphate (GDP) on their alpha subunit. However, upon coupling to an active GPCR, the alpha subunit will modify its own conformation, allowing the exchange of the GDP molecule for a guanosine triphosphate (GTP). This action, which activates the G protein, is mediated by the GPCR which acts as a guanine nucleotide exchange factor (GEF). When bound to GTP, the alpha subunit will dissociate from the beta/gamma dimer and activate its downstream effectors leading to an increase in second messenger intracellular levels. Once the effector has been activated, the alpha subunit will deactivate through its own GTPase intrinsic activity. This is done by hydrolyzing the subunit-bound GTP, converting it back to GDP. This process can also be enhanced by a family of protein called the GTPase activating protein (GAP). Once bound to GDP, the alpha subunit reassociates with the beta/gamma dimer, reforming the inactive protein once again. One thing to note in this whole mechanism is that while the alpha subunit is the main catalytic section of a G protein, there is increasing evidence that the beta/gamma dimer can also activate numerous signaling pathways (Dupre et al., 2008; Smrcka, 2008)

1.2.5 Regulation of GPCR

Regulation of GPCRs signaling can occur at the different steps of the signaling cascade. However, direct regulation of the receptor is the best way to shut down its entire signaling process. Given the numerous physiological roles that GPCRs have, a tight regulatory mechanism is necessary. Classical regulation of the receptor occurs in three distinct steps: phosphorylation, desensitization and internalization (Luttrell, 2008; Moore et al., 2007).

Protein phosphorylation is a common phenomenon that has various effects depending on where and which proteins are phosphorylated (Tobin et al., 2008). In the case of GPCRs, phosphorylation of serine (Ser) and threonine (Thr) residues at the IL3 and the CT occurs following ligand binding and subsequent G protein activation (Fig. 3 #1 and 2) (Tobin, 2008). Phosphorylation is mainly performed by proteins called GPCR kinases (GRKs), leading to homologous desensitization. GRKs are present in seven different isoforms, each having different specificity regarding which receptor it will phosphorylate (Premont and Gainetdinov, 2007). However, GPCRs does not have an exclusive relationship with a GRK. In fact, multiple GRKs can phosphorylate the same receptor. These phosphorylation events generally occur at different Ser/Thr clusters in the IL3 and CT. GRK isoform-specific effects on GPCRs activity is also possible. This has been observed with the angiotensin 1 (AT1R) and vasopressin 2 (V2) receptor where GRK2 phosphorylation leads to G protein uncoupling while GRK5/6 phosphorylation is important in the activation of the arrestin-dependent extracellular signal-regulated kinase (ERK) pathway (Kim et al., 2005; Ren et al., 2005; Wei et al., 2003).

Other proteins can also phosphorylate GPCRs and regulate their activity. Protein kinase A (PKA) and protein kinase C (PKC) are two kinases that have been demonstrated to phosphorylate and desensitize GPCRs (Tobin, 2008). This event, called heterologous desensitization, is closely similar to the negative feedback loop inhibition mechanism observed in some intracellular signaling pathways. PKA and PKC as well as GRK2 have been shown to phosphorylate the beta-adrenergic 2 receptor (β2AR) at the IL3 and CT (Tran et al., 2004; Yuan et al., 1994). PKA/PKC phosphorylation leads to a desensitization of the receptor without the recruitment of arrestin while GRK2 phosphorylation mediates arrestin recruitment and subsequent GPCR desensitization (Lohse et al., 1992). This indicated that GPCRs can also be regulated through multiple arrestin-independent pathways and opened up the possibility that GRK and β-arrestin had dual roles in GPCR signaling (more details are given below). Another aspect of GPCR regulation by kinases phosphorylation has emerged some years ago, albeit not without controversy (Lefkowitz et al., 2002). Daaka and colleagues, while studying β2AR phosphorylation pattern by PKA, noticed that the receptor could switch in its G protein coupling, exchanging from $G_{\alpha s}$ to $G_{\alpha i}$ (Daaka et al., 1997; Lefkowitz et al., 2002). This switch enabled the receptor to go from cAMP signaling to mitogen-activated protein kinase (MAPK) signaling. However, these results have been questioned by some researchers in the GPCR field (Friedman et al., 2002).

Receptor phosphorylation leads to the second event in GPCR regulation, desensitization. Desensitization, in the case of GPCRs, refers to the loss of responsiveness of the receptor for its respective ligand. The mechanisms underlying this process are not well understood but it is believed that phosphorylation of Ser/Thr at IL3 increase the total negative charge

present on the receptor, thus inhibiting G protein coupling (Premont and Gainetdinov, 2007). By effectively uncoupling the G protein from its receptor, signal transduction cannot occur properly even in the presence of the ligand. However, since phosphatases can reverse this mechanism, more definite actions need to be taken.

In that regard, phosphorylated GPCRs (on their IL3 and CT) provide a docking sites for a family of protein called arrestins (Moore et al., 2007). Moreover, ligand-induced conformational changes in the receptor also enable arrestin binding through its GPCR activation sensor sites, making it a two-step process (Fig. 3 #3). However, the general importance of GPCR phosphorylation for arrestin binding appears to be dependant on the receptor. Phosphorylated rhodopsin displayed a 10 fold-increase in arrestin affinity (Gurevich et al., 1995) while most phosphorylated non-visual GPCRs only display a 2 to 3 fold affinity increase (Gurevich and Gurevich, 2006). Moreover, phosphorylation-independent arrestin binding also occurs for some receptors as observed for the substance P and leukotriene B4 receptor (Jala et al., 2005; Richardson et al., 2003). In these studies, phospho-deficient mutant forms of the receptors were still able to bind to arrestin. This led to the hypothesis that the GPCR activation sensor sites of arrestins might provide sufficient receptor binding to permit arrestin functions (Gurevich and Gurevich, 2006).

Arrestins are divided in two different sub-classes: the visual (arrestin 1 and 4) and non-visual one (arrestin 2 and 3) (Moore et al., 2007). Arrestin 1 and 4 exclusively bind to visual GPCRs while arrestin 2 and 3 are expressed ubiquitously and act on the non-visual GPCRs. Arrestin 2 and 3 are also called β-arrestin 1 and 2 respectively, as they displayed at the time of their discovery a strong preference in binding to β-adrenergic receptors

when compared to rhodopsin. Given the numerous non-visual GPCRs that are seemingly regulated by only two arrestin proteins, various and specific binding mechanisms are essential to ensure a tight modulation of specific GPCRs activity.

Arrestin plays multiple roles inside a cell. Firstly, arrestin binding promotes internalization of the receptor (Moore et al., 2007; Premont and Gainetdinov, 2007). Arrestin, once bound to the receptor, changes conformation leading to the exposition of its CT. CT of Arrestin is a known docking site for two proteins forming clathrin coated pit (namely clathrin and AP-2). These components aggregate at the membrane, forming a pit (Fig. 3 #4). This event is followed by the invagination of the plasma membrane and subsequent internalization of the receptor inside a vesicle (Fig. 3 #4 and 5). Clathrin coated pits are also involved in the trafficking of numerous protein inside the cell, such as the trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus during the protein maturation process (Gorelick and Shugrue, 2001). However, contrarily to agonist-induced internalization, this process is arrestin-independent. Direct interaction between the receptors and members of the endocytic machinery can also occur as GPCRs possess different binding motifs at their CT. For example, the adenosine 3 receptor possesses tyrosine based and acidic di-leucine binding motifs (Marchese et al., 2008).

Once internalized, the receptor can have different fates depending on various factors (Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). One of these factors is the efficiency of arrestin binding to the receptor. Receptors binding strongly to arrestins are recycled back to the membrane at a slower rate than those who interact weakly with it. In some cases, receptors that strongly bind to arrestins can also be degraded in the lysosome,

a specialized acidic intracellular compartment (Fig. 3 #6b). As for receptors who only transiently bind to arrestins, resensitization occurs rather rapidly as receptors are recycled back to the membrane (Fig. 3 #6a). The different binding affinities for arrestins between receptors have lead to another categorization for GPCRs, namely those binding strongly (class A) to arrestins and those interacting transiently with it (Class B). While receptors appear to be either class A or class B, mutation studies using the β-adrenergic receptor demonstrated that a GPCR class switching is possible by substituting certain residues of the CT or removing some parts of it (Marchese et al., 2008).

Arrestin ubiquitination level can also direct the fate of internalized receptor (Marchese et al., 2008). Ubiquitin is small protein (8.5 Kda) that is ubiquitously expressed in eukaryotes. It is added to a lysine residues of the target protein through an enzymatic cascade that requires the sequential action of 3 enzymes. Numerous ubiquitins can be present on the same receptor by adding them on different lysine residues of the receptor or by creating poly-ubiquitin chain from an already bound ubiquitin. Receptor bound to ubiquitin is then targeted for degradation either through the proteasome (a specialized protein degradation complex) or the lysosome. It is believed that receptor ubiquitylation level is the main determinant to distinguish between proteosomal and lysosomal sorting. Mono-ubiquitylation targets receptor to the lysosome while poly-ubiquitylation targets the receptor to the proteasome.

The different affinities of GPCRs for arrestins also mediate their second property, intracellular signaling. Class A receptors, which bind strongly to arrestins, can also mediate intracellular signaling from β-arrestins. While arrestins cannot promote directly

signaling, as they do not have kinase or phosphatase activity, their scaffold properties enable the recruitment of different signaling proteins. This has been demonstrated for MAPKs and ERK where arrestin-bound tyrosine kinase c-Src was necessary for their activation (DeWire et al., 2007). Moreover, β-arrestins signaling has also been demonstrated for other MAPKs (c-Jun N-terminal kinase and p38) as well as phosphoinositide 3-kinase (PI3K) (DeWire et al., 2007).

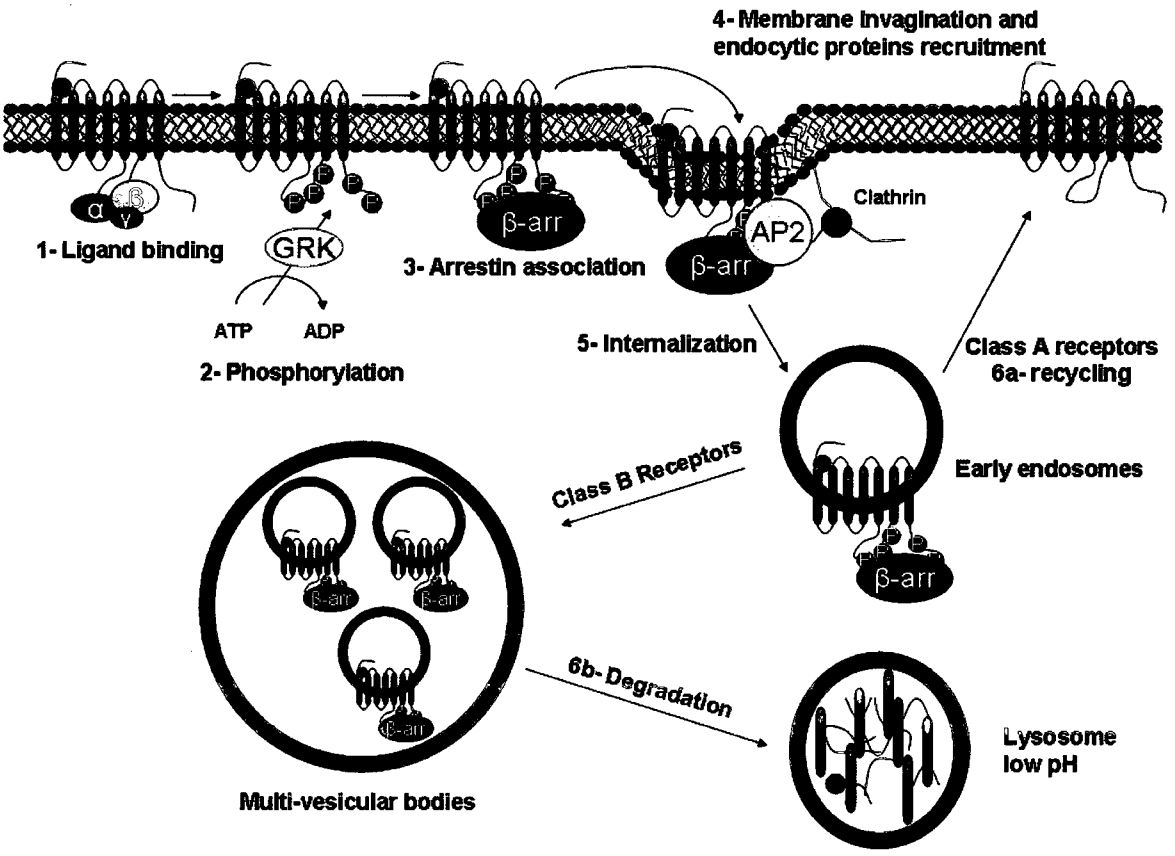


Figure 3: Classical regulation paradigm of GPCRs

Upon ligand binding (1), GPCRs modify their conformation. This change is recognized by GRKs which phosphorylate the receptor (2). Phosphorylated receptor then serve as a docking site for β-arrestin (3) leading to recruitment of endocytic proteins and plasma membrane invagination (4). Receptor-β-arrestin-endocytic proteins complex then internalize (5) and receptor is either recycled back to the plasma membrane (6a) or degraded (6b).

1.3 Dopamine

Dopamine (3,4-dihydroxyphenylethylamine) is one of the most important neurotransmitter present in the brain. Its actions through dopaminergic receptors control important physiological aspects of our life such as movement, blood pressure, reward stimuli and learning (Huang et al., 2001; Missale et al., 1998). DA is part of the monoamine neurotransmitter family, which includes other neurotransmitters such as melatonin, histamine and serotonin. As its neurotransmitter family name implies, DA is derived from the sequential enzymatic modulation of a single amino acid, namely tyrosine. Firstly, L-tyrosine is transformed into 3,4-dihydroxy-L-phenylalanine (L-Dopa) by the enzyme tyrosine hydroxylase, the rate limiting enzyme of DA generation (Kopin, 1968). L-Dopa is then transformed into DA by the enzyme L-Dopa carboxylase. DA can also be modified into norepinephrine and epinephrine more commonly known as noradrenaline and adrenaline. Once transformed into DA, the neurotransmitter is packaged into vesicles (Fon et al., 1997). These vesicles stay at the pre-synaptic site waiting to be released into the synaptic cleft following neuronal stimulation. When stimulation of post-synaptic dopaminergic receptors is not required anymore, DA is re-uptaked into the pre-synaptic neuron by the vesicular monoamine transporter (Vmat). At that point, DA either stay inside the vesicles waiting to be released once more or is degraded by the enzymes catechol-O-methyl transferase and monoamine oxidase (Kopin, 1968).

DA physiological functions are mediated through four main pathways: the mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular. The mesolimbic and mesocortical are sometimes listed as a single neuronal pathway named the mesocorticolimbal pathway.

The mesolimbic pathway begins in the ventral tegmental area and projects to the limbic system via the nucleus accumbens (Nac), amygdala and hippocampus. This pathway has been involved in reward mechanisms, mainly through two regions (the amygdala and the Nac), as well as emotional processing of stimuli. Indeed, it was previously demonstrated that usage of drugs of abuse can strongly stimulate dopaminergic neurons present in the Nac, leading to the euphoric state associated with drugs (Pierce and Kumaresan, 2006). This pathway has also been heavily involved in schizophrenia and depression. Increased dopaminergic transmission through the Nac is thought to lead to the positive symptoms of schizophrenia, such as hallucinations and the general paranoid state associated with the disease (Laviolette, 2007) . The mesocortical pathway, just like the mesolimbic pathway, is involved in reward mechanisms, connecting the ventral tegmental area to the cerebral cortex. Unlike the mesolimbic pathway, dysfunction of this pathway appears to lead to the negative aspect of schizophrenia, such as the lack of interest in normal things of life and the difficulty to make complete sentences (Laviolette, 2007).

The nigrostriatal pathway connects the substantia nigra to the striatum and is involved in voluntary movement control. Disruption of this pathway, by losing dopaminergic neurons present in the striatum, has been demonstrated to be involved in Parkinson's disease (PD) (Emilien et al., 1999; Huang et al., 2001). This pathway can also be severely impaired when diseases such as schizophrenia are treated with antipsychotics acting on blocking DA transmission as no drug specific to a dopaminergic pathway has been designed yet. The non-specificity of these drugs leads to the extrapyramidal effects often seen after treatment with antipsychotics (Mena and de Yebenes, 2006).

Finally, the tuberoinfundibular pathway is present in the hypothalamus. Dopaminergic neurons present in this particular brain region have been demonstrated to control the release of prolactin from the anterior pituitary gland (Moore, 1987). Prolactin is a peptide hormone primarily involved with lactation and to a lesser degree in regulating the menstrual cycle.

1.3.1 Dopaminergic receptors

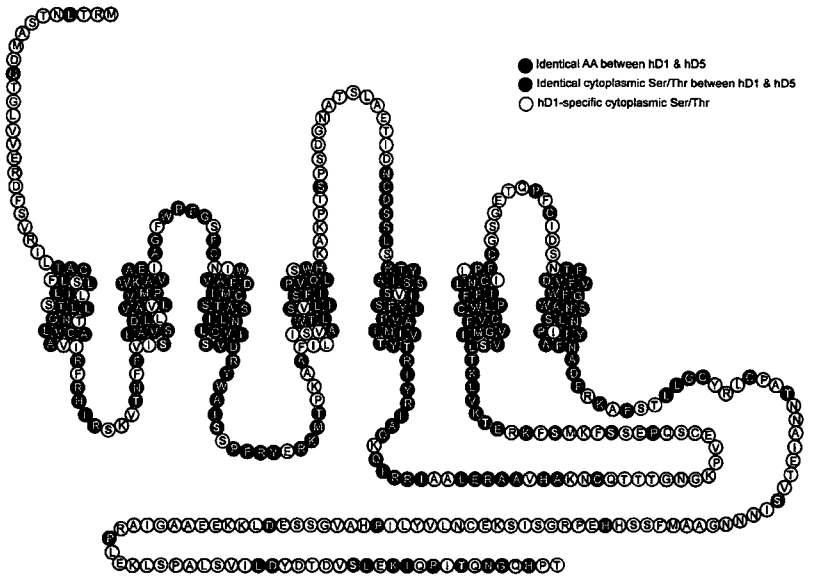
Dopaminergic receptors are members of the sub-family A15 of the rhodopsin-like GPCRs. Sub-family A15 contains other monoamine receptors such as adrenergic receptors and 5-hydroxytryptamine receptors. Dopaminergic receptors are further subdivided in two distinct subtypes: the D1-like receptors (consisting of D1 and D5 receptors) and the D2-like receptors (consisting of D2, D3 and D4 receptors) (Missale et al., 1998; Neve et al., 2004). This classification is based on structural and G protein coupling homology. More details on each subtype will be given in the following sections.

1.3.2 D2-like receptors

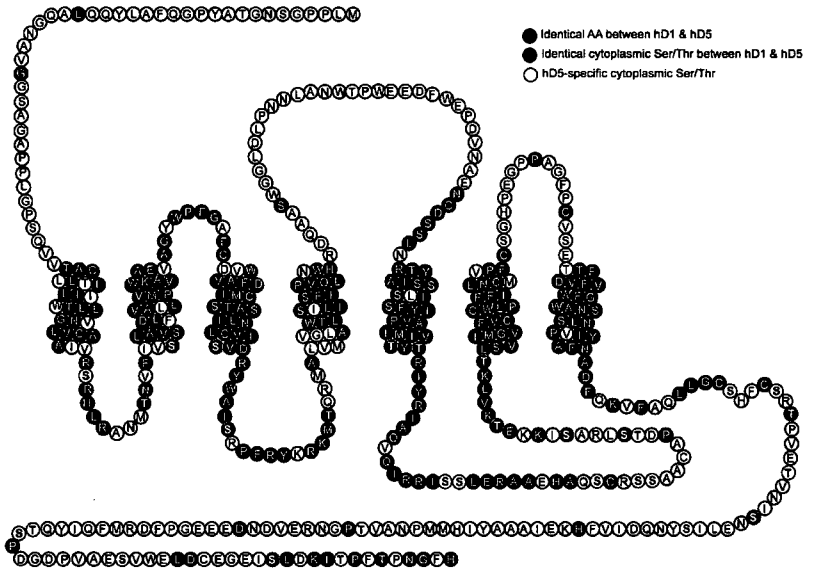
First clues about the existence of D2-like receptors subtype came around the late seventies when Caron and colleagues discovered that DA stimulation in the anterior pituitary gland led to a decrease in prolactin secretion without a concomitant increase in cAMP (Caron et al., 1978). This result was surprising considering that previous observations made indicated that DA led to an accumulation of cAMP through AC activation in the neostriatum (Kebabian et al., 1972). D2 receptor (D2R) was the first dopaminergic receptor to be cloned and characterized in 1988 (Bunzow et al., 1988) .

This discovery was later followed by the cloning of two other D2-like receptors: the D3 (Sokoloff et al., 1990) and the D4 receptors (Van Tol et al., 1991). Characterization studies determined that D2-like receptors predominantly couple to $G_{\alpha i}$ protein and in some cases $G_{\alpha o}$, leading to the inhibition of AC activity. D2-like receptors all possess a similar structure with a high degree of sequence homology. In fact, D2-like receptors share about 60 % homology in amino acids primary sequence. They possess a short N-terminal chain, a large IL3 and a very short CT. Thus, it is believed that D2-like receptors regulation is almost exclusively controlled through interactions with its IL3.

D2-like receptors also share distinct properties compared to other GPCRs as their genes contain introns. This enables the generation of different form of each receptors. Of these different forms, two stand out particularly. Splicing at the IL3 coding region of the D2R gene leads to the encoding of two proteins of 443 and 472 amino acids called the short and long isoforms respectively (D2L and D2S) (Dal Toso et al., 1989). This led to the hypothesis that these two isoforms might couple to different G proteins. However, both isoforms were found to have the same signaling and pharmacological properties. Later on, neuronal localization studies demonstrated that D2S and D2L receptor had distinct localization pattern in the synaptic cleft. D2S receptors were found to be pre-synaptic while D2L receptors were post-synaptic. Further functional studies showed that D2S was the receptor regulating DA release in the synaptic cleft (called autoreceptor) (Usiello et al., 2000). D2R, along with D1 receptor, is the most highly expressed dopaminergic receptor in the brain (Missale et al., 1998). It is present in the prefrontal cortex, the striatum and the Nac. D3 and D4 receptors (D3R and D4R) are less expressed than D2R and show a more precise pattern of expression (Missale, 1998).



human D1R



human D5R

Figure 4: Amino acid sequences of D1 and D5 human dopaminergic receptor
Identical amino acids and identical intracellular serine and threonine residues between D1R and D5R are highlighted with black circles and red filled circles, respectively. Specific serine and threonine intracellular residues for each receptor are highlighted by empty red circles.

1.3.3 D1-like receptors

D1-like receptors are comprised of two distinct receptors: D1R and D5R (presented in Fig. 4). First evidence of a stimulatory dopamine receptor came in 1972 when it was observed that incubation of mice caudate nucleus homogenates with DA led to the activation of AC (Kebabian et al., 1972). This was later followed by the molecular cloning of D1R (Dearry et al., 1990; Monsma et al., 1990; Sunahara et al., 1990; Zhou et al., 1990). Contrarily to D2-like receptors, D1R gene does not contain introns in the coding region, thus leaving out the possibility of splice variants. In the early nineties, another member of the D1-like subtype was discovered and cloned around the same time by four different groups (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991; Weinshank et al., 1991). This receptor was named D5R (also called D1B in the rat by Tiberi et al. and D1β by Weinshank et al.), as its discovery came after the cloning of the three D2-like receptors. As was the case with D1R gene, D5R gene does not contain introns.

Just like D2-like receptors, D1R and D5R have different brain expression pattern. Early localization studies were rather difficult as no discriminating compound between D1R and D5R exist. However, the use of mRNA probes still led to some interesting discoveries. Studies using probes against D1R or D5R demonstrated that D1R is a lot more abundant in the brain than D5R. D1R mRNA was found to be present in the prefrontal cortex, the striatum and the nucleus accumbens while D5R mRNA was found to be strongly present in the hippocampus and to a lesser extent in the prefrontal cortex (Missale et al., 1998). Later on, the creation of D1R and D5R specific antibodies was a great addition to localization studies. Using those antibodies, D1R was observed in the

substantia nigra even though no D1R mRNA was previously detected (Missale et al., 1998). These observations led to the hypothesis that the D1R was transported and expressed in projecting neurons of the substantia nigra, a claim later confirmed by retrograde transport and lesions studies (Gerfen et al., 1990; Harrison et al., 1990) . Results obtained from a D5R anti-receptor antisera study closely matched the mRNA profile observed by others, as D5R protein expression was detected in the hippocampus and the prefrontal cortex (Ariano et al., 1997). However, those results were not duplicated by another study (Montague et al., 2001). Using the D1-like selective antagonist SCH23390 to map D5R binding sites in D1R knockout (KO) mice brain, Montague et al. saw only a small amount of D5R staining in the hippocampus. It is possible that the method used to detect D5R in this study was not sensitive enough to detect all of D5R binding sites, leading to the discrepancies in the results. Another study focusing on D1R and D5R localization led to some surprising results (Bergson et al., 1995). Electron microscopy done on neurons co-expressing D1R and D5R showed that these two receptors have a distinct neuronal intracellular localization pattern. While D1R was found mostly on dendritic spines, D5R was mostly seen on the shaft. This observation suggests that these two receptors have different physiological roles inside the same neuron. It is important to note that although localization of D1-like receptors is focused mostly in the brain, D1-like receptors have also been observed in the blood vessels as well as in the kidney (Emilien et al., 1999; Missale et al., 1998).

Cloning and characterization studies of these two receptors led to some interesting discoveries. As was the case with D2-like receptors, D1R and D5R share a common structure (Fig. 4). They both possess a long IL3, although not as long as D2-Like

receptors, and a long CT. D1R and D5R were also found to share a high homology in their TMDs (Fig. 4). In fact, TMD sequence homology between D1R and D5R is the highest observed for dopaminergic receptors at around 80% (Missale et al., 1998). Despite that fact, D1R and D5R differ greatly in their ligand binding properties (Sunahara et al., 1991; Tiberi et al., 1991). As stated before, GPCR TMDs have been demonstrated to be an important docking site for ligands. In that regard, DA 10 fold higher affinity for D5R over D1R is quite surprising. Moreover, competition studies showed that generally, D1-like agonists have a higher affinity for D5R than D1R. However, the increase in binding affinity is not shared by all D1-like ligands as inverse agonists displayed a higher affinity for D1R than D5R.

Differences in signaling properties were also detected between D1R and D5R (Sunahara et al., 1991; Tiberi et al., 1991). D5R constitutive activity as well as its G protein coupling is enhanced compared to D1R. In fact, G protein coupling is around 10 times higher for D5R than D1R, as measured by their EC50 value (half maximal effective concentration) in a dose-response assay. Using the same assay, it was also demonstrated that DA-mediated maximal activation of AC is higher for D1R than D5R. These results led to the suggestion that D5R might be a more constitutive active version of D1R, a hypothesis that is still being debated by the scientific community.

D1-like receptors are considered stimulatory receptors. Just like other stimulatory GPCRs, D1-like receptors have been demonstrated to couple to $G_{\alpha s}$ protein. However, D1-like receptors also have the ability to couple to other G proteins. First evidences of this claim came with D1R localization studies. D1R expression observed in the

neostriatum led to some questioning as $G_{\alpha s}$ expression in that particular brain region is below detection level. Later on, $G_{\alpha olf}$ was demonstrated to be the main G protein present in the neostriatum where it mediates D1R signaling (Neve et al., 2004; Zhuang et al., 2000). Some studies also observed D1R coupling to other G protein such as $G_{\alpha i}$ and $G_{\alpha q}$ (Huang et al., 2001). However, no clear evidence of similar observations by other groups has been made to date. D1R also have a preference in regard to which Gγ subunit it couples to (Neve et al., 2004). Studies have revealed that γ7 is the predominant subunit bounded to D1R when activated. Moreover, γ7 subunit appears to bind preferentially to the β1 subunit, thus indicating that the D1R receptor signals mostly through a β1/γ7 dimer. D5Rs also appear to couple to different G protein subunits. D1-like receptor activation was demonstrated to lead to an increase in intracellular calcium level and phosphoinositide turnover independently of D1R (Friedman et al., 1997). This process is thought to be mediated by Gαq protein coupling with D5R. However, this observation have not been shared with other groups, who failed to observed phosphoinositide turnover following D5R activation leading to doubts about the legitimacy of this claim (Sunahara et al., 1991; Tiberi et al., 1991).

While G protein coupling is occurs mainly through the IL3, other receptor regions also mediate some of the differences observed between D1R and D5R. D1R and D5R both possess two arginine residues that can be glycosylated in their N-terminal chain. However, a study made by Karpa and colleagues demonstrated that glycosylation is not as equally important for both D1-like receptors. It was shown that loss of glycosylation sites for D1R does not impair its membrane recruitment in transfected cells contrarily to

D5R (Karpa et al., 1999). Their CT also mediates functional differences between D1R and D5R. This aspect will be further discussed in section 1.4.

D1-like receptors mediate their action through several intracellular signaling pathways (Huang et al., 2001; Missale et al., 1998; Neve et al., 2004). As explained earlier, D1-like receptors couple to $G_{\alpha s}$ or $G_{\alpha olf}$ and activate AC leading to the formation of cAMP from adenosine triphosphate (ATP). cAMP is an important second messenger in mammals and its accumulation is a key component in the activation of PKA. PKA is a serine-threonine kinase implicated in numerous intracellular signaling pathways (Neve et al., 2004). One of the most studied D1-like dependant signaling pathway downstream of PKA is the activation of dopamine activated protein of 32 kda (DARPP-32). DARPP-32 has been involved in various neurological disorders such as schizophrenia and drug addiction (Svenningsson et al., 2005; Svenningsson et al., 2004). It is tightly regulated by phosphorylation at key residues, some of them phosphorylated by PKA (Svenningsson et al., 2004).

D1-like receptors also control intracellular calcium level through different pathways (Missale et al., 1998; Neve et al., 2004). First, D1R can interact directly with calcium channels present at the plasma membrane. This interaction was demonstrated between N-type calcium channels and D1R (Kisilevsky et al., 2008). Stimulation of D1R with different agonists led to a decrease in calcium current, an effect that was abolished with D1R inverse agonists. Moreover, membrane presence of Cav2.2 calcium channel in human embryonic kidney 293T cells (HEK293T) was up-regulated when co-transfected with D1R compared to cells transfected with Cav2.2 alone. This effect was caused by

direct interaction between the two proteins as addition of a D1R intracellular loop 2 peptide diminished the amount of calcium channels at the membrane. D1-like receptors can also control intracellular calcium channel by coupling to $G_{\alpha q}$ and activating the PLC pathway (Missale et al., 1998; Neve et al., 2004). Increase in IP3 concentration, a second messenger from this pathway leads to the release of calcium from the ER. Ca^{2+} release can then activate other downstream effectors, such as PKC. However, there are still doubts in the scientific community about the coupling of D1-like receptors to $G_{\alpha q}$ proteins.

Dopamine receptors interaction with other proteins is an important component of their activity. In that regard, dopamine receptors dimerization with other GPCRs appears to partially modulate their regulation. Receptor dimerization in living cells was first demonstrated with the β-adrenergic receptor in 2000 (Angers et al., 2000). Since then, D1R has been demonstrated to form heterodimers with D2R (Dziedzicka-Wasylewska et al., 2006) and D3R (Marcellino et al., 2008) in neurons. D1R-D2R heterodimers formation leads to the activation of intracellular calcium signaling pathways. Interestingly, clozapine (an antipsychotic used in the treatment of schizophrenia) appears to antagonize the formation of the heterodimer upon binding to D1R and D2R (Faron-Gorecka et al., 2008). D1R-D3R heterodimers were observed in HEK293 cells using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET). D1R-D3R heteromerization increased D1R agonists affinity in cells and in striatum membrane preparation. Moreover, D1R-D3R heteromerization also led to D1R mediated behavioral effect in mice through activation of D3R. D1R dimerization with non-dopaminergic GPCRs has been demonstrated with μ-opioid (MOR) (Juhasz et

al., 2008) and adenosine A1 receptor (A1R) (Gines et al., 2000; Torvinen et al., 2002) to name a few. D1R can also interact with the N-methyl-D-aspartate receptor (NMDAR) (Lee et al., 2002). This direct interaction between D1R and the NR1 and NR2 subunit of NMDAR regulate NMDAR currents in hippocampal neurons. Moreover, interaction between the two receptors has anti-apoptotic effect through the activation of the PI3K pathway. As for D5R, interaction has been shown with the gamma-aminobutyric acid receptor (GABAR), an important receptor in synaptic transmission modulation (Liu et al., 2000).

1.3.4 D1-like receptor physiological functions: insights from knock-out mice

Physiological relevance of the two different D1-like receptor came into question after their respective characterization since D1R and D5R were demonstrated to share the same functional characteristics. Physiological studies were difficult to conduct since no compound outside of DA displayed more than a 10-fold selectivity between the two receptors. However, advances in molecular biology enabled the creation of mice lacking the D1R or D5R by gene knockout (Holmes et al., 2004).

The first D1-like KO mice line generated was D1R KO in 1994 (Drago et al., 1994; Xu et al., 1994). D1R KO mice appeared normal, albeit with slight growth retardation and a fairly low survival rate after weaning. This was not due to a lack of motivation to seek food as mice can survive if provided with easy access to food. However D1R KO mice did display some abnormalities. Xu and al. observed a strong hyperlocomotion phenotype in the open field apparatus as well as a delay in locomotor habituation. D1R KO mice

have also been described as having deficit in motor coordination using the rotarod test. However, some other groups failed to see a deficit in locomotion, sometimes even reporting hypolocomotion instead of hyperlocomotion (Holmes et al., 2004). In fact, diverging observations appears to be the norm rather than the exception with respect to dopaminergic receptors KO mice. A couple of factors can account for these discrepancies. First, differences in experimental protocols can lead to diverging observations. Since these experiments increase the amount of stress imposed on the mice, divergence between individual mouse stress levels could lead to different results. It is especially true when looking at mice exploration patterns in the open field apparatus as overly stressed mice tends to move along the edges of the box. Secondly, genetic background pattern also appears to play an important role in behavioral observations. Even though different lines of KO mice were all backcrossed with the conventional wild type (WT) mouse (a C57BL/6 mouse), sizable genetic variations in the resulting KO mice lines were observed (Holmes et al., 2004). These genetic variations could explain the diverging results obtained when using different mice lines in behavioral experiments.

D1R KO mice also responded quite differently to D1-like agonists and inverse agonists. However, no difference between KO and WT mice was observed when stimulated with D2-like specific compounds. Response to different drugs of abuse was also monitored (Holmes et al., 2004). D1R KO mice showed a drastic decrease in the behavioral and hyperlocomotion inducing effects of chronic uptake of psychostimulants when compared to WT. Even tough psychostimulants failed to mediate their usual effects on KO mice, conditioned-placed preference (an experiment studying reward mechanisms) is unaffected between KO and WT mice (Holmes et al., 2004; Karlsson et al., 2008).

Cognitive studies were also done to evaluate D1R role in this process (Holmes et al., 2004). D1R KO mice displayed a decrease in efficiency when subjected to the Morris water maze, a common tool to study cognitive functions. This apparent decrease in memory could be partially explained by the fact that KO mice showed a decrease in long-term potentiation (LTP) in the prefrontal cortex, a phenomenon linked to memory development.

D5R KO mice were created a couple of years after D1R KO mice (Hollon et al., 2002; Holmes et al., 2001). As seen with D1R KO mice, D5R KO mice appeared normal, with no obvious changes in their physiognomy except that aging mice display high blood pressure over time. Contrarily to D1R KO mice, Holmes et al. failed to see change in locomotion paradigms suggesting that implication of D5R in locomotion is rather limited. D5R also seems to have a limited role in mediating agonist and cocaine-induced hyperlocomotion. However, some studies still led to some interesting discoveries concerning D5R physiological function. As was explained earlier, D5R is strongly expressed in the hippocampus. Hippocampal acetylcholine (ACh) level as well as ACh release following D1-like agonist stimulation were strongly reduced in D5R KO mice (Laplante et al., 2004). Given the role of cholinergic signaling in regulating learning and memory, these findings shed new light on a possible physiological role for D5R. However, no behavioral studies as of yet found a defect in hippocampal-mediated cognition in D5R KO mice (Holmes et al., 2004). More studies are needed to elucidate this mystery.

1.3.5 Dopaminergic receptors and diseases

Dopaminergic receptors have numerous physiological roles in humans. In fact, various diseases have been linked to dysregulation of dopaminergic pathways (Emilien et al., 1999; Huang et al., 2001; Missale et al., 1998). Parkinson's disease is a prevalent neurological disorder amongst the elderly. In fact, close to 1% of human over 70 year old suffers from PD. PD is a neurodegenerative disease that is characterized by various symptoms such as dyskinesia, tremor and muscular rigidity. Loss of control over voluntary movement is the most common and recognizable trait of PD. Other symptoms include gait and loss of posture, usually leading to the so-called hunchback pose of PD patients. Symptoms are believed to occur following the loss of projecting DA neurons located in the striatum, a specialized region of the brain involved in movement control. In fact, PD most serious symptoms are observed after about 80 % of striatal DA is lost (Emilien et al., 1999). Treatment of PD is rather limited in its efficacy as neuronal replacement is currently impossible. Currently, the most widely used treatment for treating PD is over-stimulation of the remaining DA neurons through ingestion of L-Dopa (also called levodopa), a DA precursor. While this treatment if effective in increasing the amount of DA present in the striatum, important issues with this technique still exist. Firstly, since the ingestion is not specific to a brain region, treatment with L-Dopa can lead to adverse induction of other dopaminergic pathways. In fact, some patients can experience side effects such as nausea, confusion or hallucinations, all believed to be mediated by the activation of D2R (Huang et al., 2001). Another problem with L-Dopa use is its loss of effectiveness over time (5 to 10 years of usage) and the increase in the severity of side effects. Loss of effectiveness appears to occur when DA

neurons are not present in sufficient number to properly uptake L-Dopa and generate enough DA for the required signal output. Moreover, loss of receptor responsiveness leads to the on-off state seen over time with the treatment (Huang et al., 2001) . These undesired effects led to the search of novel compounds to treat PD. In that regard, the creation of the first full synthetic D1-like agonist, dihydrexidine (DHX), is very promising. DHX has been use with success with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkey, a common primate model for PD studies. DHX treatment drastically decreased PD symptoms without most of the side effects seen when using levodopa, making it a promising drug for the future treatment of PD (Huang et al., 2001) .

Dopaminergic signaling abnormalities have also been observed in another neurological disease: schizophrenia. Schizophrenia is defined as two distinct phases referred to as the positive and negative phase. The positive phase includes symptoms such as hallucinations and the general paranoid state of schizophrenics. Those symptoms are thought to occur when dopaminergic receptor activity increases in the mesolimbic pathway, more specifically, at the nucleus accumbens (Laviolette, 2007). Negative symptoms of schizophrenia include lack of joy, lack of motivation, poverty of speech, severe cognitive deficits as well as difficulty to interact with other human beings leading to withdrawal. These symptoms are thought to be caused by a decrease in dopaminergic synaptic transmission in the mesocortical pathway. As was the case with PD, treatment of schizophrenia is possible, but not without side effects (Mena and de Yebenes, 2006). Most of the anti-psychotics on the market to treat schizophrenia acts on the D2R through a common mechanism. A recent study by Masri and al. have demonstrated that a majority

of antipsychotic decrease β-arrestin 2 receptor binding leading to a decrease in D2R activity down-regulation (Masri et al., 2008). Since D2R inhibits dopaminergic signaling, an increase in D2R activity is able to limit the positive symptoms of schizophrenia by downregulating dopaminergic signaling at the Nac. However, increased activity from the D2R also leads to the apparition of extra-pyramidal effects in patients, leading to a PD-like phenotype. Even though most of the drugs used to treat schizophrenia are aimed at D2R, D1-like receptors involvement in the disease state is still possible. D1-like receptor stimulation in the prefrontal cortex is an important regulator of memory formation. However, this effect is mediated through a limited range of activation as both under or over stimulation of prefrontal DA receptors leads to a decrease in working memory (Huang et al., 2001). Thus, D1-like receptors may be involved in the memory impairment seen in schizophrenics.

Drug addiction is an increasing problem amongst the population. Over the years, interest over dopaminergic receptors involvement in drug addiction has grown considerably. As described in the previous section, cocaine-induced hyperlocomotion have been shown to be mediated by D1R in mice. Cocaine blocks the action of the DA transporter and inhibits DA reuptake (Koob and Bloom, 1988) leading to an increase in dopaminergic signaling. While not using the same mechanisms as cocaine, a majority of drugs of abuse are thought to lead to an increase in dopaminergic signaling (Huang et al., 2001). Increased dopaminergic transmission in the striatum and Nac leads to the cocaine-induced hyperlocomotion and hallucinations caused by hallucinogens respectively. Moreover, DA stimulation in the Nac is also important for the reinforcing effects of addictive drugs. The nucleus accumbens is strongly involved in reward mechanisms and

drugs of abuse increase DA stimulation in this particular region following their uptake. Thus, during drug withdrawal, DA stimulation returns to baseline level. However, since high DA stimulation of the Nac leads to the "beneficial" effects of drug use, drug addicts seek re-uptake of drugs to return to that state. This drug-seeking behavior is a key component in creating the addictive properties of drugs of abuse. Moreover, drugs also have a perverse effect since dose-dependant desensitization can also occur. This event generates the necessity of an increase in drug uptake to reach the same effect as previous usage.

1.4 Importance of CT and specific CT residues for D1-Like receptors

It has been demonstrated that GPCRs different regions have specific functions in mediating the receptor diverse physiological roles. Moreover, it appears that specific residues located in these sections are key components in ensuring receptor-specific activity and pharmacological selectivity. Characterization of D1R and D5R led to the discovery of significant differences between the two receptors pharmacological and signaling properties (Sunahara et al., 1991; Tiberi et al., 1991). These differences, especially the ones pertaining to ligand affinity, were thought for a long time to be mediated by their TMDs as this region is involved in ligand binding of a majority of the GPCRs. However, little difference exists between the TMD primary sequence of D1R and D5R (see fig. 4). Our lab, along with others, investigated whether other regions of these receptors could be involved.

Surprisingly, intracellular regions of the receptors (loops and CT) were demonstrated to mediate the functional differences observed between D1R and D5R (Charpentier et al., 1996; Demchyshyn et al., 2000; Iwasiow et al., 1999; Jackson et al., 2000; Tumova et al., 2004). We first investigated the region between TMD 6 and the CT (referred in the study as the terminal receptor locus (TRL)) (Iwasiow et al., 1999) by designing chimeric D1R and D5R where their respective TRL were swapped. While this mutation did not have a strong effect on functional receptor expression level, DA affinity was significantly altered. D1R harboring D5R-TRL displayed a 10 time increase in DA affinity while D5R harboring D1R-TRL DA affinity dropped around 6-fold, results that closely mimic DA affinity of the TRL donor. Affinity for a non-specific antagonist (flupentixol) as well as two D1-like inverse agonists (SCH23982 and (+)-butaclamol) were unchanged by the mutation. Swapping of signaling properties was also observed for constitutive and DA-mediated maximal activation of AC between the two receptors. D1R harboring D5R-TRL displayed a 3-fold increase in constitutive activity while D5R harboring D1R-TRL constitutive activity dropped 3-fold. Finally, swap of the TRL between D1R and D5R also led to a swap of each receptor G protein coupling efficiency, as observed by the exchange of their EC50 value. These results put forward for the first time the role of D1R and D5R TRL in regulating their functional properties

To further specify the region that was behind these observations, our group decided to construct new mutant forms of D1R and D5R, swapping only the CT between one another (Jackson et al., 2000). As was the case with the TRL swap, CT exchange between the receptors led to DA affinity swapping as well as an exchange of their constitutive activity and DA potency. Surprisingly, DA-mediated maximal activation of AC increased

for both mutants. D1R harboring D5R CT also displayed a strong decrease in its functional expression level, a result not observed with the D5R/D1R CT mutant. These results, except for the difference in functional expression level for one of the mutant, were also confirmed by a study from another group (Demchyshyn et al., 2000).

Later on, our group decided to look at specific sections of the CT. To accomplish that, truncations or swaps of CT regions were performed on D1R and D5R (Chaar et al., 2001; Jackson et al., 2002; Kim et al., 2004; Tumova et al., 2004). This led to the same observations as the ones detailed above. Interestingly, loss of CT sections decreased D1R DA affinity while increasing its constitutive activity. In fact, the increase was CT-length dependant as bigger truncations led to the most drastic effects (Chaar et al., 2001). D1R lacking almost the entire CT displayed a decrease in G protein coupling efficiency as well as an increase in DA-mediated maximal activation of AC. Truncation of the CT also had an impact in the functional receptor expression levels as heavily truncated receptor were less expressed at the cell membrane. An increase in intracellular staining was also observed for two of the mutants using immunofluorescence. However, this effect could not be solely explained by receptor intracellular retention as strong plasma membrane staining was detected for all the mutants. These results shed new light on the importance of specific CT regions in modulating the functional properties of D1-like dopaminergic receptors.

Other studies also established a role for the CT in receptor regulation and trafficking. Studies from our group have demonstrated that certain CT regions of D1R were essential for its phosphorylation, desensitization and endocytosis (Jackson et al., 2002).

Truncation of D1R CT at proximal sites (residue 379 or 351) greatly reduced receptor phosphorylation level following DA stimulation while truncation at a more distal site (residue 425) had a significantly weaker effect. Moreover, D1R CT truncation led to a decrease in D1R agonist-mediated desensitization. Truncation of the CT also had important consequences in DA-mediated endocytosis. While Δ425 and Δ379 mutants still displayed DA-mediated endocytosis, Δ351 mutant did not endocytose efficiently following DA stimulation. Moreover, Δ351 mutant displayed a strong intracellular staining even when non-stimulated, suggesting that a specific sequence important for D1R intracellular transport is located between residue 351 and 379 of its CT. Reduction of D1R DA-mediated phosphorylation after CT truncation was also observed by another group (Kim et al., 2004).

Studies have shown that D1R CT can interact directly with arrestin 2 and 3 (Macey et al., 2005). Arrestin3 interaction was strongly increased following agonist stimulation while arrestin2 remained the same, an effect concomitant with the internalization of the receptor. This suggests that D1R CT plays a direct role in its internalization following agonist stimulation through an arrestin3 dependant mechanism. Other evidences of D1R CT implication in its own trafficking came from a study demonstrating D1R direct binding to γ-COP, an important member of the clathrin coated pit complex (Bermak et al., 2002). Results from another study also suggest that D1R CT is important in its trafficking during its maturation process through association to a membrane-associated ER protein named dopamine receptor interacting protein 78 (DRIP78) (Bermak et al., 2001). It was demonstrated that overexpression of DRIP78 can lead to receptor retention in the ER.

D1 receptor also regulate other proteins through interaction with its CT. Direct interaction have been demonstrated with NMDAR (Lee et al., 2002) as well as N-type calcium channels (Kisilevsky et al., 2008). In fact, direct interaction between D1R CT and NMDAR leads to a decrease in neuron potentiation. It is thus possible that D1R activation modulates long-term potentiation/depression, two important processes in memory formation. In the case of calcium channels, physical interaction between D1R and the channels regulate their synaptic distribution and calcium entry into synapses.

Direct interaction between the CT of D1R and other proteins is also important in mediating D1R activity (Zhan et al., 2008; Zhang et al., 2007). In fact, D1R CT binding to the protein postsynaptic density-95 (PSD-95) led to a decrease in cAMP formation following agonist stimulation. Moreover, PSD-95 binding led to an increase in constitutive internalization, a dynamin-dependent process. Protein interaction with D1R also mediates cross-talk between D1R and D3R. ALG-2 interacting protein 1 (AIP1) has been demonstrated to bind to both D1R CT and D3R. Moreover, D1R binding to AIP1 appear to modulate its transport to the membrane as functional receptor expression levels were markedly decreased when both proteins where coexpressed in HEK293 cells. Even though no direct complex between D1R, AIP1 and D3R has been described, the possibility that heteromer formation occurs with the help of other proteins is an intriguing concept. Most studies have focused on D1R. However, a recent report indicated that D5R CT can interact with the second intracellular loop of the GABAa receptor, regulating its activity (Liu, 2000). As more research is done on D5R, others interacting partners should begin to emerge.

1.5 Rationale and hypotheses

Modulation of dopamine response in the central nervous system has been an important area of research in neuroscience for decades. Interest in DA has grown over time as evidences suggest a role of the neurotransmitter in incurable neurological disorders such as PD and schizophrenia (Emilien et al., 1999; Laviolette, 2007). Therefore, understanding of the components regulating the activity of DA receptors is paramount to gain insight on how to potentially treat these diseases. In that regard, recent studies done by our group and others have led to some interesting discoveries (Demchyshyn et al., 2000; Iwasiow et al., 1999; Jackson et al., 2002; Jackson et al., 2000). The CT of D1-like receptors was found to be an important modulator of their ligand binding pocket (Jackson et al., 2000). In fact, swapping of D1R and D5R CT led to an almost complete swap of their respective DA affinity (Jackson et al., 2000). A swap in respective constitutive activity was also noted for D1R and D5R when individual CTs were swapped (Jackson et al., 2000). Moreover, complete or partial loss of the CT impaired to different degrees D1R desensitization (Jackson et al., 2002). CT of GPCRs, as mentioned above, is an important part of receptor intracellular trafficking during their maturation process or following agonist stimulation (Bermak et al., 2001; Moore et al., 2007; Spiegel, 1996). As such, understanding the role of the specific CT residues governing those aspects of a receptor life cycle is important for further comprehension of diseases such as PD. Studies done on the β-adrenergic receptors have implied an important role of cytoplasmic serine and threonine in the regulation of GPCR activity. Phosphorylation of these residues has been demonstrated to be important for the desensitization, arrestin binding and

internalization of a variety of GPCRs following agonist stimulation (Moore et al., 2007; Premont and Gainetdinov, 2007). Since β-adrenergic receptors are close phylogenic cousins of dopaminergic receptors, one could predict that Ser/Thr residues of their CT are also important for the pharmacological and regulation properties of D1-like receptors. Moreover, few studies have been done to distinguish D1R and D5R subtype-specific regulation properties. Given the lack of studies done on the subject as well as results obtained from β-adrenergic receptors and our own studies, I propose the following hypotheses and objectives.

Hypotheses:

1- Serine and threonine residues located at the C-terminal tail of human dopaminergic D1 and D5 receptors are important modulators of their subtype-specific pharmacological properties.

2- Serine and threonine residues located at the C-terminal tail of human dopaminergic D1 and D5 receptors regulate differentially their DA-stimulated signaling properties.

Objectives:

1- Determine the overall influence of Ser/Thr residues located in the CT on D1R and D5R receptor binding function.

2- Determine the role of Ser/Thr residues on the modulation of D1-like receptor constitutive activity and dopamine-mediated G protein coupling properties.

3- Establish the functional importance of Ser/Thr residues of CT for the short-term desensitization and long-term regulation of D1-like receptors.

Materials and methods

2.1 Materials

N-[methyl-^{3}H]-SCH23390 (73-78 Ci/mmol); herein referred to as [^{3}H]-SCH23390 and [^{14}C]-cAMP (250-275 mCi/mmol) were acquired from GE Healthcare (Baie d'Urfé, QC, Canada). [2,8-^{3}H]-adenine (24-27 Ci/mmol) was purchased from PerkinElmer (Woodbridge, ON, Canada). Dopamine hydrochloride, cis-flupenthixol dihydrochloride, (+)-butaclamol hydrochloride, thioridazine hydrochloride and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Dihydrexidine hydrochloride was acquired from Tocris Bioscience (Ellisville, MO, USA). Gentamicin and Trypsin-HCl 0.25% were obtained from Invitrogen (Burlington, ON, Canada). Phosphate buffer saline (PBS) and minimal essential media (MEM) were purchased from Wisent (Saint-Bruno, QC, Canada). Fetal bovine serum (FBS) was obtained from PAA laboratories (Etobicoke, ON, Canada). Restriction enzymes were acquired from Fermentas Life Sciences (Burlington, ON, Canada) and New England Biolabs (Pickering, ON, Canada)

2.2 Generation of D1R and D5R expression constructs

DNA constructs coding for phospho-deficient human D1R and D5R (HD1RRD1R and HD5RRD1R) were custom made in vector pUC57 by Genscript Corp. (New Jersey, USA). All serine and threonine residues located in D1R and D5R intracellular regions were mutated to alanine and valine, respectively (see figure 4 for exact locations of intracellular serine and threonine residues). Phospho-deficient tail (PDT) mutants were derived from those constructs using a restriction digestion approach. WT-D1R (cloned in pCMV5 vector) and HD1RRD1R were digested by BmgB I and Xba I while WT-D5R

and HD5RRD1R (cloned in pCMV5 vector) were digested by Eag I and Hind III. Subsequent steps in PDT mutants generation are similar to those detailed below.

Flag and HA epitope were added to the extracellular amino terminus of PDT-D1R and PDT-D5R using a restriction digest approach. Flag-tagged WT-D1R, HA-tagged WT-D1R and PDT-D1R were digested with Hind III and Xba I. Meanwhile, Flag-tagged WT-D5R, HA-tagged WT-D5R and PDT-D5R were cut with BmgB I and Hind III. Fragments from PDT-D1R (745 base pairs), Flag-tagged WT-D1R (5200 bases pairs), HA-tagged WT-D1R (5200 base pairs), PDT-D5R (802 base pairs), Flag-tagged WT-D5R (5100 base pairs) and HA-tagged WT-D5R (5100 base pairs) were excised from an agarose gel and purified using a Qiagen purification kit according to the manufacturer protocols. Purified DNA inserts from PDT-D1R and PDT-D5R were ligated to their respective linearized HA/Flag vectors overnight at 4°C using an insert/vector ratio of 3:1. Ligation product was then desalted and transformed in *E. Coli* XL1-Blue by electroporation. DNA sequences were verified using DNA automated sequencing.

2.3 Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Tissue Culture Collection) were cultured at 37°C and 5% CO_2 in MEM with Earle's salt supplemented with 10% (v/v) FBS and 20 μg/ml gentamicin. Cells were seeded in 100 mm petri dishes at a density of 2.5 x 10^6 cells/dish. Cells were then transiently transfected with a total amount of 5 μg/dish of receptor DNA using a calcium-phosphate method, as described elsewhere (Tumova et al., 2004). When a reduced amount of receptor DNA was used for

transfection, empty vector (pCMV5) DNA was used to adjust the total amount of DNA transfected to a total of 5 μg per dish.

2.4 Radioligand binding assay

2.4.1 Preparation of membranes

Following transfection (18-24 hours), HEK293 cells were washed at room temperature with PBS, trypsinized, reseeded in 150 mm dishes and grown for 48 hours. Prior to membrane preparation, cells were washed with cold PBS and scraped in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA). Lysed cells were centrifuged at 40,000 g for 20 minutes at 4°C. Membrane pellets were homogenized in lysis buffer using a Brinkman Polytron (17 000 rpm for 15 seconds) and centrifuged again as described above. Final pellets were homogenized in resuspension buffer (1 M Tris-HCl, pH 7.4, 0.5 M EDTA). Fresh membrane aliquots were employed immediately for saturation studies or were frozen in liquid nitrogen and stored at -80°C until use for competition studies.

2.4.2 Binding assay

Fresh or frozen membranes (thawed on ice) were diluted in resuspension buffer (50 mM Tris-HCl. pH 7.4, 120 mM NaCl) and mixed using a Brinkman Polytron (17 000 rpm for 15 seconds). Binding assays were performed in binding buffer (Final concentration in assay: 50 mM Tris-HCl, pH 7.4; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$ and 1 mM EDTA, pH 8.0) with 100 μl of membranes in a total volume of 500 μl using [^{3}H]-SCH23390 as radioligand. Saturations studies were done using fresh

membranes and increasing concentrations of [^{3}H]-SCH23390 ranging from 0.01 to 7.0 nM in the absence (total binding) or the presence of 10 μm cis-flupentixol (non-specific binding). For competition studies (measuring displacement of [^{3}H]-SCH23390), frozen membranes were thawed on ice and incubated with a constant concentration of [^{3}H]-SCH23390 (between 0.4 – 0.8 nM) and increasing concentrations of cold dopamine, dihydrexidine, thioridazine or (+)-butaclamol. Competitions studies using DA were done in the presence of 0.1 mM ascorbic acid (AA). All other drugs used for competition studies were diluted in water. Binding assays were incubated 90 min at room temperature and terminated using rapid filtration through glass fiber filters (GF/C, Whatman). Filters were washed three times with cold washing buffer (50 mM Tris-HCl, pH 7.4 and 100 mM NaCl) and bound radioactivity determined by liquid scintillation counting (Beckman LS 6500). Protein concentrations were determined using the Bio-Rad assay kit with bovine serum albumin as standard. Equilibrium dissociation constant (K_d), inhibitory constant (K_i) and maximal binding capacity (Bmax, pmol/mg of membrane proteins) were determined using non linear regression curve fitting analyses with GraphPad Prism 5.02 software for Windows (GraphPad Prism software, San Diego, CA USA)

2.5 Whole cell cAMP assay

To measure activation of AC by wild-type and mutant receptors, whole cell cAMP assays were performed. Following transfection (18-24 hours), cells were trypsinized and reseeded in 6 or 12-well dishes. The day after, the media was removed and cells incubated in fresh MEM containing 5% FBS (v/v), 20 μg/ml gentamicin and 1-2 μci/ml of [^{3}H]-adenine for 18-24 h. The day of the experiment, labeling media was replaced with

fresh 20 mM HEPES-buffered MEM containing 1mM IBMX in absence (0.1 mM of AA) or presence of DA (in 0.1 mM AA) for 30 minutes at 37°C and 5% CO_2. At the end of the incubation period, the medium was aspirated, 1ml of lysis solution (2.5% (v/v) perchloric acid, 0.1 mM cAMP and [^{14}C]-cAMP (25 nCi, 8000-12,000 dpm)) was added to each well and cells were incubated 30 minutes at 4°C. Lysates are then transferred in tubes containing 0.1 ml of 4.2M KOH (neutralizing solution) and sedimented by low-speed centrifugation (1500 rpm) for 10 minutes at 4°C. The amount of intracellular [^{3}H]-cAMP was then determined from supernatants purified by a sequential chromatography using Dowex and alumina columns as described previously (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 relative adenylyl cyclase activity (expressed as CA/TU x 1000). Increasing amount of DA (0 to 10 μM) was used to generate dose-response curves, which were fitted to a four-parameter logistic equation using GraphPad Prism 5.02 software for Windows (GraphPad Prism software, San Diego, CA, USA). Functional receptor expression levels (Bmax) were determined using a saturating concentration of [^{3}H]-SCH23390 (~ 6.0 nM) as described in section 2.4.2

2.6 Desensitization assay

Cells cultured in [^{3}H]-adenine labeling medium were treated with 0.1 mM AA (control) or 10 μm DA (treated) for 5 min at 37°C and 5% CO_2. After the first incubation, cells were washed twice with 2ml of room temperature PBS. Following washes, 2 ml of 20 mM HEPES-buffered MEM containing 1mM IBMX was added to each well and cells stimulated with DA (0-10 μM) for 10 min at 37°C and 5% CO_2. Cells were then lysed

and cAMP purified as described in section 2.5. Basal levels of cAMP production were subtracted prior to fitting dose-response curves using a four-parameter logistic equation.

2.7 Long-term regulation assay

For receptor long-term regulation studies, cells were seeded in 100 mm dishes at a concentration of 2.5 x 10^6 cells/dish. The next day, cells were incubated with 0.1 mM AA, 100 μM DA or 1 μM thioridazine (antipsychotic drug) for different periods of time (15 min, 1 h and 24 h). Following the incubation, cells were washed twice with 5 ml of cold PBS and scraped in cold lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA). Lysed cells were centrifuged at 40,000 g for 20 minutes at 4°C. Membrane pellets were homogenized in lysis buffer using a Brinkman Polytron (17 000 rpm for 15 seconds). Homogenates were centrifuged again twice as described above. Final pellets were homogenized in resuspension buffer (1 M Tris-HCl, pH 7.4, 0.5 M EDTA). The receptor expression levels (Bmax) were determined using a saturating concentration of [^{3}H]-SCH23390 (~ 6.0 nM) as described in section 2.4.2.

2.8 Data analysis

Equilibrium and inhibitory dissociation binding constants (K_d and K_i) and Bmax values from saturation studies are expressed as geometric mean with 95% upper and lower confidence intervals. All other data are presented as arithmetic mean ± standard error unless stated otherwise. Dose-response curves were best-fitted using a four-parameter logistic equation using GraphPad Prism 5.02 for Windows (GraphPad Prism Software, San Diego, CA, USA). Desensitization assay curves were corrected for basal values and

fitted with an operational model of pharmacological agonism using a receptor depletion equation of GraphPad Prism to determine signal transduction efficiency (τ value) following DA pre-treatment. Unconstrained, shared and constrained parameters (bottom or constitutive activity, top or DA-mediated maximal stimulation, log EC50, hill slope and τ value) were used to determine if differences between best-fitted values were statistically different. The t test and one-way ANOVA followed by Newman-Keuls post test were used to compare two or multiple groups, respectively.

Results

3.1 D1-like receptor Ser/Thr residues of CT are important for high receptor membrane expression

The equilibrium dissociation constant (K_d) and maximal number of binding sites (Bmax) of wild type and mutant forms of D1-like receptors for the radioligand [^{3}H]-SCH23390 were determined using saturation studies. Sufficient expression level of the two mutant receptors was needed to carry out our subsequent experiments. Maintenance of [^{3}H]-SCH23390 high affinity for PDT mutants was also important as it indicates that the folding conformation mediating optimal binding to this compound was not altered. This is critical for enabling competition studies determining the role of Ser/Thr residues of CT in controlling affinity of dopaminergic drugs as [^{3}H]-SCH23390 is the only radioligand currently available for D1-like receptors.

Both mutants retained the ability to bind to [^{3}H]-SCH23390 with high affinity compared to respective WT as assessed by K_d values (Table 1). However, Bmax values for PDT-D1R and PDT-D5R were markedly decreased relative to their WT receptor (Table 1 and Fig. 5).This decrease was more robust in cells expressing PDT-D5R (Table 1 and Fig. 5). In fact, Bmax values for PDT-D1R and PDT-D5R were decreased by 40% and 70% respectively (Fig. 5). Nonetheless, both mutant receptors were expressed at a sufficiently high level to perform the next series of experiments.

	WT-D1R	PDT-D1R	WT-D5R	PDT-D5R
K_d (nM) (n=6)	0.49 [0.36-0.67]	0.43 [0.34-0.55]	0.57 [0.46-0.72]	0.41 [0.28-0.60]
Bmax (pmol/mg of membrane proteins) (n=6)	10.5 [6.37-17.4]	5.99* [4.11-8.72]	11.4# [6.79-19.3]	2.48*,#,& [1.65-3.73]

Table 1: Equilibrium dissociation constant and maximal binding capacity values of [3H]-SCH23390 for wild type and mutant receptors

Equilibrium dissociation constant (Kd) and maximal binding capacity (Bmax) values of [3H]-SCH23390 for wild type and mutant receptors expressed in HEK293 cells. Shown are geometrical means and 95% confidence intervals. The statistical significance of differences between receptors was assessed using one way-ANOVA and Newman-Keuls post-test. *p<0.05 when compared to WT-D1R, #p<0.05 when compared to PDT-D1R and &p<0.05 when compared to WT-D5R.

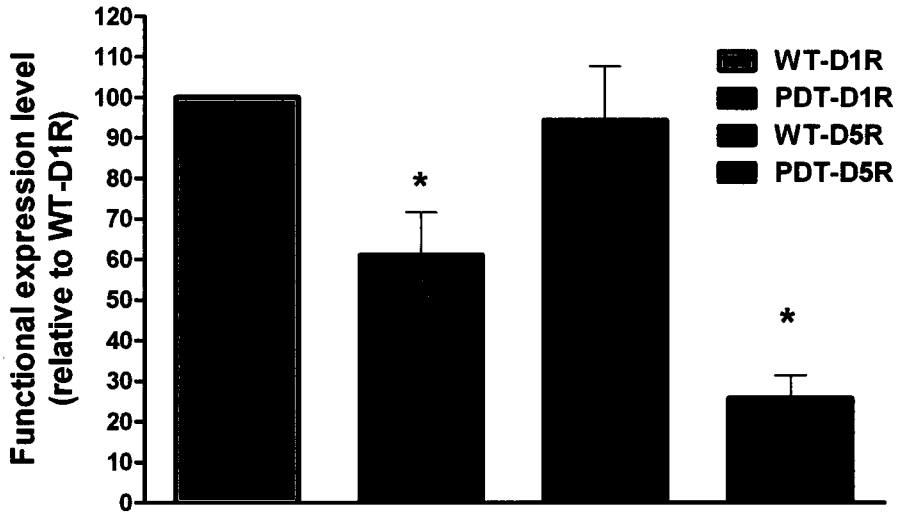


Figure 5: Functional receptor membrane expression level of D1-like receptors relative to WT-D1R

Functional receptor membrane expression level (Bmax) calculated relative to WT-D1R value (n=6). Results were analyzed using a one-sample t-test against a hypothetical value of 100%. *p<0.05 when compared to 100%.

3.2 Ser/Thr residues of CT modulate D5 receptor agonist affinity

Given that our previous results indicated that the CT of D1-like receptors modulates the distinct binding properties of D1 and D5 receptors (Jackson et al., 2000), we investigated the impact of mutating all Ser/Thr residues of the CT on ligand binding function. Competition studies were done with two different agonists, namely dopamine and dihydrexidine as well as two inverse agonists, thioridazine and (+)-butaclamol. A 10 fold difference in agonist affinity (measured by their difference in inhibitory constant (K_i) values) was observed between WT-D1R and WT-D5R, as previously reported (Sunahara et al., 1991; Tiberi et al., 1991) (Table 2). While no change in agonist affinity was observed between PDT-D1R and WT-D1R, a significant increase was detected for PDT-D5R (Table 2). PDT-D5R displayed an increased affinity for DA (5-fold) and DHX (4-fold) in comparison to WT-D5R (Fig. 6). Moreover, a small increase in thioridazine affinity for PDT-D5R was also observed; an effect not seen with (+)-butaclamol (Table 2 and Fig. 6). As seen with agonists, no significant difference in inverse agonists affinity was observed between WT-D1R and PDT-D1R (Table 2 and Fig. 6).

K_i (nM)	WT-D1R	PDT-D1R	WT-D5R	PDT-D5R
Dopamine (n=5)	5280 [4560-6120]	3580 [1960-6510]	555*,# [246-1250]	116*,#,& [33.6-402]
Dihydrexidine (n=5)	455 [376-550]	364 [285-464]	33.8*,# [15.5-73.6]	8.59*,#,& [3.29-22.4]
Thioridazine (n=3)	43.6 [27.3-69.4]	41.8 [30.3-57.7]	126*,# [87.3-181]	82.0*,#.,& [53.2-126]
(+)-Butaclamol (n=3)	4.59 [1.56-13.5]	4.89 [2.27-10.5]	21.6*,# [14.9-31.5]	17.6*,# [8.96-34.7]

Table 2: Inhibitory constant values of dopaminergic drugs for wild type and mutant receptors
Inhibitory constant (Ki) values of dopaminergic drugs for wild type and mutant receptors expressed in HEK293 cells. Shown are geometrical means and 95% confidence intervals. The statistical significance of differences between receptors was assessed using one way-ANOVA and Newman-Keuls post-test. *p<0.05 when compared to WT-D1R, #p<0.05 when compared to PDT-D1R and &p<0.05 when compared to WT-D5R.

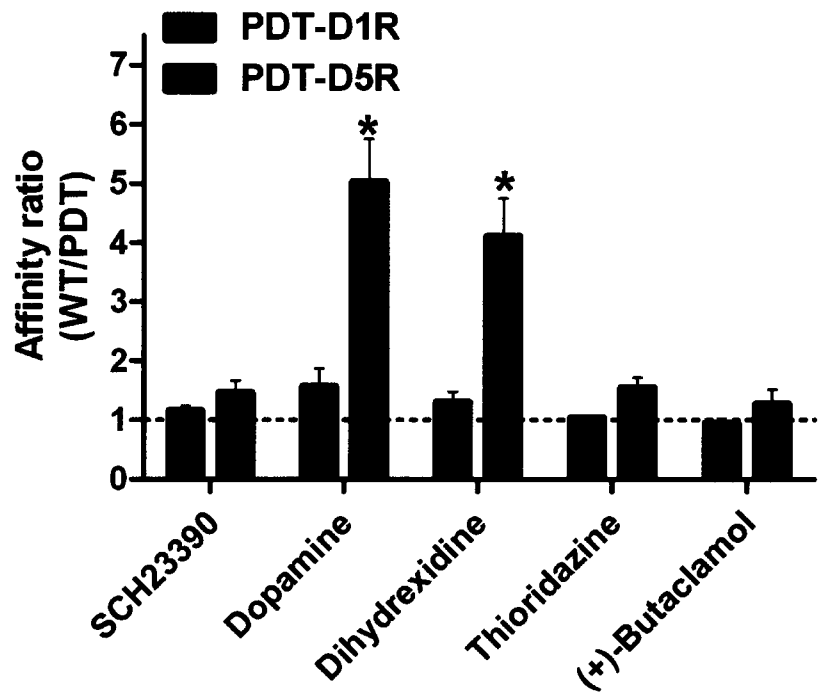


Figure 6: Affinity ratio values of dopaminergic drugs for PDT mutants relative to their respective WT
Affinity ratio values of dopaminergic drugs for PDT mutants calculated relative to their respective WT (n=3-5). Bars represent arithmetic mean ± SE. Results were analyzed using a one-sample t-test against a hypothetical value of 1. *p<0.05 when compared to 1 (dashed line).

3.3 Epitope-tagged PDT receptors membrane expression and agonists affinity are similar when compared to untagged PDT receptors

To perform future studies such as confocal microscopy and immunoblotting, epitope-tagged versions (Flag and HA) of each mutant receptor were generated (see section 2.2 for more details). To ensure that no significant differences could be observed between tagged and untagged receptors, binding and signaling studies were performed. As assessed by saturation studies, tagged receptors did not exhibit any significant difference in K_d values for [^{3}H]-SCH23390 when compared to untagged receptors (Table 3). No difference in Bmax values was also observed between tagged and untagged PDT-D5R receptors (Table 3). As for PDT-D1R receptors, only Flag-PDT-D1R exhibited a small but significant decrease in its Bmax value when compared with untagged PDT-D1R (Table 3).

Competition studies were also performed to compare agonists affinity between tagged and untagged PDT receptors (Table 4). Only dopamine and dihydrexidine were used as larger differences in affinities were reported between untagged forms of PDT-D1R and PDT-D5R using these two agonists when compared to inverse agonists (Table 2). No difference in affinity for both agonists was observed between tagged and untagged receptors (Table 4). PDT-D5R tagged and untagged receptor still exhibited a significantly higher affinity for agonist (~15 fold) than PDT-D1R (Table 4).

	Kd (nM)	Bmax (pmol/mg of membrane proteins)
PDT-D1R	0.51 [0.48-0.54]	8.26 [5.93-11.5]
Flag-PDT-D1R	0.41 [0.19-0.89]	4.59* [4.35-4.85]
HA-PDT-D1R	0.48 [0.43-0.52]	8.21 [5.45-12.4]
PDT-D5R	0.51 [0.41-0.63]	3.29* [2.40-4.51]
Flag-PDT-D5R	0.42 [0.28-0.61]	2.11* [1.25-3.56]
HA-PDT-D5R	0.52 [0.32-0.84]	1.85* [0.89-3.75]

Table 3: Equilibrium dissociation constants and maximal binding capacity values of [3H]-SCH23390 for tagged and untagged mutant receptors

Equilibrium dissociation constants (Kd) and maximal binding capacity (Bmax) values of [3H]-SCH23390 for tagged and untagged mutant receptors expressed in HEK293 cells (n=3-4). Shown are geometrical means and 95% confidence intervals. The statistical significance of differences between receptors was assessed using one way-ANOVA and Newman-Keuls post-test. *p<0.05 when compared to PDT-D1R.

Ki (nM)	Dopamine	Dihydrexidine
PDT-D1R	6569 [2299-18700]	413 [159-1140]
Flag-PDT-D1R	5357 [1158-24780]	384 [134-1098]
HA-PDT-D1R	7028 [2333-21170]	345 [300-397]
PDT-D5R	117* [76.7-179]	7.23* [4.36-12.0]
Flag-PDT-D5R	118* [30.4-459]	16.1* [4.61-56.5]
HA-PDT-D5R	138* [64.5-294]	9.49* [3.95-22.8]

Table 4: Inhibitory constant values of dopaminergic agonists for tagged and untagged mutant receptors

Inhibitory constant (Ki) values of dopaminergic agonists for tagged and untagged mutant receptors expressed in HEK293 cells (n=3-4). Shown are geometrical means and 95% confidence interval. The statistical significance of differences between receptors was assessed using one way-ANOVA and Newman-Keuls post-test. *p<0.05 when compared to PDT-D1R.

3.4 Ser/Thr residues of CT modulate D5 receptor constitutive activity

We next investigated the constitutive activity (ability to mediate intracellular signaling in the absence of agonists) of each receptor. However, discrepancies in Bmax values between WT and mutant receptors made the comparison very difficult. Importantly, a linear relationship exists between the extent of receptor number (Bmax) and the extent of GPCR constitutive activity (Samama et al., 1993; Tiberi and Caron, 1994). As seen in figure 7, despite not being as abundant as WT-D5R, PDT-D5R still displayed similar constitutive activity and an increased DA-mediated maximal stimulation of AC (Fig. 7). On the other hand, PDT-D1R did not display any major difference in constitutive activity and DA-mediated maximal stimulation when compared to WT-D1R (Fig. 7). These observations suggest that PDT-D5R may exhibit a stronger constitutive activity than WT-D5R, if expressed at a similar Bmax value. To test this hypothesis, HEK293 cells were transfected with various amount of receptor's DNA to obtain a similar expression level (see section 2.3 for more details). As hypothesized, PDT-D5R constitutive activity was found to be about 2.5 fold higher than WT-D5R (Fig. 8). PDT-D1R did not display any significant difference in its constitutive activity compared to WT-D1R at similar Bmax values (Fig. 8).

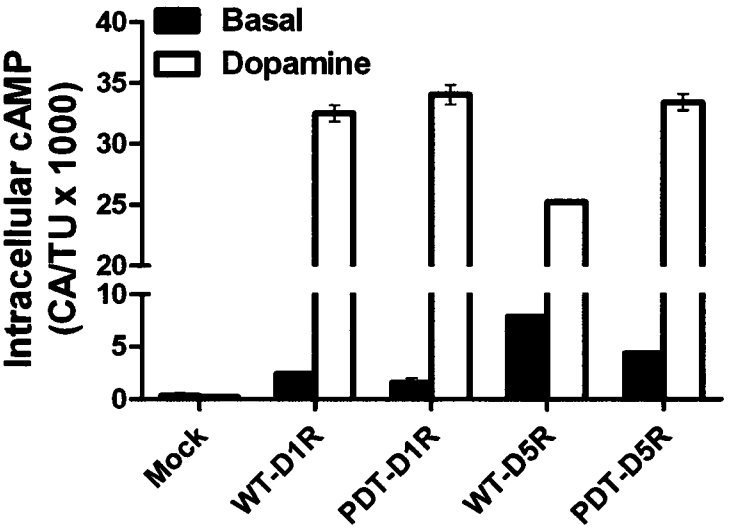


Figure 7: Representative example of a cAMP assay
HEK293 cells were transfected with empty vector (pCMV5) or receptor constructs. Cells were incubated in culture media containing IBMX in the presence of ascorbic acid (basal condition) or DA (10 μM) for 30 minutes. Bars represent arithmetic mean ± SE. Bmax values (pmol/mg of membrane proteins) were as follows: WT-D1R (11.5), PDT-D1R (8.23), WT-D5R (9.83) and PDT-D5R (3.15).

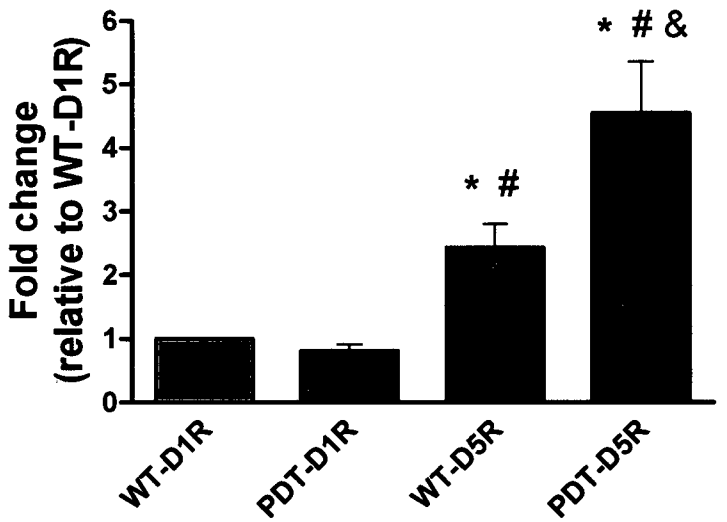


Figure 8: Constitutive activity of WT or mutant receptors relative to WT-D1R
Constitutive activity of WT or mutant receptors normalized relative to WT-D1R (n=7). Cells were incubated in culture media containing IBMX in the presence of ascorbic acid for 30 minutes. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (2.36 ± 0.32), PDT-D1R (2.94 ± 0.42), WT-D5R (2.55 ± 0.29) and PDT-D5R (2.05 ± 0.28). Bars represent arithmetic mean ± SE. *$p<0.05$ when compared to a value of 1 (WT-D1R) using a one-sample t test. #$p<0.05$ when compared to PDT-D1R and &$p<0.05$ when compared to WT-D5R using a one-way ANOVA followed with Newman-Keuls post-test.

3.5 Mutations of Ser/Thr residues of CT of D1R and D5R differentially modulate their DA-mediated maximal activation

We next explored the agonist-dependent coupling properties of PDT mutants using dopamine dose-response curves (Fig. 9 and Table 5). It is important to note that the EC50 value (DA concentration eliciting 50% of the maximal response) was used as an indicator of dopamine potency. As with its constitutive activity, PDT-D1R did not display any difference in dopamine potency relative to the one measured in cells expressing WT-D1R (Table 5). No difference in DA-mediated maximal stimulation of AC between WT-D1R and PDT-D1R was also noted (Fig. 9 A and B and Table 5). On the other hand, PDT-D5R displayed a strong increase in maximal activation of AC relative to WT-D5R (Fig. 9 C). In fact, the increase amounted to approximately 250 % of WT-D5R value (Fig. 9 D). Meanwhile, DA potency in cells expressing PDT-D5R was not altered significantly in comparison to WT-D5R (Table 5).

3.6 Epitope-tagged PDT receptors display similar constitutive activity and DA-mediated maximal stimulation than untagged PDT receptors

Constitutive activity and DA-mediated maximal stimulation of AC were also assessed with tagged and untagged receptor using whole cell cAMP assay (Fig. 10). No significant difference in constitutive and maximal stimulation of AC was observed between tagged and respective untagged receptors (Fig. 10).

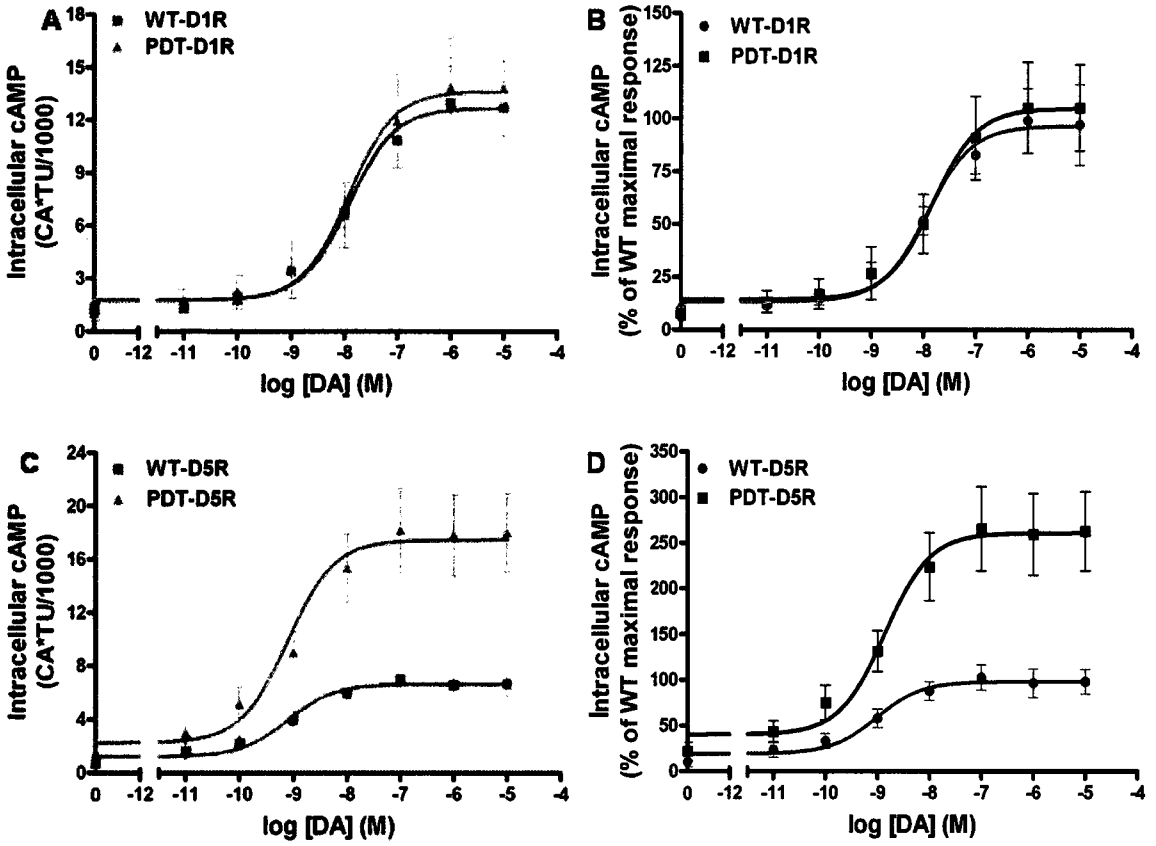


Figure 9: Average dose-response curves for dopamine of WT and mutant D1-like receptors
A and C, Average dose-response curves for dopamine in HEK293 cells expressing WT-D1R and PDT-D1R (A) or WT-D5R and PDT-D5R (C) using raw data (n=5). Each point represents arithmetic mean ± SE. Curves were given a hill slope of 1.0 as no significant difference in hill slope was observed between wild type receptors and their respective mutant using constrained and unconstrained fits. B and D, Average dose-response curves for dopamine relative to respective WT Emax values in HEK293 cells expressing WT-D1R and PDT-D1R (B) or WT-D5R and PDT-D5R (D) (n=5). Each point represents arithmetic mean ± SE. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (1.84 ± 0.37), PDT-D1R (1.53 ± 0.43), WT-D5R (1.53 ± 0.21) and PDT-D5R (1.58 ± 0.38).

	WT-D1R	PDT-D1R	WT-D5R	PDT-D5R
EC50 (nM)	11.7 [4.30-31.8]	14.9 [3.75-59.1]	0.97 [0.29-3.21]	1.39 [0.40-4.87]
Emax (CAxTU/1000)	12.7 [11.1-14.3]	13.8 [11.4-16.2]	6.71 [5.91-7.51]	17.9* [15.5-20.2]

Table 5: Average EC50 and Emax values from dose-response curves
Average EC50 and Emax values from averaged dose-response curves displayed in figure 9 A and C (n=5). Shown are best-fitted values with 95% confidence intervals in brackets. Significance of results between wild type and respective mutant receptor was analyzed using constrained and unconstrained curve fitting analysis. No significant difference in EC50 values was observed between mutants and respective mutant.
* $p < 0.05$ when compared to corresponding wild-type value.

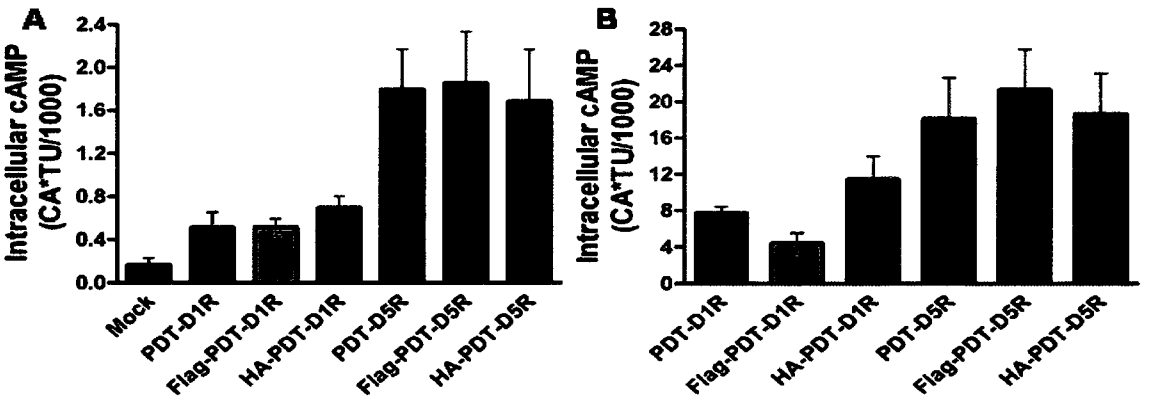


Figure 10: Basal and DA-mediated maximal activation of AC of tagged and untagged mutant receptors Basal (A) and DA-mediated maximal activation of AC (B) of tagged and untagged mutant receptors expressed in HEK293 cells (n=5). Cells were incubated 30 minutes with ascorbic acid (basal) or 10 μM dopamine (maximal activation) in the presence of IBMX. Significance of differences was assessed using one way-ANOVA and Newman-Keuls post-test. No significant difference in basal or DA-mediated maximal activation of AC between tagged mutants and their respective untagged receptor was observed. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: PDT-D1R (1.26 ± 0.26), Flag-PDT-D1R (1.38 ± 0.33), HA-PDT-D1R (1.61 ± 0.49), PDT-D5R (2.00 ± 0.59), Flag-PDT-D5R (2.14 ± 0.76) and HA-PDT-D5R (1.34 ± 0.33)

3.7 Regulation of D1R and D5R by desensitization

3.7.1 Establishing a receptor desensitization assay

To address how the loss of Ser/Thr residues of the CT impact D1-like receptor responsiveness following a brief exposure to dopamine, desensitization assays were performed in intact HEK293 cells. First assays were done in 12 well-dishes. However, high variability obtained with the results did not allow us to make compelling conclusions (Fig 11). This pitfall can possibly be explained by the low cell number from the losses of cells following successive washes. To circumvent these issues, we repeated these assays using 6-well dishes. We tested the desensitization pattern of each receptor following an exposure to 10 μM DA for 5 min. Subsequently, cells were washed twice and stimulated again with different DA concentration (1 nM, 10 nM and 10 μM). These DA concentrations were selected to probe the desensitization of wild type and mutant forms of D1R and D5R at doses mediating submaximal (~EC50) and maximal production of intracellular cAMP levels. Interestingly, striking differences in the extent of desensitization were observed between D1R and D5R (Fig. 12). While WT-D1R responsiveness produced by 10 μM DA was desensitized by 20%, WT-D5R exhibited a desensitization of approximately 50% (Fig. 12). Surprisingly, desensitization was not abolished in cells expressing PDT mutants (Fig. 12)

It is worth mentioning that these results were obtained using a washing procedure whereby each well was washed twice with 2ml of PBS at room temperature to remove DA following the five-minute DA pretreatment. To rule out that the increased D5R desensitization was not associated with its 10-fold higher affinity for DA when compared

to D1R (Table 2) thus lowering the efficiency of PBS washes for DA removal, we tested the effect of using larger volume of PBS (2 or 4 ml) during the washing procedure (Fig. 13). As seen in figure 13, the larger volume of PBS during washes did not significantly alter the desensitization of wild type and mutant receptors.

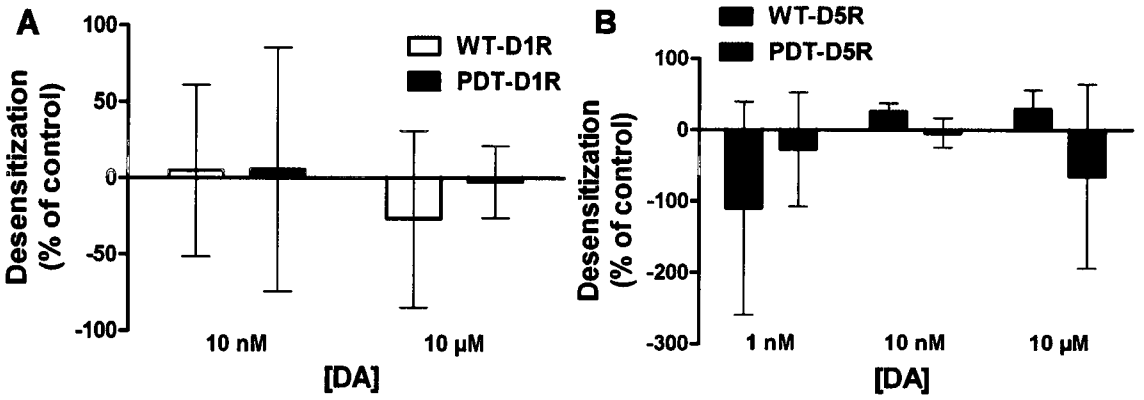


Figure 11: Desensitization of WT and PDT receptors in 12-well dishes
Desensitization of WT and PDT receptors in 12-well dishes (n=3-6). Bars represent arithmetic mean ± SE. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (1.84 ± 0.64), PDT-D1R (1.92 ± 0.34), WT-D5R (3.30 ± 2.62) and PDT-D5R (1.73 ± 0.29)

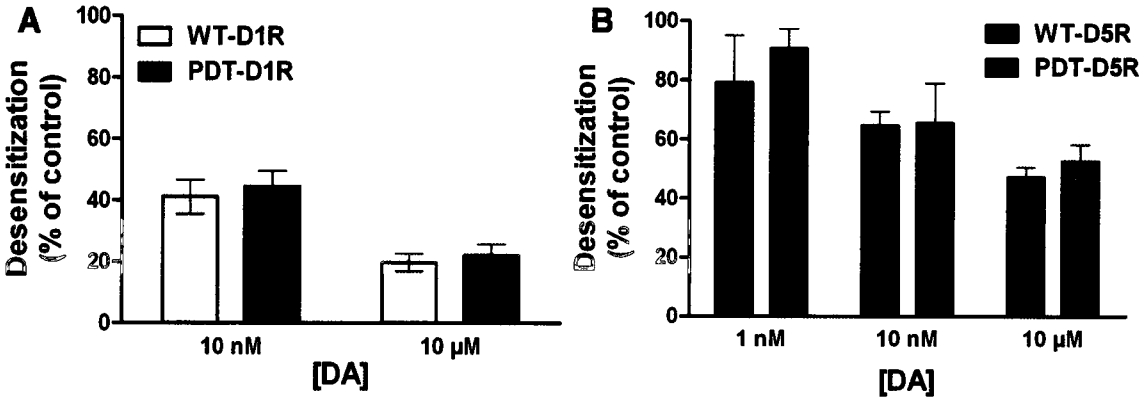


Figure 12: Desensitization of WT and PDT receptors in 6-well dishes
Desensitization of WT and PDT receptors in 6-well dishes (n=5). Bars represent arithmetic mean ± SE. Bmax (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (1.28 ± 0.22), PDT-D1R (1.48 ± 0.21), WT-D5R (1.50 ± 0.31) and PDT-D5R (2.04 ± 0.20).

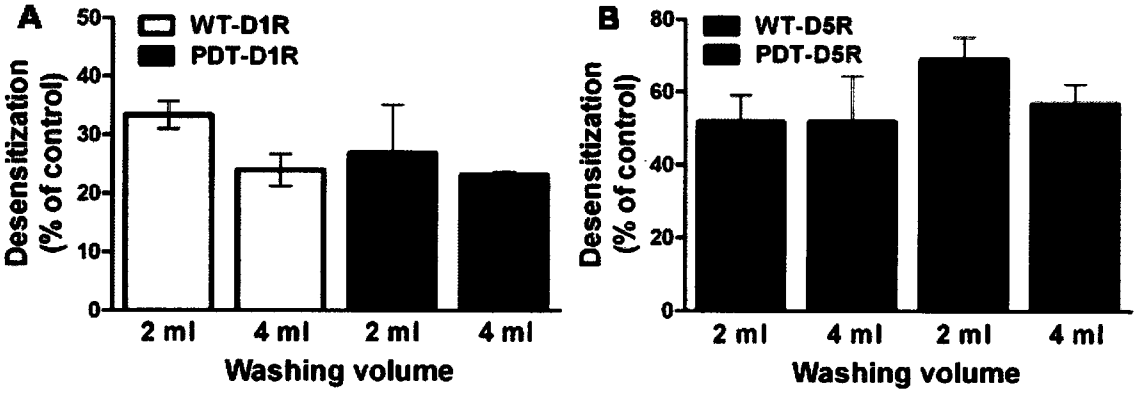


Figure 13: Influence of washing volume on short-term desensitization assay
Desensitization assay at 10 µM DA for WT-D1R and PDT-D1R (A) or WT-D5R and PDT-D5R (B) (n=3). Cells were washed twice with either 2ml or 4 ml of PBS 1X. Bars represent arithmetic mean ± SE. Significance of difference was analyzed using one way-ANOVA and Newman-Keuls post-test. No significant difference in desensitization was observed between 2ml and 4ml wash for all construct. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (1.85 ± 0.32), PDT-D1R (2.32 ± 0.84), WT-D5R (0.82 ± 0.19) and PDT-D5R (1.77 ± 0.43).

3.7.2 Loss of Ser/Thr residues of CT differentially regulate short-term DA-induced desensitization of D1R and D5R

While assays performed in 6-well dishes indicated that wild type and mutant D1R and D5R undergo rapid desensitization, it remained unclear whether mutations of putative Ser and Thr phosphorylation sites on CT modulate desensitization pattern of D1-like receptors. To address this issue in a more compelling manner, dose-response curves to DA were performed following the 5 min desensitization period to assess the functional role of Ser/Thr residues of CT in mediating G protein uncoupling (translated in a rightward shift in EC50 and a reduced Emax) of D1R and D5R. As seen in figure 12, WT-D5R display a stronger desensitization than WT-D1R following DA stimulation (Fig. 14 and Table 6) and a robust rightward shift in EC50 value (Table 6). Indeed, desensitized WT-D5R exhibited a ~16-fold rightward shift in EC50 shift while desensitized WT-D1R displayed a 4-fold shift (Table 6). No significant difference in the extent of EC50 rightward shift was observed between desensitized WT-D5R and PDT-D5R (Table 6). However, a decrease in the extent of EC50 rightward shift of about 2 fold was observed in desensitized cells expressing WT-D1R and PDT-D1R (Table 6). All receptors exhibited an inhibition of their DA-mediated maximal response following DA pretreatment (Table 6). WT-D1R and PDT-D1R displayed an Emax drop of ~15% while WT-D5R and PDT-D5R had a Emax decrease of 65% (Table 6). No significant difference in Emax drop was observed between wild type and respective mutant receptors (Table 6).

To measure the extent of desensitization of wild type and mutant forms of D1R and D5R, we also analyzed the dose-response curves with the operational model of agonism using

GraphPad Prism algorithm for receptor depletion (Fig. 15). Previously, the operational model of agonism has been applied to agonist-induced desensitization of β2AR and opioid receptors. It was established that β2AR desensitization was explained by a reduction in the signal transduction efficacy determined by the transduction constant τ (tau) values (Lohse et al., 1990). Moreover, it was shown that the reduced signal transduction efficacy of desensitized β2AR was in line with a diminution of signal transduction efficacy following β2AR depletion using alkylating agents. Comparing the τ values obtained using this model, PDT-D1R displayed a 2-fold decrease in its DA-induced desensitization relative to WT-D1R (Fig. 15). In contrast, desensitization of WT-D5R and PDT-D5R leads to a similar decrease in their signal transduction efficacy (Fig. 15). As results from figure 14 and table 6 pointed out, D5 receptor desensitization is higher than that of D1 receptor (Fig. 15). In fact, τ values suggest that receptor D5R activity was almost completely abolished following DA pretreatment (Fig. 15).

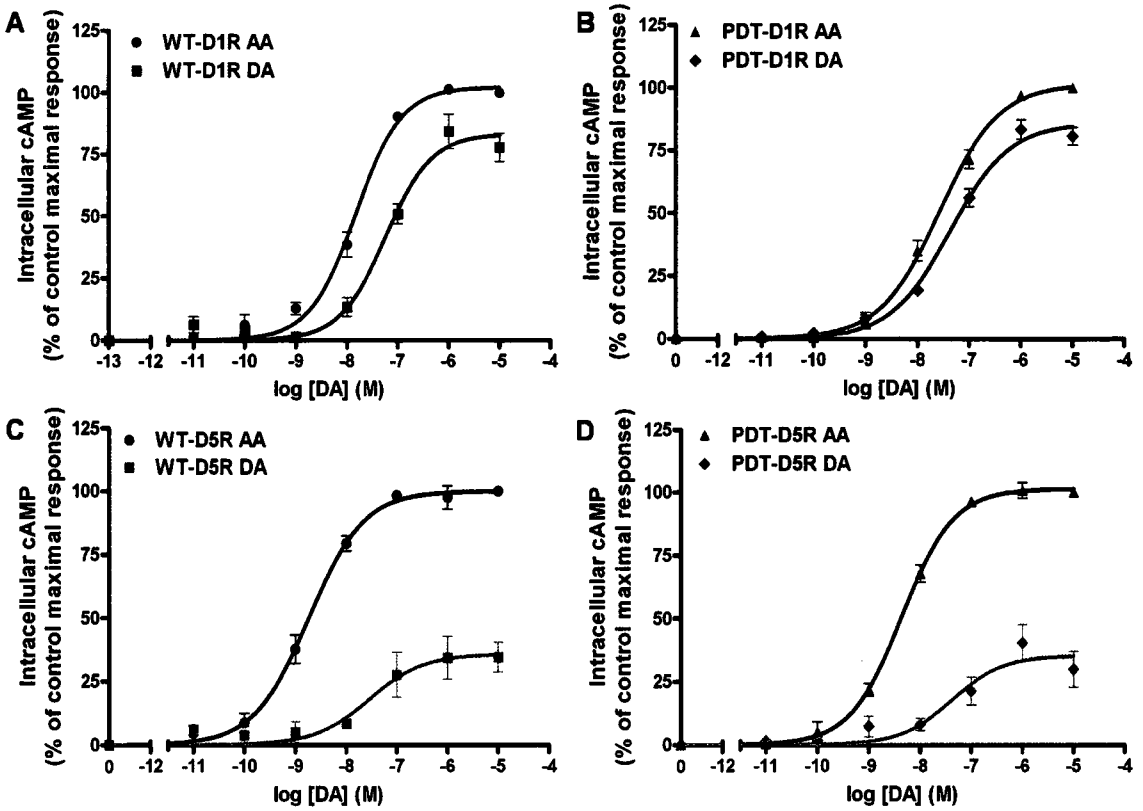


Figure 14: DA-induced dose-response curves after a previous short-term DA incubation
Desensitization of WT-D1R and PDT-D1R (A and B) or WT-D5R and PDT-D5R (C and D) (n=5). Curves represent AA or DA pretreated HEK293 cells relative to AA stimulated Emax. Basal values were subtracted and curves were simultaneously analyzed using GraphPad Prism. Each point represents arithmetic mean ± SE. Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (2.40 ± 0.97), PDT-D1R (1.83 ± 0.24) WT-D5R (1.68 ± 0.32) and PDT-D5R (1.24 ± 0.24).

	EC50 (nM) Control	EC50 (nM) Treated	Emax Control	Emax Treated
WT-D1R	**14.9** [11.0-20.3]	**57.5*** [39.0-84.7]	**102** [97.1-108]	**83.3*** [77.3-89.3]
PDT-D1R	**26.9** [20.6-35.1]	**42.6*** [31.1-58.4]	**102** [97.2-106]	**85.8*** [81.3-90.3]
WT-D5R	**1.84** [1.23-2.75]	**28.6*** [8.72-93.6]	**100** [94.5-106]	**35.8*** [29.3-42.3]
PDT-D5R	**4.36** [3.14-6.07]	**41.4*** [15.3-112]	**102** [96.5-106]	**35.4*** [29.6-41.1]

Table 6: Best-fitted EC50 and Emax values of desensitization assay curves
Shown are best-fitted EC50 and Emax values with 95% confidence intervals of desensitization assay curves. *p>0.05 when compared with control value. Statistical significance of differences between best-fitted control and treated values was assessed using unconstrained and constrained curves fitting analysis.

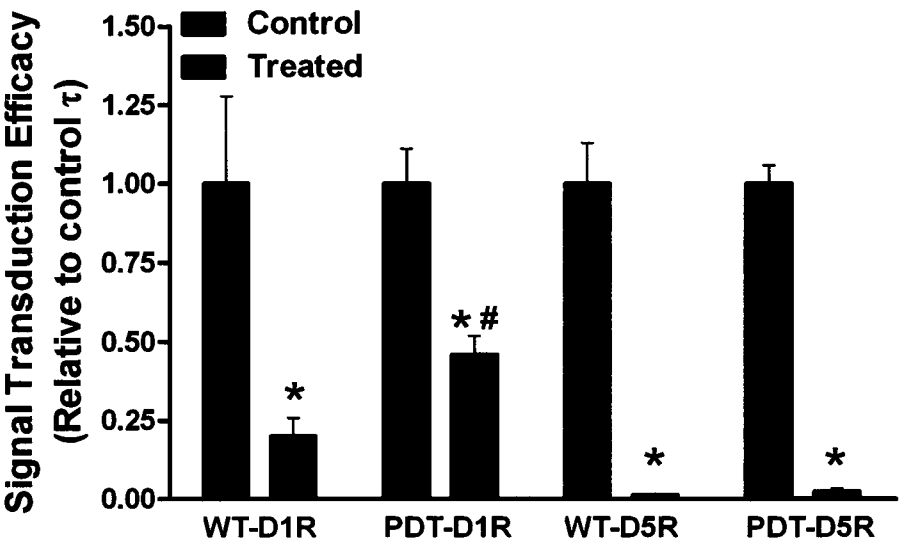


Figure 15: Signal transduction efficacy (τ) relative to control values of WT and PDT mutants
Signal transduction efficacy (τ) expressed relative to control values obtained for WT and PDT mutants (n=5). Curves shown in figure 14 were simultaneously analyzed using an operational model of agonism using unconstrained and constrained τ values. Best-fitted τ value for control condition was set to 1 and expressed with 95% confidence intervals. *$p<0.05$ when compared to respective control value and #$p<0.05$ when compared to respective WT.

3.8 Ser/Thr residues of CT are critical in long-term regulation of D1-like receptors by DA

Another important aspect of modulation of receptor signaling is down-regulation. Following prolonged treatment with agonists, GPCRs endocytose and degrade, leading to a reduction of the number of available binding sites (Bmax) (Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). To test the role of Ser and Thr residues of CT in the down-regulation of D1R and D5R, HEK293 cells were stimulated with 100 μM of DA at three time points (15 min, 1 h and 24 h). The 15-min time point was chosen to validate that differences in desensitization observed in figure 14 and table 6 was not explained by a rapid down-regulation of receptors. The other two time points were chosen to monitor down-regulation at its earliest onset (1 h) or after long-term treatment (24 h). Significant differences in down-regulation pattern were observed between receptors (Fig. 16). WT-D1R only displayed DA-induced down-regulation at 24 h of DA treatment while down-regulation of WT-D5R is only detected at 15 min and 1 h of DA treatment (Fig. 16). Moreover, differences were also observed between WT receptors and their respective PDT mutant (Fig. 16). PDT receptors failed to display any down-regulation at the three time points used in the assay (Fig. 16). In fact, both mutant receptors displayed a significant up-regulation of Bmax levels following exposure to DA for 24 h (Fig. 16). This effect was even stronger for PDT-D5R than PDT-D1R. PDT-D5R exhibited a 3.5-fold increase in its Bmax value compared to approximately 1.5-fold for PDT-D1R (Fig. 16). The difference observed between long-term regulation of receptors after 24 h of DA treatment was not due to ascorbic acid (vehicle for DA) as Bmax values detected at 15 min and 24 h were not significantly modified for all receptors incubated with ascorbic acid (Fig. 17).

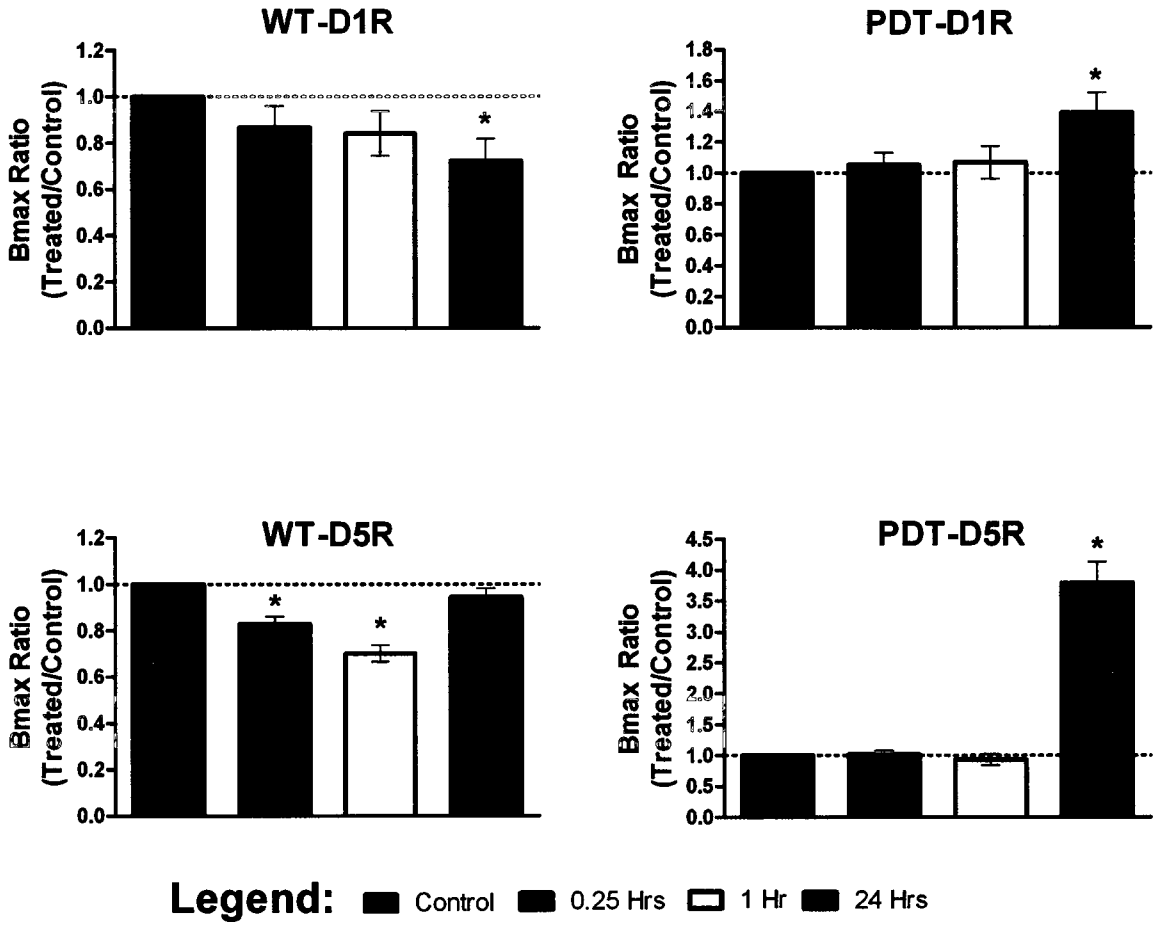


Figure 16: Regulation of membrane expression of WT and PDT D1-like receptors following DA treatment for 15 min, 1 h and 24 h

Regulation of WT and PDT D1-like receptors following DA treatment for 15 min, 1 h and 24 h (n=5-7). Control (15 min and 24 h stimulation with ascorbic acid) Bmax values were set at 1. DA-stimulated values for the three time points were normalized against control values. Value lower than 1 signifies receptor down-regulation while a value above 1 represents receptor up-regulation. Bars represent arithmetic mean ± SE. Control Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (2.52 ± 0.55), PDT-D1R (1.57 ± 0.15), WT-D5R (1.25 ± 0.15) and PDT-D5R (1.28 ± 0.15) *$p<0.05$ when compared to a hypothetical value of 1 using a one-sample t-test.

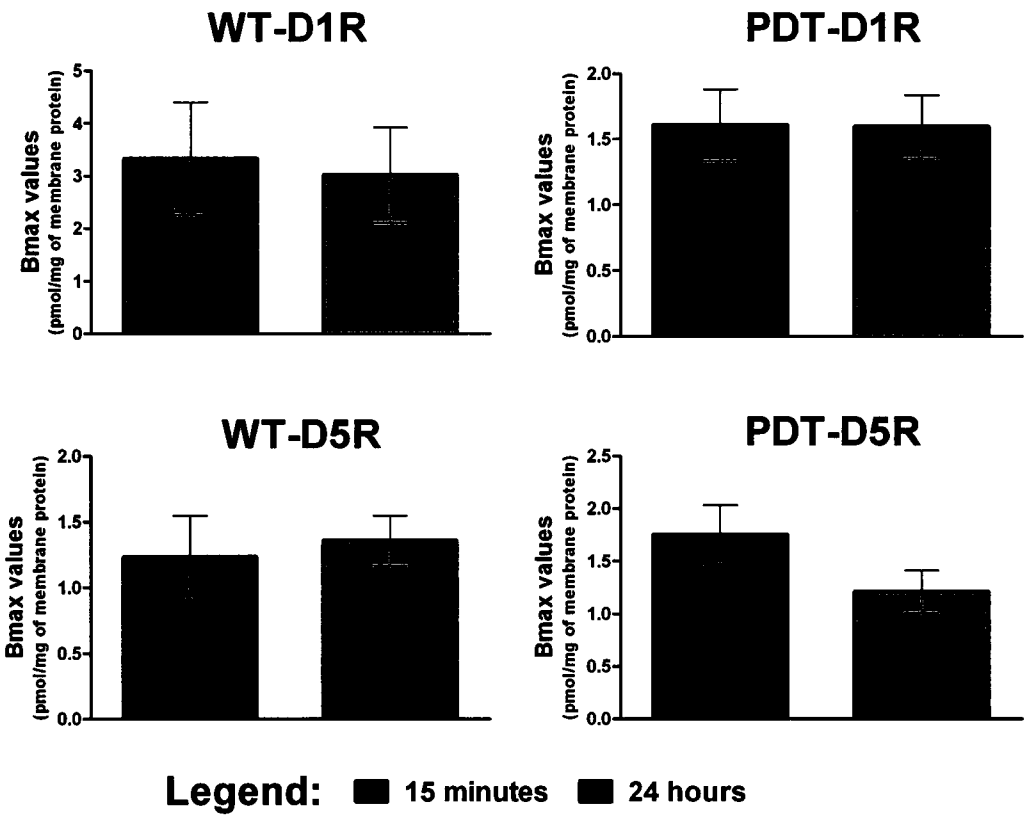


Figure 17: Control Bmax values of down-regulation assay following exposure to ascorbic acid for 15 min and 24 h

Control Bmax values of down-regulation assay (figure 16) following exposure to ascorbic acid for 15 min and 24 h (n=5-7). Bars represent arithmetic mean ± SE. Significance of difference was assessed using a one-sample t test. No difference in Bmax was detected between each receptor treated for 15 min and 24 h with ascorbic acid. (n=5-7)

3.9 Long-term regulation properties of WT and PDT receptors are controlled in a ligand-specific manner

To test the effect of drug specificity, down-regulation assays were done using a dopaminergic receptors inverse agonist (thioridazine). HEK293 cells were stimulated for 24 h with 100 μM DA and 1 μM thioridazine. In contrast to DA, thioridazine treatment had no effect on Bmax levels of D1R and D5R (Fig. 18). However, PDT receptors still displayed up-regulation when treated with thioridazine, albeit to a significantly lower level than with DA (Fig. 18). Moreover, differences in long-term regulation pattern between thioridazine and DA were significant for both WT receptors (Fig. 18).

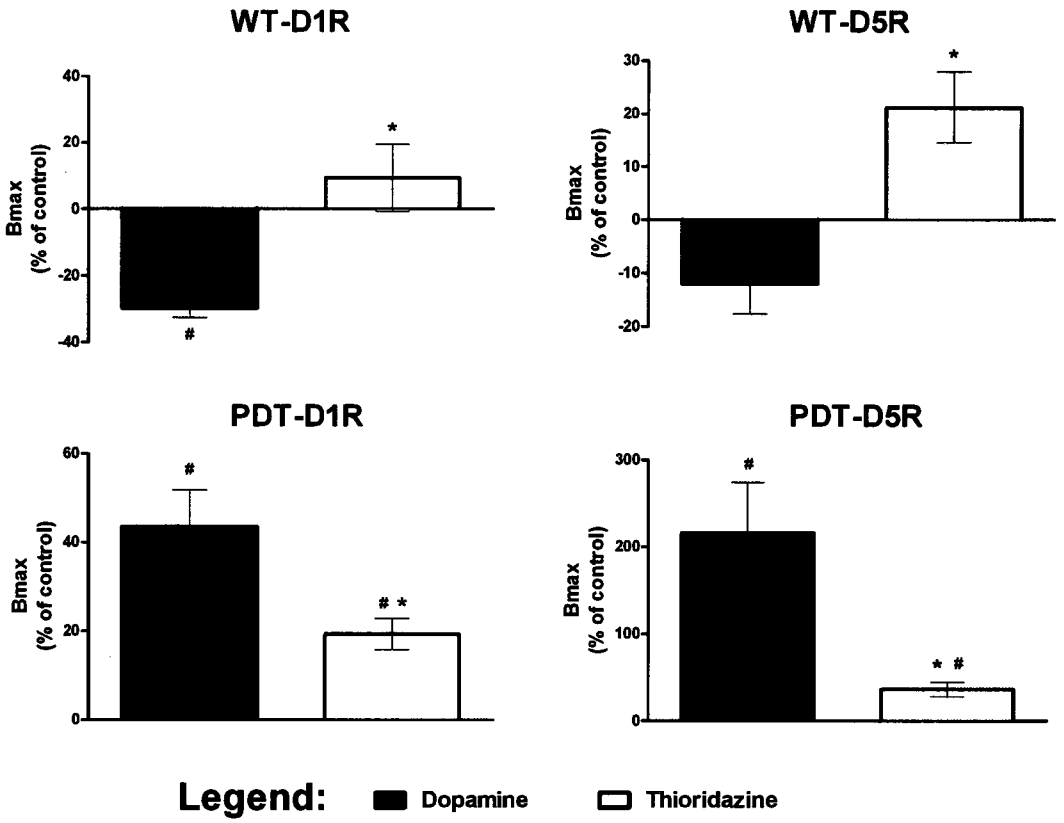


Figure 18: Regulation of WT and PDT D1-like receptors following 24 h treatment with DA or thioridazine

Regulation of WT and PDT D1-like receptors following 24-h treatment with DA or thioridazine (n=4). DA and thioridazine treated value were normalized against control (24 h treatment with ascorbic acid) and reported as the percentage of change relative to Bmax control value. Negative value signifies receptor down-regulation while a positive value represents receptor up-regulation. Bars represent arithmetic mean ± SE. Control Bmax values (pmol/mg of membrane proteins ± SE) were as follows: WT-D1R (1.14 ± 0.10), PDT-D1R (1.20 ± 0.31), WT-D5R (1.65 ± 0.16) and PDT-D5R (1.98 ± 0.28) *$p<0.05$ when compared to DA-treated values using a one-sample t-test and $^{\#}p<0.05$ when compared to zero (no effect) using a one-sample t-test.

Discussion

4.1 Importance of the CT in regulating D1-like receptors folding and plasma membrane expression

Protein maturation is a complicated and tightly regulated process. Strict folding restrictions as well as post-translational modifications are essential for receptor placement into the plasma membrane. Thus, modification at the primary level of GPCRs can have drastic consequences in the receptor maturation process. Results obtained with PDT mutants attest to that as both mutants receptors displayed Bmax values significantly lower than their WT counterpart (Table 1). This effect was very important for PDT-D5R who displayed a decrease close to 75% in Bmax values compared to WT-D5R (Table 1 and Fig. 5). Our results imply that removal of Ser/Thr of the CT potentially modify D1-like receptors three dimensional structure. This structural change could be recognized by the ER quality control machinery and lead to an increase of receptor degradation. Mutant receptors could also have difficulty binding to molecular chaperone such as DRIP78, limiting their capacity to mature efficiently (Bermak et al., 2001; Wang et al., 2008). As well, it is possible that mutant receptors cannot bind to proteins responsible for their shuttling from the Golgi apparatus/ER to the plasma membrane, leading to strong ER retention and decreased Bmax value (Bermak et al., 2002). This decrease in Bmax value following truncations or mutations of the CT of GPCRs was also observed by our and other groups previous studies (Chaar et al., 2001; Jackson et al., 2002; Kim et al., 2004; Lamey et al., 2002; Schroder et al., 2009). It is particularly interesting that in three of those studies (Chaar et al., 2001; Jackson et al., 2002; Schroder et al., 2009), immunofluorescence assay performed on the mutant receptors displayed a stronger intracellular immunostaining than for WT. This observation further suggests that

sequences modulating plasma membrane localization of D1-like receptors are present in their CT.

4.2 Rethinking the conventional role of cytoplasmic regions of GPCRs

As mentioned in section 1.2.1, specific regions of GPCRs have been demonstrated to play predetermined roles in receptor signaling output and regulation. These functions are accepted by the GPCR scientific community and are considered to be almost universal for all GPCRs. However, previous results by our lab and results obtained from this study tend to demonstrate a more complex regulation of D1-like receptors pharmacological properties (Chaar et al., 2001; Charpentier et al., 1996; Demchyshyn et al., 2000; Iwasiow et al., 1999; Jackson et al., 2000; Lamey et al., 2002; Tumova et al., 2003; Tumova et al., 2004). Our results arguably present an interesting starting point to rethink the conventional views about specific roles of cytoplasmic regions of GPCRs in ligand binding pocket function.

4.3 Ser/Thr residues of CT regulate the binding pocket of D1-like receptors

Previous studies done by our lab pointed to the CT as a modulator of D1-like receptor affinity for agonists. Results obtained during my master degree further specify the residues implicated in those observations. Results from table 2 and figure 6 clearly demonstrate a gain of affinity for DA as well as DHX for PDT-D5R as well a small gain in agonist affinity for PDT-D1R. Moreover, a small increase in affinity for thioridazine was also noted for PDT-D5R. These results imply that the presence of D1R and D5R

Ser/Thr residues of CT is important for the formation and maintenance of D1-like receptor binding pocket either directly or indirectly.

It is important to note that while crystal structures of different GPCRs have been made (Cherezov et al., 2007; Jaakola et al., 2008; Palczewski et al., 2000; Rasmussen et al., 2007), only one of them included the CT in their crystal structure (rhodopsin). Moreover, some of them (Cherezov et al., 2007; Jaakola et al., 2008; Rasmussen et al., 2007) even removed or importantly modified the IL3 when performing the crystallography. These omissions are due to the high mobility of these intracellular regions that does not permit the structural rigidity required for X-ray crystallography (Kobilka and Schertler, 2008). Thus, no current crystal structure is available to directly assess the role of the CT on the ligand binding pocket. Nonetheless, our results clearly demonstrate the importance of considering the CT when analyzing GPCR crystallography structure, especially when analyzing their binding pocket. Recent results from our lab also have pointed out to a role for TM1 specific residues in generating compound binding specificity towards D1R or D5R (D'Aoust and Tiberi, 2010). Crystal structures have demonstrated the proximity of TM1 and TM7 in the binding pocket. Being that TM7 is attached to the CT, it is thus possible that alterations in the CT could change the orientation of TM7 and TM1. This would lead to an increased exposition of TM1 residues, generating the higher affinity state for agonists seen with PDT-D5R and to a lesser extent PDT-D1R.

Another recent report could help explain my observations (Ahn et al., 2009). Using a computational model, Ahn et al. indicated a possible localization of the cannabinoid one receptor (CB1R) CT alongside TMDs. This localization would enable direct interaction

with the different TMDs, principal components of the ligand binding pocket of GPCRs. This would be particularly interesting if the same model could be applied to D5R given the important gain in affinity for DA and DHX observed with PDT-D5R. However, it is important to note the limitations of this comparison. CB1R CT is significantly shorter than D5R (72 amino acids versus 109). A longer CT could prove to be more difficult to place inside the cell cytoplasmic membrane. Moreover, CB1R CT possesses numerous hydrophobic amino acids, permitting the arrangement of the tail into alpha helixes. Alpha helixes forms the majority of TMDs, permitting their intra-membranous localization (Kristiansen, 2004). In that respect, the replacement of CT serine and threonine residues by alanine and valine (two hydrophobic amino acids), could have provided the necessary overall hydrophobicity to permit the placement of the CT alongside the TMDs. Further assessment of the position of the CT relative to the TMD will be important to fully determine the extent of CT involvement on ligand binding pocket formation.

4.4 Ser/Thr residues of CT modulate DA-independent and dependent D5R signaling output

As with DA affinity, previous results from our group demonstrate the importance of the CT in governing D1R and D5R subtype-specific constitutive activity (Chaar et al., 2001; Iwasiow et al., 1999; Jackson et al., 2000; Tumova et al., 2003; Tumova et al., 2004). As seen from figure 8, a strong increase in constitutive activity could be observed from PDT-D5R but not PDT-D1R. In fact, constitutive activity of PDT-D1R is almost identical to WT-D1R. It is possible that the mutations-induced conformational change for D5R is more pronounced than for D1R, eliciting a stronger constitutive signaling output. This effect could come from a change in the thermodynamic equilibrium for PDT-D5R. By

augmenting the adoption of the R* active receptor conformation, mutations of Ser/Thr of CT could increase the total number of receptors at the membrane with the ability to couple to G protein, leading to the increase in constitutive activity.

However, it is also possible that the constitutive activity increase from PDT-D5R is emanating from a higher capacity of the receptor to couple to G protein and its other signaling partners. Evidences point out to a pre-coupling of G proteins and AC to GPCRs, forming a signalosome (Rebois and Hebert, 2003). Therefore, it is possible that D5R CT mutations increase the signalosome formation frequency simply by limiting the physical hindrance normally caused by the CT. This would imply that CT movement is due to its lack of phosphorylation or by the removal of possible hydrogen bonds between the CT and other intracellular regions. It is important to note that hydrogen bonds are usually pretty weak and are thus less likely to serve as a strong docking link between the CT and IL3. Moreover, while the idea of potential loss of CT negative charges modulating CT position from IL3 is intriguing, no evidence points towards constitutive or agonist-induced D5R phosphorylation. In fact, no report of D5R phosphorylation has currently been published as opposed to D1R, which displays constitutive and agonist-induced phosphorylation (Jackson et al., 2002; Kim et al., 2004; Lamey et al., 2002).

As was the case with constitutive activity, PDT-D5R DA-mediated maximal activation of AC was greatly augmented compared to WT-D5R, a result not duplicated with PDT-D1R (Fig. 9). This increase in DA-mediated maximal activation of AC by PDT-D5R closely match the observations made with the constitutive activity (3-fold increase) consistent with the idea that PDT-D5R display a greater capacity to adopt the R* conformation,

generating a "super-active" version of WT-D5R. This observation is closely similar to recent reports with the chemoattractant receptor homologous molecule expressed on T helper type 2 cells (CRTH2) (Schroder et al., 2009) and D1R (Chaar et al., 2001). Both groups noticed an increase in signaling when the CT was completely removed leading to the proposition that the CT act as a signaling inhibitor for the WT receptor. Given the results from my study, it could be possible that the observations made with PDT-D5R are due to a loss of this effect following Ser/Thr removal.

4.5 WT-D1R and WT-D5R display drastically different short-term desensitization patterns

Short-term desensitization properties of WT-D5R are ill-defined (Jarvie et al., 1993; Le Crom et al., 2002; Le Crom et al., 2004). Results reported in figure 14 and table 6 show striking differences in agonist-induced desensitization between WT-D1R and WT-D5R. Indeed, based on Emax and τ value (Fig. 14 and 15 and Table 6), WT-D5R short-term desensitization is drastically higher than WT-D1R. Moreover, G protein uncoupling following DA-treatment is much more important for WT-D5R than WT-D1R (16-fold vs. 4-fold) (Table 6). These results imply a more robust regulation of D5R signaling output. This strong regulation could also explain the weaker DA-mediated activation of AC for WT-D5R than WT-D1R observed by others and in the present study (Sunahara et al., 1991; Tiberi et al., 1991) (Fig. 9 and Table 5). It is important to consider this aspect as our short-term desensitization assay is designed to have two periods of DA stimulation of 5 and 10 minutes respectively. In that respect, the first 5 minutes DA stimulation almost completely abolishes D5R signaling output. Thus, it is entirely possible that D5R signaling output during our maximal stimulation assay, which last 30 minutes, is affected

by D5R desensitization. In that regard, D1R would continue to produce cAMP throughout the 30 minutes while D5R cAMP production is greatly slowed down by its desensitization.

While the assay is performed in HEK293 cells, the differences in short-term desensitization between the two receptors could also potentially indicate a greater need for CNS neurons to regulate D5R signaling over D1R. Little is known about D5R physiological role as its brain localization is relatively punctuate (Ariano et al., 1997; Missale et al., 1998). Its high mRNA expression level in the hippocampus as well as its regulation of acetylcholine release in this brain region was hypothesized to implicate WT-D5R in playing an important role in cognitive functions (Holmes et al., 2004). However, studies regarding D5R brain function are currently lacking in order to evaluate the physiological relevance of short-term desensitization differences between D1R and D5R.

4.6 Ser/Thr residues of CT modulate D1R but not D5R short-term desensitization

Interesting observations were made from desensitization studies of PDT receptors (Fig. 14 and 15 and Table 6). PDT-D5R did not display any difference in its short-term desensitization when compared to WT-D5R. This is especially surprising considering the extent of WT-D5R desensitization. These results indicate that D5R desensitization is not dependent upon phosphorylation of its CT. This goes against the canonical model of GPCR regulation where phosphorylation of the CT and IL3 is essential for arrestin binding, G protein uncoupling and internalization of the receptor (Luttrell, 2008).

However, this model was based mostly on studies done with β-adrenergic receptors. At that time, results from these studies were thought to be a universal rule amongst GPCRs. While this desensitization model has been proven to be right with most receptors (Luo et al., 2008; Luttrell, 2008; Premont and Gainetdinov, 2007; Ricks and Trejo, 2009), more and more evidence begin to emerge to cast some doubts over the universality of this canonical model. Some examples of phosphorylation-independent regulation were demonstrated for D1R, D2R, the μ-opioid receptor and the metabotropic glutamate 5 receptor (mGluR5) (Chu et al., 2010; Jackson et al., 2002; Lan et al., 2009; Namkung et al., 2009; Ribeiro et al., 2009). These results were surprising considering that MOR could go through phosphorylation-induced desensitization when stimulated with D-Ala(2), N-Me-Phe(4), Gly(5)-ol]-enkephalin but not with morphine (Chu et al., 2010). Interesting results also came from D2R studies (Lan et al., 2009; Namkung et al., 2009). In this case, loss of phosphorylation sites in its IL3 did not impair D2R agonist-induced desensitization (Namkung et al., 2009). In fact, impairment of D2R desensitization was observed only when mutating a certain section of its IL3 (residues 212 to 215), devoid of phosphorylation sites (Lan et al., 2009). This region was demonstrated to be important for arrestin binding (Lan et al., 2009). These different phospho-independent desensitization mechanisms could be an interesting point to investigate in light of the drastic differences in short-term desensitization observed between WT-D1R and WT-D5R.

Opposite results from PDT-D5R were seen with PDT-D1R (Fig 14 and 15 and Table 6). PDT-D1R displayed a decrease in its short-term desensitization when compared to WT-D1R (Fig. 14 and 15 and Table 6). This suggests that in contrast to D5R, D1R short-term desensitization is dependent on the phosphorylation of its CT. However, some

desensitization remained after the removal of putative D1R CT phosphorylation sites indicating that CT phosphorylation is not essential for D1R short-term desensitization (Fig. 14 and 15 and Table 6). These results are in agreement with previous results from our group (Jackson et al., 2002). Removal of CT of D1R led to a complete abrogation of phosphorylation without completely eliminating desensitization (Jackson et al., 2002). These results combined with those obtained during my Master studies indicate that the observations made previously by Jackson et al. were likely explained by the loss of Ser/Thr residues of D1R CT.

Our results also come in contradiction with the model for D1R desensitization put forward by Kim et al (Kim et al., 2004). In their model, D1R CT acts as lid to inhibit arrestin IL3 binding. Once stimulated with an agonist, DIR CT is phosphorylated and move away from the IL3, enabling arrestin binding. While our results suggest that D1R CT phosphorylation modulate some of its desensitization, the fact that desensitization still occurs even with PDT mutants suggests that other desensitization mechanisms are in play. According to the model proposed by Kim et al., PDT mutants should not be able to desensitize given the lack of CT phosphorylation. While phosphorylation is the main regulatory process for arrestin binding to GPCRs, studies have shown that arrestin can bind to receptors in a phosphorylation-independent manner (Gurevich and Gurevich, 2006, 2008; Namkung et al., 2009). These studies suggest that arrestin detects and binds to the activated receptor conformation. This is an especially interesting avenue for the desensitization of D1-like receptors as both receptors possess phosphorylation-independent desensitization mechanisms. This phenomenon could also help explain the discrepancies observed between WT-D1R and WT-D5R with respect to their own

desensitization. D5R, being a more constitutively active receptor than D1R, could adopt an active conformation with a higher affinity for arrestin, increasing its binding. This would then lead to stronger desensitization for D5R, as seen in figure 14. Further studies are needed to uncover the molecular mechanisms underlying D5R desensitization.

4.7 Differences in the down-regulation kinetics of D1R and D5R

According to the canonical model of GPCR regulation, arrestin receptor binding leads to receptor internalization followed by its recycling back to the plasma membrane or its degradation (Hanyaloglu and von Zastrow, 2008; Luttrell, 2008; Marchese et al., 2008). Our studies suggest that D1R and D5R down-regulate following long-term DA treatment (≥1h) with distinct kinetics and amplitude (Fig. 16). While WT-D1R displayed down-regulation only after 24 h DA exposition, WT-D5R DA-induced down-regulation was observed at 15 min and 1 h treatment but was not detected at 24 h (Fig. 16). The underlying molecular mechanisms for the lack of detectable WT-D5R down-regulation at 24 h are unclear. A possible explanation could be that WT-D5R down-regulation is somewhat inhibited when DA treatment is prolonged. This would lead to the increase of functional membrane expression level of the receptor through *de novo* synthesis. This inhibition could possibly be mediated through prolonged PKA activation. However, PKA has been demonstrated to regulate GPCR protein expression by inhibiting DNA translation through phosphorylation of regulatory transcription factors (Bohm et al., 1997). On the other hand, podophyllotoxin (a microtubule disrupter) treatment of cells has been shown to increase melanocortin-4-receptor expression (a G_s-coupled GPCR) through PKA activation (Chen and Xie, 2009).

Another explanation could come from changes in the intracellular routing of D5R. Intracellular sorting is a complex and tightly regulated mechanism (Bohm et al., 1997; Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). While some receptors display a defined endocytic fate (recycling or degradation) following agonist stimulation, others can alternate between the two possibilities. Studies done on β-adrenergic receptors demonstrated that endocytic routing can be switched (Marchese et al., 2008). Following prolonged agonist stimulation (approximately 20 hours), β2AR are targeted to lysosome for degradation instead of their usual recycling pattern. Given the pronounced down-regulation of WT-D5R observed after 1 hour DA stimulation, it is possible that D5R prolonged DA stimulation leads to a switch in its intracellular sorting. This would permit the functional expression level of the receptor to return closer to control level, as seen in figure 16.

4.8 Mutations of Ser/Thr residues of CT abolish D1-like receptor down-regulation

PDT receptors exhibited drastically different long-term regulation pattern than their respective WT counterpart (Fig. 16). In fact, PDT receptors failed to down-regulate at any of time points studied (Fig. 16). Moreover, PDT receptors both displayed an up-regulation of their Bmax values following 24 hours DA stimulation (Fig. 16). This up-regulation was stronger for PDT-D5R (~4-fold) than PDT-D1R (~1.5-fold) (Fig. 16). Notably, this effect was DA-dependent as treatment with ascorbic acid alone (DA vehicle) has no impact on Bmax values (Fig. 17).

Therefore, our results suggest a more specific role of CT phosphorylation in long-term DA-induced trafficking of the receptor. This is surprising considering that both receptors were still able to desensitize despite mutations of Ser/Thr phosphorylation sites of CT (Fig. 14 and Table 6). Results from figure 16 suggest that endocytic proteins recruitment, necessary for receptor internalization and down-regulation, is a phosphorylation-dependent process. Phosphorylation-dependent intracellular trafficking was recently reported for D1R (Mason et al., 2002). Mutation of a putative PKA phosphorylation site in the IL3 of D1R modified DA-induced D1R intracellular localization without affecting the receptor internalization rate (Mason et al., 2002). Phosphorylation of the CT has also been demonstrated to be important for GPCRs internalization, such as the protease-activated receptor 2 (PAR2) (Ricks and Trejo, 2009). In this report, Ricks and Trejo demonstrated that PAR2 phosphorylation modulates the internalization mechanisms employed for its internalization. WT receptors internalized through the canonical dynamin, clathrin and β-arrestin pathway. However, phosphorylation-deficient CT mutant displayed internalization through a dynamin but not clathrin and β-arrestin pathway. This differential regulation of internalization mechanisms by CT phosphorylation could help explain the differences observed in long-term DA-mediated regulation of PDT mutants and WT receptors. This lack of phosphorylation could also impair the post-endocytic fate of the receptor, as observed with D2R (Namkung et al., 2009). Namkung et al. demonstrated that phosphorylation of IL3 of D2R is necessary for proper recycling of the receptor (Namkung et al., 2009). These results indicate that phosphorylation of GPCRs is important for receptor internalization and intracellular sorting.

My results can be potentially explained by an alternative model of desensitization/long-term regulation of D1-like receptors by DA (Fig. 19). In contrast to the model presented by Kim et al., D1-like receptors when activated by DA would adopt a specific conformation leading to arrestin binding (Fig. 19 #1 and 2). This event would mediate another conformation change, enabling the phosphorylation of the CT of the receptor by GRKs (Fig. 19 #3). Phosphorylated D1-like receptor would then act as a docking site for endocytic proteins leading to its internalization and proper post-endocytic fate (Fig. 19 #4 and 5). This alternative model accommodates the desensitization and down-regulation properties observed for PDT-D1R and PDT-D5R. In this model, the CT of D1-like receptors would not serve as a lid to inhibit arrestin access to the IL3 but would act instead as a docking site for endocytic proteins.

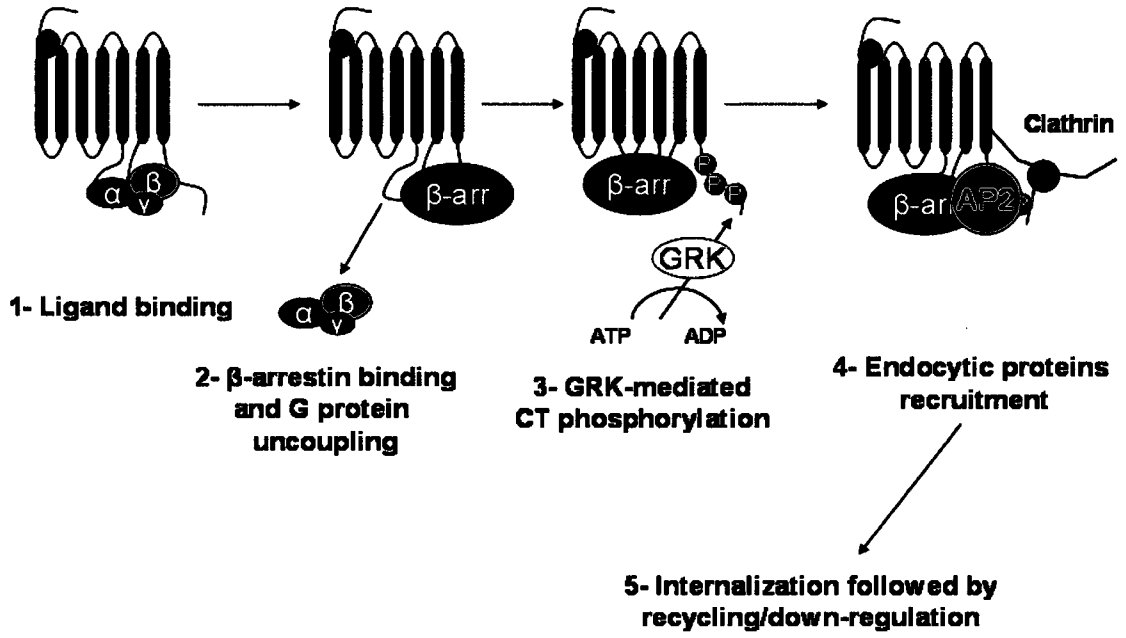


Figure 19: Alternative model of regulation of D1-like receptors

The up-regulation observed with PDT receptors is puzzling (Fig. 16). DA is not known to act as a pharmacological chaperone and cannot cross the plasma membrane in the absence of DA transporter due to its hydrophilic nature. While an increase in transcription for the mutant receptors following prolonged DA stimulation would be a logical explanation, it is also very unlikely. The expression vector used to clone cDNA encoding for the mutant receptors does not possess transcriptional regulatory sequences. In fact, this expression vector contains the immediate early promoter region of the human cytomegalovirus (CMV). The CMV promoter is constitutively active and thus protein expression derived by this promoter is not subjected to a regulation by transcription factors.

I propose three hypotheses to explain the up-regulation of PDT mutants following long-term treatment with DA (Fig. 20). Firstly, as stated previously, conformation of PDT mutants may favor a strong ER retention, delaying their expression at the cell surface. Importantly, my data show that the mutant receptors expressed at the cell surface are functional as they elicit adenylyl cyclase activity (Fig. 9). However, these PDT mutants may display an internalization-prone conformation either due to an instability of their conformation or by facilitating the docking of endocytic proteins leading to constitutive internalization and to their potential intracellular sequestration or degradation (Fig. 20A). DA binding to PDT receptors could possibly stabilizes an active conformation that reduces endocytosis and degradation (Fig. 20B). Over time, the decrease in receptor degradation would increase the functional receptor expression level (Bmax) at the plasma membrane as receptor shuttling from ER to the cell surface would not be altered (Fig. 20B).

Secondly, extracellular DA can generate reactive oxygen species (ROS) (Cookson, 2003; Tabrizi et al., 2000; Xu et al., 2002). ROS have been demonstrated to be especially hurtful to cells, most often leading to their death (Colapinto et al., 2006; Jiang et al., 2008). ROS generation has also been demonstrated to increase plasma membrane permeability (Pun et al., 2009). This may enable the entry of extracellular components such as intact DA into the cell (Fig. 20C). Once inside the cell, DA could serve as a pharmacological chaperone acting on receptors retained in the ER (Fig. 20C). DA-induced stable conformation of PDT receptor would facilitate the shuttling from the ER to the plasma membrane. At the plasma membrane, receptors would also have the added benefit of having extracellular DA to maintain the stabilization of their conformation. As with the previous hypothesis, this would decrease receptor degradation, translating into a higher Bmax value. Thirdly, ROS have also been demonstrated to activate intracellular signaling pathways (Brown and Griendling, 2009). Therefore, ROS may lead to activation of intracellular signaling pathways promoting up-regulation of PDT receptors (Fig. 20D). It is also possible that DA, through PDT receptors, activates intracellular signaling pathways responsible for the up-regulation of PDT receptors (Fig. 20D).

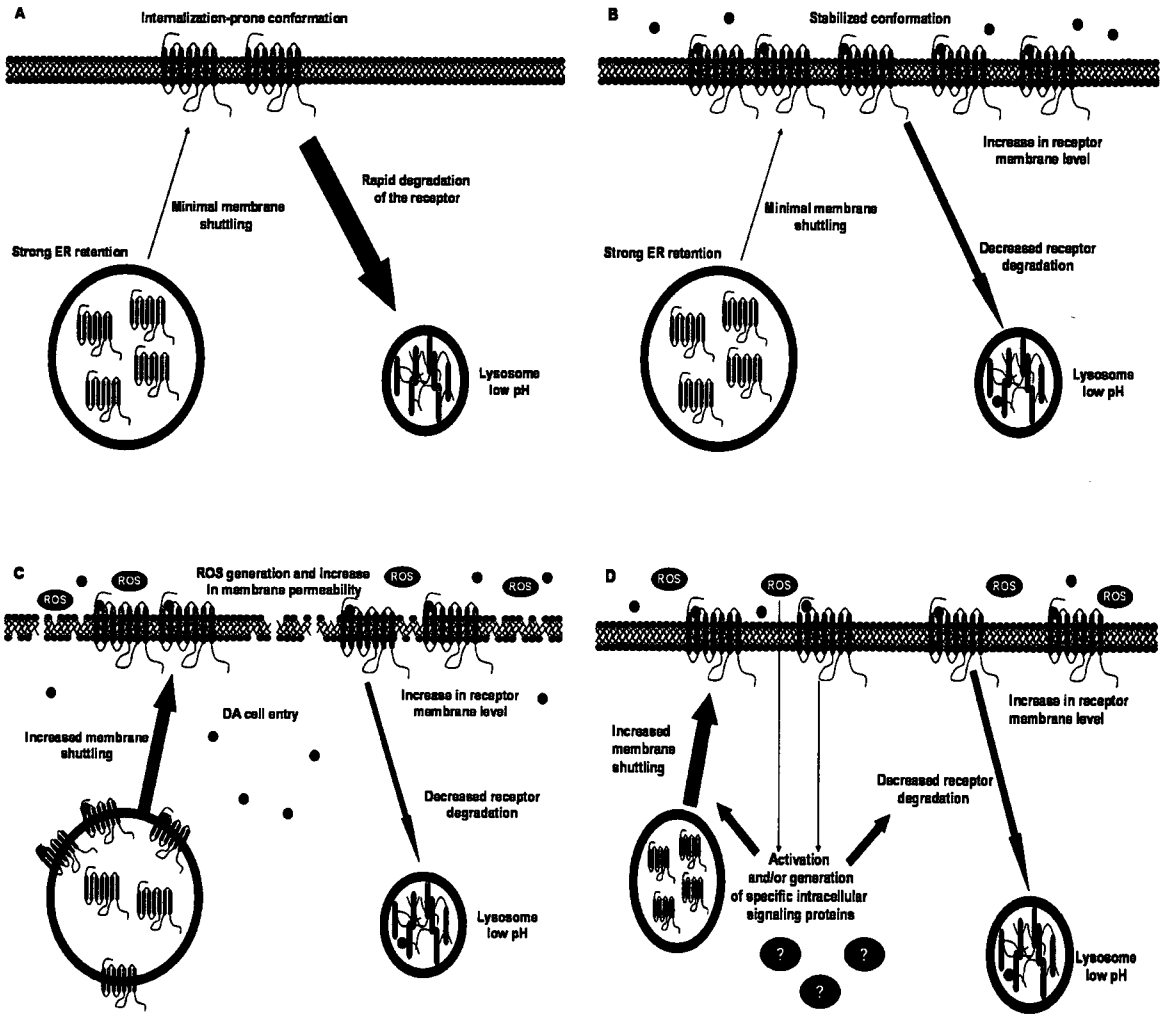


Figure 20: Main hypotheses for PDT receptors down-regulation results
A: Internalization-prone receptors at the membrane are rapidly degraded. B: Extracellular DA stabilizes PDT receptors conformation at the membrane and decreases their internalization/degradation. C: Extracellular DA degradation generates ROS. ROS increase plasma membrane permeability, permitting the entry of DA into the cell. Intracellular DA stabilizes receptor conformation at the ER, leading to an increased shuttling to the membrane. Extracellular DA also stabilizes receptor conformation at the plasma membrane. D: DA stimulation of PDT receptors and/or ROS induces specific intracellular signaling pathways. Activation of these pathways has a stimulatory effect on receptor shuttling from the ER to the plasma membrane and/or an inhibitory effect on receptor degradation, leading to an increase in membrane receptor level.

4.9 Down-regulation properties of D1-like receptors are agonist specific

In contrast to DA, thioridazine (inverse agonist/antipsychotic) 24 hours treatment did not elicit WT and PDT receptors down-regulation (Fig. 18). Moreover, PDT mutants receptors displayed a slight up-regulation when stimulated with thioridazine (Fig. 18). Recently, our lab showed that thioridazine behaves as an inverse agonist, which stabilizes the inactive conformation (R state) of D1R and D5R, a conformation not believed to lead to receptor internalization (D'Aoust and Tiberi, 2010). Therefore, the lack of down-regulation of WT and PDT receptors induced by long-term treatment with thioridazine may be explained by a reduction in the constitutive internalization of the receptors.

Differences between DA and thioridazine-induced long-term regulation of PDT receptors could be explained by the inability of thioridazine to lead to one of the three hypotheses presented in figure 20. Firstly, receptor conformation adopted following inverse agonist binding is quite different than with agonist binding (Park et al., 2008). Therefore, thioridazine stimulation may not lead to the stabilization of PDT receptors structure. Secondly, thioridazine stimulation is not known to generate ROS nor is it known to stimulate intracellular signaling pathways, eliminating the remaining two possibilities.

4.10 Insights gained into diseases mechanisms and potential treatments from PDT mutant receptors results

Drugs aimed to control dopaminergic receptor signaling output are already on the market. However, drugs currently being used all share the common characteristic of targeting the receptor ligand binding site. Our results argue for a potential novel target for D1-like

receptor targeted drugs : Ser/Thr residues of the CT. Phosphorylation site blockers have been developed for some receptors, such as tyrphostin AG1296 acting on the platelet-derived growth factor receptor (Kovalenko et al., 1997). This compound, which masks phosphorylation sites present on the receptor, has shown some potential in treating pulmonary fibrosis and corneal neovascularization as assessed in rats and mice respectively (Dell et al., 2006; Rice et al., 1999).

An intriguing feature of using a drug targeted at Ser/Thr residues of CT is the possibility of inhibiting D1-like receptor down-regulation. This could be an interesting avenue to treat PD as one of its hallmark is the neurodegeneration of dopaminergic neurons projecting in the striatum (Emilien et al., 1999). This loss of striatal DA neurons leads to a decrease in DA signaling output. This is particularly important in PD pathology as DA signaling in the striatum elicit movement control. Currently, treatment of PD consists mainly of L-Dopa ingestion (a DA precursor) to increase signaling in the striatum. Thus, being able to limit D1R down-regulation and desensitization using phosphorylation sites blockers (as an adjuvant) could increase L-Dopa induced DA signaling. Another drawback of current PD treatment is the side effects associated with it. Our results demonstrate that removing Ser/Thr residues of CT of D1-like receptors lead to an increase in agonist affinity (Table 2). Usage of phosphorylation sites blockers may potentially allow the use of lower doses of L-Dopa for PD treatment, diminishing the side effects observed with L-Dopa therapy.

It is also interesting to note that D1R trafficking has been demonstrated to be modified in Parkinsonian and dyskinetic L-Dopa treated monkeys (Guigoni et al., 2007). D1R

membrane expression level was increased for Parkinsonian and dyskinetic L-Dopa treated monkeys compared to control (Guigoni et al., 2007). Our results obtained with the long-term regulation of PDT-D1R by DA, which lead to an increased membrane expression of this mutant (Fig. 16), potentially suggest that regulation of CT phosphorylation mediates this effect. Altered CT phosphorylation may increase the number of D1R available at the membrane, leading to the dyskinesia associated with L-Dopa treatment of PD.

Increased signaling in the mesolimbic pathway is thought to be the predominant cause of schizophrenia. Schizophrenia treatment currently utilizes D2-like antagonist with moderate success (Masri et al., 2008). However, numerous studies point to D1-like receptors as being important pharmacological targets (Laviolette, 2007). It could be possible to use phosphorylation site blockers to increase D1-like receptors activity, further complementing D2-like antagonists effect. It is known that D1-like and D2-like receptors have opposite signaling output in cells. By simultaneously activating D1-like receptors and inhibiting D2-like receptors, it would be possible to enhance the benefits emanating from the treatment.

As discussed in section 1.3.4, D5R KO mice display hypertension. A recent report could help shine some light on this observation (Li et al., 2008). Li et al. demonstrated that D5R activation led to the degradation of the angiotensin 1 receptor through a proteosomal pathway. This observation is really interesting given that the increase in blood pressure in D5R KO mice was attributable to an elevated expression level of AT1R in the kidney (Li et al., 2008). Hypertension is major risk factor for cardiovascular diseases and stroke.

Moreover, its prevalence amongst Canadian population has increased around 60% from 1995 to 2005, making it one of the most important health issue of the 21st century (Tu et al., 2008). Usage of phosphorylation sites blockers appears to be a valuable strategy to treat hypertension. As my results demonstrated, removal of D5R CT putative phosphorylation sites increase its constitutive and DA-mediated maximal activation of AC. This increase in activity could be translated in an increase of AT1R degradation, helping regulate blood pressure level in hypertensive patients.

D5R has also been implicated in drug addiction using KO mice (Argilli et al., 2008). Cocaine-induced LTP in the ventral tegmental area, a process that is important in establishing drug addiction in users, was found to be dependant on D5R activation of NMDAR (Argilli et al., 2008). Results obtained during my Master studies demonstrate the importance of phosphorylation sites of D5R CT in regulating its activity (Fig. 9). Therefore, if phosphorylation of D5R is important for its activity, impairment of this biochemical process in humans could play a role in the degree of susceptibility to develop addiction following intake of illicit drugs.

D5R have also been implicated in the hippocampal acetylcholine release (Hersi et al., 2000; Laplante et al., 2004). ACh is a neurotransmitter implicated in learning and memory. In fact, decrease of ACh release is one of the symptoms associated with Alzheimer's disease (AD) (Thathiah and De Strooper, 2009). Given the role of Ach in the central nervous system and the strong localization of D5R in the hippocampus (a specialized brain region in learning and memory), a treatment could be designed by targeting CT of D5R. Use of phosphorylation sites blockers could help increase D5R

activity, thus increasing acetylcholine release in the hippocampus. This increase in ACh release could help eradicate or at least slow down AD symptoms progression by stimulating brain cognitive functions.

4.11 Future studies

While work done during my thesis has provided a greater understanding on D1R and D5R subtype-specific modulation of their pharmacological and regulatory properties, much more work is required to expand our knowledge. Immunofluorescence studies will need to be carried out to investigate the internalization properties of the PDT mutants. This will enable us to monitor DA-induced internalization of the receptors, if PDT receptors internalize at all. It would also enable us to observe the post-endocytic fate of the receptor and help us pinpoint the accurate cause for DA-induced PDT receptor up-regulation. Immunofluorescence could also help detect PDT receptors potentially retained in the ER, at the same time helping in validating our hypothetic model for their lower Bmax value.

Other dopaminergic compounds could also be tested in the regulation experiments. This would help in determining whether D1R and D5R are modulated by compound-specific regulatory mechanisms as observed for MOR. Moreover, the use of agonists which do not produce ROS for our long-term regulation assay could help discriminate between the three hypotheses for PDT up-regulation presented in figure 20.

Experiments could be done to evaluate specific CT regions of D1R and D5R in modulating the effect observed with PDT mutant. It would also be interesting to assess

whether opposite effects on pharmacological and regulation properties of D1-like receptors could be observed using phospho-mimetic mutations instead of phospho-deficient mutations. Replacing serine and threonine by aspartate and glutamate, which add negative charges to the CT, may mimic phosphorylation at those residues.

4.12 Conclusion

Dopamine is one of the most important neurotransmitter in the central nervous system. Knowledge of D1-like receptor pharmacology is important to our understanding of the phenotypic expression of neurological diseases linked to a compromised D1-like receptor function and in helping devising new therapeutic approaches. Results presented in my thesis point to an important role for Ser/Thr residues of the CT in modulating several aspects of D1-like receptors signal transduction and regulation. Results obtained also give an insight into potential receptor dysfunction leading to disease state. This study provides an interesting starting point to develop novel drugs targeted at another region than the ligand binding pocket of GPCRs. Future work will be needed to specify CT regions and/or residues that are responsible for the drastic effects observed in the various assays performed during my Master studies.

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