The Role of the Central Region of the Third Intracellular Loop of D1-Class Receptors in Signalling

by

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Abstract

The D1-class receptors (D1R, D5R) each possess distinct signaling characteristics; however, pharmacological selectivity between them remains elusive. The third intracellular loops (IL3) of D1R and D5R harbour divergent residues that may contribute to their individual signalling phenotypes. Here we probe the function of central region of IL3 of D1R and D5R using deletion mutagenesis. Radioligand binding and whole cell cAMP assays suggest that the N-terminal and C-terminal moieties of the central IL3 oppositely contribute to the constitutive and agonist-dependant activity of D1-Class receptors. Whereas the N-terminal deletions ablated constitutive activity and decreased DA-induced activation, C-terminal deletions induced robust increases. These data, interpreted in concert with structural predictions generated from homology modeling implicate the central IL3 as playing an important role in the activation and subtype-specific characteristics of the D1-class receptors. This study may serve as a basis for the development of novel drugs targeting the central IL3 region.

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List of Abbreviations

$5HT1_AR$: Serotonin 1_A receptor

α_{2A} AR: α_{2A} adrenergic receptor

A_1R: A_1 adenosine receptor

$A_{2A}AR$: A_{2A} adenosine receptor

AA: Ascorbic acid

AC: Adenylyl cyclase

ACTH: Adrenocorticotropic hormone

ADHD: Attention deficit hyperactivity disorder

ATII: Angiotensin II

β_1AR: Beta-adrenergic 1 receptor

β_2AR: Beta-adrenergic 2 receptor

B_{max}: Maximal binding capacity

C1,2: Cytoplasmic domain 1, 2 (G protein)

CAM: Constitutively active mutant

cAMP: Cyclic adenosine monophosphate

CB_1R: Cannabinoid 1 receptor

COMT: Catechol-o-methyl transferase

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

DAT: Dopamine transporter

DHX: Dihydrexidine

DOPAC: 3,4 dihydroxyphenylacetic acid

DRD1: Dopamine receptor 1 gene

DRD5: Dopamine receptor 5 gene

DRIPs: Dopamine receptor interacting proteins

EC50: Half-maximal effective concentration

EL: Extracellular loop

ER: Endoplasmic reticulum

FAB: Fragment antigen binding (antibody fragment)

FLUP: *cis*-flupenthixol

FRET: Fluorescence resonance energy transfer

FSH: Follicle-stimulating hormone

G protein: Guanine nucleotide-binding protein

GDP: Guanosine diphosphate

GPCR: G protein-coupled receptor

GTP: Guanosine triphosphate

HVA: Homovanillic acid

IL: Intracellular loop

IP_3: inositol triphosphate

KO: Knock-out

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

M1, 2: Membrane spanning domain 1, 2 (G protein)

MAO: Monoamine oxidase

MPP^+: 1-methyl-4-phenylpyridinium

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

NAc: Nucleus accumbens

NE: Norepinephrine

NT: N-terminal domain

PD: Parkinson's disease

PFC: Prefrontal cortex

PIP_2: phosphotidylinositol biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PMA: Phorbol-12-myristate-13-acetate

PP1: Protein phosphatase 1

PP2: Protein phosphatase 2

PRL: Prolactin

RET: Resonance energy transfer

RGS: Regulators of G protein signalling

ROS: Reactive oxygen species

SKF: SKF83566

SCH: SCH23390

Ste2p: Yeast pheromone α-factor receptor

THIO: Thioridazine hydrochloride

TM: Transmembrane domain

TRL: Terminal receptor locus

Vmat: Vesicular monamine transporter

VTA: Ventral tegmental area

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Introduction

1.1 Dopaminergic Signalling

1.1.1 - Principles of Dopaminergic Neurotransmission

Over 50 years ago, Arvid Carlsson suggested that the catecholamine 3-hydroxytyramine, or dopamine (DA), was a neurotransmitter. It was the first of many contributions that earned him the 2000 Nobel Prize in Physiology or Medicine (Iversen and Iversen, 2007). Prior to this landmark discovery, DA was thought only to be an intermediate in the synthesis of norepinephrine (NE). DA is produced in presynaptic neurons and stored in vesicles near the presynaptic terminal. It is derived from the amino acid L-tyrosine, transiently existing as 3,4-dihydroxy-L-phenylalanine (L-Dopa) before being transformed into DA by the enzyme aromatic L-amino acid decarboxylase or Dopa decarboxylase. The rate limiting step in the synthesis process is the production of L-Dopa by tyrosine hydroxylase (**Figure 1**) (Elsworth and Roth, 1997). DA is then taken up into storage vesicles by the vesicular monoamine transporter (Vmat) where it can then be further processed into NE (Verheij and Cools, 2008).

Upon stimulation by an action potential, an influx of Ca^{2+} enters the nerve terminal, causing vesicles storing DA to fuse with the plasma membrane and release their cache into the synaptic cleft. Freshly synthesized intracellular DA can also be released prior to storage in vesicles (Verheij and Cools, 2008). This occurs if intracellular concentrations of DA are high and is facilitated by reverse transport of the dopamine transporter (DAT) on the synaptic membrane (Sulzer et al., 1993). DA released into the synaptic cleft is then able to act on postsynaptic receptors to propagate a signal or to negatively feedback by activating autoreceptors of the presynaptic cells to inhibit further

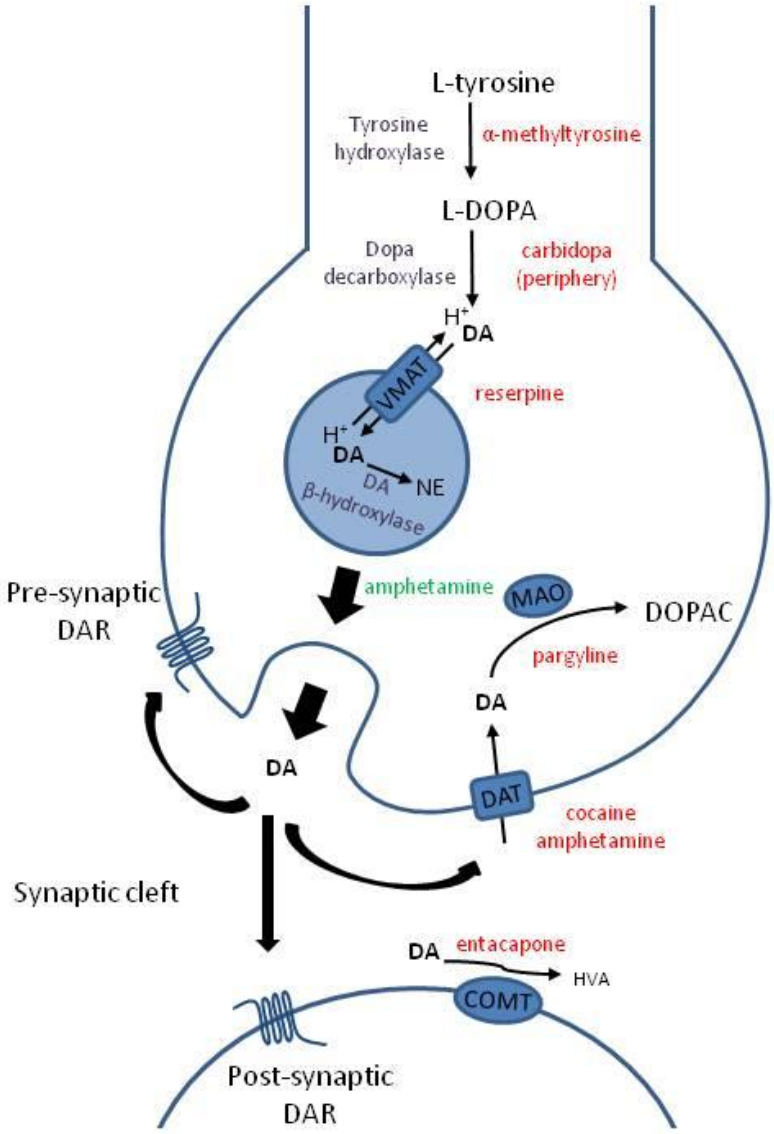


Figure 1. Schematic of dopamine synthesis, storage and signalling. Tyrosine hydroxylase converts L-tyrosine to L-Dopa. L-Dopa is processed into DA which is stored in vesicles by VMAT. Action potentials cause vesicles to fuse with synaptic membrane and release DA into synapse. DA acts on pre and post-synaptic receptors before being degraded by COMT or transported back into the cell by DAT. Once back in the cell, DA may be recycled or degraded by MAO. Drugs inhibiting (red) or enhancing (green) pathways are shown.

neurotransmitter release. When its signalling function is complete, DA is either degraded on the postsynaptic membrane by catechol-O-methyl transferase (COMT), diffuses out of the synaptic cleft, or is actively transported back into the presynaptic terminal via DAT. Once back in the presynaptic cell, DA can be recycled to the presynaptic vesicles or metabolized into DOPAC by monoamine oxidase (MAO)(Elsworth and Roth, 1997).

Pharmacological intervention is possible at virtually every step of DA synthesis, release and reuptake (**Figure 1**). During the synthesis of DA, α-methyltyrosine and carbidopa can be employed to inhibit the activity of tyrosine hydroxylase and L-Dopa decarboxylase, respectively. Vesicular storage of bioamines is obstructed by reserpine, while their release from vesicles is enhanced by amphetamine. Furthermore, both amphetamine and cocaine block the reuptake of DA by DAT. Degradation of DA by COMT and MAO can be inhibited by entacapone and pargyline, respectively. These drugs have been used both therapeutically, and to characterize the physiological functions of DA.

DA exerts its actions on a cell through its cognate receptors, of which 5 subtypes have been identified; D1-D5 (Bunzow et al., 1988, Grandy et al., 1989, Dearry et al., 1990, Sunahara et al., 1990, Zhou et al., 1990, Sunahara et al., 1991, Tiberi et al., 1991, Van Tol et al., 1991). DA receptors are G protein-coupled receptors (GPCRs) and are classified as either D1-class, consisting of D1R and D5R; or D2-class, consisting of $D2R_{long}$, $D2R_{short}$, D3R and D4R. The D1 and D2-classes differ on the genetic level, in that the D1-class receptors are intronless, whereas the D2-class genes contain introns which facilitate splice variants (Missale et al., 1998). The long and short isoforms of D2R differ by a 29 amino acid stretch in the third intracellular loop and display distinct expression and signalling characteristics. Variants of the D3R and D4R have also been described but are either non-

functional or display little divergence in function (Beaulieu and Gainetdinov, 2011). The classification of DA receptors however, is not based on their differences in gene structure, but was determined by the receptors action upon adenylyl cyclase (AC) (Kebabian and Calne, 1979). AC activation results in the production of cyclic adenosine monophosphate (cAMP). The D1-class receptors act to stimulate the activation of AC, whereas the D2-class inhibit its activation.

Signal transduction from the DA receptor to AC is facilitated by either the $G\alpha_{s/olf}$ proteins, in the case of the D1-class, or the $G\alpha_{i/o}$ proteins, in the case of the D2-class receptors (Missale et al., 1998). By altering the level of cAMP through their effects on AC, DA receptors effectively control the activation of protein kinase A (PKA) and its downstream effector proteins which initiate a cellular response through ion channels, gene transcription and other neurotransmitter mechanisms (Greengard, 2001a). At the centre of signalling downstream of DA receptors and PKA is the protein, dopamine and cAMP regulated phosphoprotein M.W. 32 kDa (DARPP32). DARPP32 is phosphorylated by PKA on Thr 34, which leads to inhibition of protein phosphatase 1 and broad effects on a number of signalling pathways. Phosphorylation of DARPP32 is the major link that allows DA to influence the overall function of the cell through interactions with other receptors and ion channels within the cell (**Figure 2**). A myriad of cellular machinery has evolved to exert sophisticated control of DARPP32, as it represents a hub for signal amplification (Greengard, 2001a). Whereas activation of D1-class receptors leads to phosphorylated DARPP32, D2Rs can affect the phosphorylation state of DARPP32 through both dampening cAMP production, and by increasing intracellular Ca^{2+}, thereby

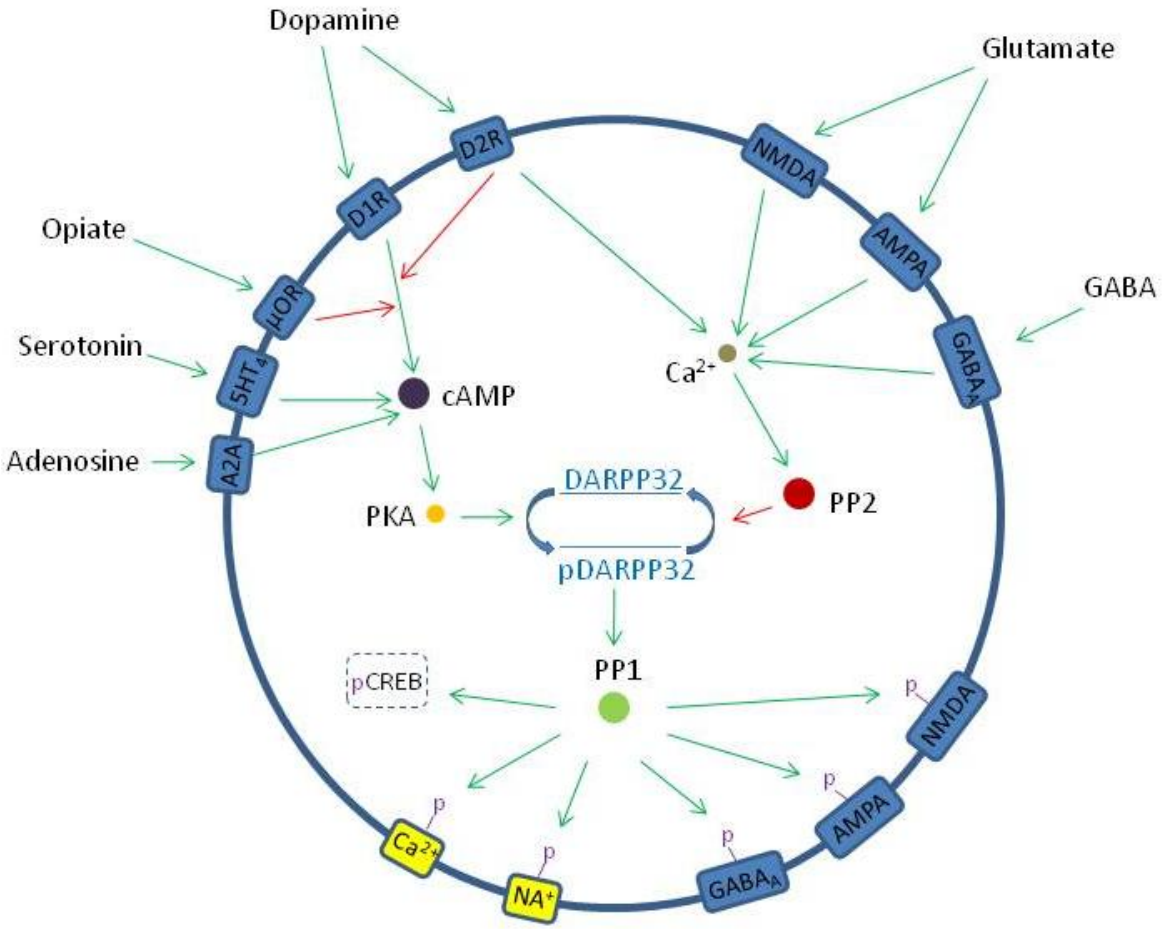


Figure 2. DARPP32 is a central hub of dopamine signalling. Activation of D1R causes an increase in cAMP and activates PKA. PKA phosphorylates DARPP32 on Thr34 thereby activating it. DARPP32 in turn activates PP1 which can act to phosphorylate receptors involved in fast synaptic transmission, ion channels and transcription factors. Other receptors act to enhance or inhibit the production of cAMP. D2R acts in direct opposition to D1R by stifling cAMP production and stimulating intracellular Ca^{2+} which activates PP2 and dephosphorylates DARPP32, thereby inactivating it.

activating protein phosphatase 2B (PP2) which dephophorylates DARPP32 at Thr 34 (Greengard, 2001a). Additionally, DA receptors have been demonstrated to act through other pathways affecting intracellular calcium release. It has been shown that a D1R/D2R receptor dimer activates the PLC pathway in neurons via stimulation of $G\alpha_q$ (Ng et al., 2010). Similarly, evidence exists that D5R can also couple to $G\alpha_q$ as part of a heterodimer with D2R and as a homodimer, though this remains to be widely accepted (So et al., 2009, Beaulieu and Gainetdinov, 2011).

Dopamine, therefore, does not itself partake in fast synaptic transmission which is mediated by glutamate and GABA, but instead modulates it through what is termed "slow synaptic transmission" (Greengard, 2001b). DA signalling can both directly affect GABA and glutamate receptors, and indirectly modulate their signalling by altering the cellular environment through effects on ion channels and other effector proteins (Neve et al., 2004). Although dopaminergic signalling does occur in peripheral tissues, it is predominantly found in the brain. It is through slow synaptic transmission that DA can modulate the tonic and burst firing of DA neurons. Although DA neurons constitute less than 1% total neurons in the brain, they exert strong modulation over physiological processes. This occurs through 4 major pathways (**Figure 3**).

1.1.2 - The Nigrostriatal Pathway

In the early days of DA research, one of the first observations made was the loss of the dopamine system in the basal ganglia of Parkinson's disease (PD) patients (Iversen and Iversen, 2007). This association helped to identify the role that DA plays in the

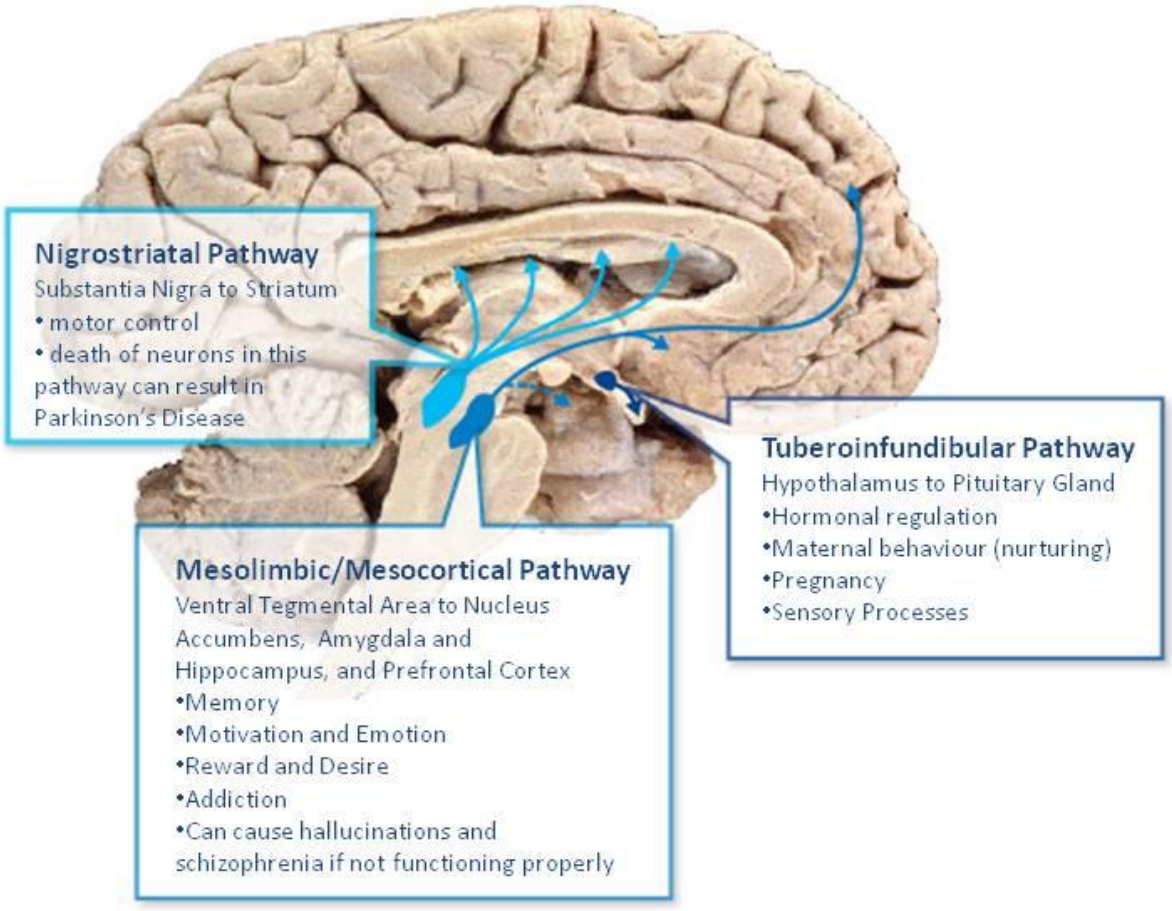


Figure 3. The 4 major dopaminergic pathways of the brain and their functions.

Genetic Science Learning Center, University of Utah, http://learn.genetics.utah.edu (Center, 2012)

control of voluntary movement. The nigrostriatal pathway connects the substantia nigra to the caudate putamen (dorsal striatum). It functions to control and initiate movement planning by modulating descending cortical inputs as they are directed back up to the cortex via the thalamus. This occurs through what are known as the direct and indirect pathways of the basal ganglia. D1Rs in the dorsal striatum are involved in the direct pathway, whereas D2Rs act both pre and postsynaptically and function in the indirect pathway. Degeneration of the DA neurons originating in the substantia nigra pars compacta in PD leads to decreased stimulation of DA receptors involved in the direct and indirect pathways and therefore decreased output from the thalamus to the frontal cortex (Kandel ER, 2000). Similarly, the modulation of these pathways is responsible for the tardive dyskinesia induced by classic antipsychotic drugs and L-Dopa induced dyskinesia, which result from antagonism of D2Rs and agonism of the D1Rs, respectively (Mailman et al., 2001, Marsden, 2006). In order to probe the significance of DA neurons *in vivo*, models in which DA neurons are selectively targeted have been developed. Though it has its limitations, one of the most commonly employed behavior models of disorders affected DA in the basal ganglia is the unilateral selective lesion of ascending DA neurons in animal models using 6-hydroxydopamine. This lesion induces rotational movement in the animal and provides an observable behaviour for experimental testing. Another more specific experimental model for PD is the MPTP lesion. MPTP is a synthetic chemical that is bioactivated by MAO-B into MPP+, a neurotoxin that causes degeneration within DA neurons and astrocytes (Marsden, 2006). These models have been employed to successfully identify the changes in DA circuitry in the PD basal ganglia and highlight the role of D1-class receptors in L-Dopa induced dyskinesia (Berthet and Bezard, 2009, Thiele

et al., 2012). Although new genetic models are being developed to better understand the disease, these lesion models remain a mainstay of PD and DA neuron research today.

1.1.3 - The Mesolimbic/Mesocortical Pathway

The mesolimbic pathway extends from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), ventral striatum and amygdala, whereas the mesocortical links the VTA to cortex. As such, these pathways are associated with attention, reward, and cognition and are therefore linked to ADHD, addiction and schizophrenia. Both D1R and D2R mediate the action of this pathway. It is thought that inputs to the core of the NAc encode information about the salience of events. Encoding the reward or incentive of a particular event is considered to be the function of the associated burst firing of dopamine neurons. Schizophrenic delusions, known as the positive symptoms of the disease, may be the result of problems with this encoding process and the misperception of the salience of events (Iversen and Iversen, 2007). Antipsychotic drugs exert their action through inhibition of D2-like receptors, which with the observed increase in DA release with amphetamines, led to the development of the dopamine hypothesis of schizophrenia; psychosis associated with hyperdopaminergia (Meltzer and Stahl, 1976). However, the negative symptoms of schizophrenia, the cognitive deficits and social withdrawl, have been shown to be linked to a deficit of DA signalling in the prefrontal cortex (PFC). D1R stimulation within the frontal cortex displays an inverted U-shape relationship with working memory, indicating that tight control of D1R signalling is required for efficient cognitive processing (Williams and Castner, 2006). The mostly D1-class selective full agonist dihydrexidine (DHX) has been shown to increase perfusion to the prefrontal cortex of schizophrenic patients and therefore may be therapeutically relevant (Mu et al., 2007).

Partial agonists at D2R are also drugs that are being pursued, as these drugs would exert decreased activation at D2Rs in the hyperdopaminergic striatum and increased D1R activation in the hypodopaminergic PFC. New lines of evidence also suggest that the D1R/D2R heteromer is also a therapeutically relevant to schizophrenia and may be involved in a third basal ganglia pathway (Perreault et al., 2011a, Perreault et al., 2011b).

One of the most frequently diagnosed conditions in children is attention deficit hyperactivity disorder (ADHD). The physiological mechanisms of ADHD are poorly understood but have been correlated with polymorphisms in the genes of D1R, D4R, D5R and DAT (Bobb et al., 2005). Interestingly, ADHD is successfully treated with the methylphenidate (Ritalin), an inhibitor of DAT. Ritalin increases release of DA, suggesting that a hypodopaminergic state may be the underlying cause of ADHD. This drug has been found to have low risk for the development of dependence unlike the closely related amphetamine and methamphetamine, possibly because unlike its more addictive relatives, it only affects the synaptic release of DA (Iversen and Iversen, 2007).

Drugs of abuse have been shown to cause an increase in DA within the VTA, NAc and other mesolimbic areas triggering the reward system of the brain. Amphetamines cause an indiscriminate increase in release and synthesis of DA, while cocaine blocks DA reuptake. Other drugs such as opiates and alcohol mediate DA increase through other pathways. D5R has been shown to be involved in sexual reward in rats, increasing receptivity in females and intromission in males (Marsden, 2006). Addiction, or the physiological need to seek and experience pleasure despite negative consequences, develops through long term increases of mesolimbic DA and has been shown to involve all five of the DA receptors (Le Foll et al., 2009).

1.1.4 - The Tuberoinfundibular Pathway

Dopaminergic neurons also control the secretion of the peptide hormone prolactin (PRL) from lactotrophs through the tuberoinfundibular pathway. This pathway runs from the paraventricular and arcuate nucleus of the hypothalamus to the median eminence and intermediate lobe of the pituitary (Marsden, 2006, Pivonello et al., 2007). Regulation of PRL is achieved through the activation of D2Rs in the anterior pituitary, which results in decreased PRL release (Ben-Jonathan and Hnasko, 2001). D2R agonists are used to treat hyperprolactinemia, a condition which also arises as a side effect of treatment with conventional antipsychotics (Ben-Jonathan and Hnasko, 2001, Marsden, 2006). Additionally, there is evidence that DA modulates the release of other peptide hormones of the anterior pituitary such as growth hormone, luteinizing hormone and follicle stimulating hormone (FSH), adrenocorticotropic hormone (ACTH) and thyroid-stimulating hormone. This places DA in an important role in the control of the adrenal-hypophyseal axis. Furthermore, in pituitary tumours secreting ACTH and cortisol, treatment with cabergoline, a D2R selective agonist, is able to induce remission in 30% of cases (Pivonello et al., 2007).

1.1.5 – Dopaminergic Signalling in the Periphery

The discovery that DA acts as a neurotransmitter has led to its extensive study within the central nervous system. Much less established are the roles that DA signalling plays peripherally. DA receptors are found in the intestines, blood vessels, cochlea, adrenal glands and in the kidneys (Missale et al., 1998, Maison et al., 2012). D1-class receptors are usually expressed on post-junctional membrane and result in vasodilation

when stimulated. D2-class receptors are for the most part found pre-junctionally on post-ganglionic sympathetic effector neurons but also post-junctionally (Zeng et al., 2008). These receptors cause the inhibition of noradrenaline release which leads to decreased vasoconstriction responses (Doggrell, 2002). Recently it was found that DA may play a protective role in the cochlea of mice, helping to control noise-induced excitotoxicity (Maison et al., 2012).

In the kidney, DA is synthesized from L-Dopa on tubule membranes and mediates natriuretic responses through autocrine and paracrine actions. D2-class receptors mediate increased glomerular filtration following amino acid influx and it is thought that the D3R may be the specific receptor involved (Zeng et al., 2008). Accompanying an increased filtration rate is enhanced renal bloodflow, mediated through the D1-class receptors. In the renal tubules, DA opposes anti-natriuretic mediators such as angiotensin-II (ATII) and facilitates some the natriuretic actions of extra-renal hormones like atrial natriuretic peptide (Doggrell, 2002).

Natriuresis via DA occurs through stimulation of D1-class receptors, which inhibits Na^+ reabsorption by inactivating Na^+/K^+ATPase in the entire nephron and other Na^+ channels in the proximal tubules. ATII acts in complete opposition to the D1-class receptors, inhibiting cAMP production and activating PLC and different protein kinase C (PKC) isoforms (Doggrell, 2002, Zeng et al., 2008). D1R inhibits the anti-natriuretic effects of ATII through a direct interaction which is disrupted in hypertensive rats (Zeng et al., 2003). On the other hand, stimulation of D5R but not D1R was found to induce degradation of the angiotensin II type 1 receptor, suggesting that D5R specific drugs may therefore be useful in treating hypertension (Gildea et al., 2008, Li et al., 2008).

Dysfunction of DA signalling in the kidneys can result in hypertension, diabetes mellitus, reduced renal filtration and renal failure. Studies of $D1^{-/-}$ knock-out (KO) mice have reiterated the role of this receptor in the development of hypertension due to its effects on Na^{+} balance (Zeng et al., 2008). $D5^{-/-}$ mice also are hypertensive, however, this has been shown to originate through a CNS pathway involving glutamatergic, vasopressin, oxytocin and adrenergic receptors (Zeng et al., 2008).

DA receptors are also found in vascular cells throughout the body. Stimulation of the D1-class receptors by low concentrations of DA causes vasodilation in resistance vessels. The D1-like receptors show similar distribution in the vasculature, therefore differences in DA affinity and basal signalling may be important. Circulating concentrations of DA are too low to stimulate DA receptors found in the heart, however it is high enough to stimulate β adrenergic receptors and increase contractility. (Zeng et al., 2008). DA is used as a positive inotrope for the acute treatment of myocardial infarction, as at low doses it increases cardiac output without constricting blood flow to tissues (Doggrell, 2002).

D1R, D2R, D4R and D5R are expressed in the normal adrenal medulla and cortex, with evidence indicating their participation in the control of aldosterone secretion through a functional interaction with the angiotensin II system (Pivonello et al., 2004). Dopamine has also been shown to modulate the secretion of cortisol and androstenedione. Similar to pituitary tumours, it has been hypothesized that treatment of adrenal tumours may be possible with dopaminergic drugs (Pivonello et al., 2007). As mentioned in the above sections, drugs targeting DA signalling are currently in clinical use, however, many studies have highlighted that subtype specific targeting of DA receptors may hold greater

therapeutic potential (Beaulieu and Gainetdinov, 2011). In order to further develop specific and effective dopaminergic treatments, an intimate knowledge of receptor structure is required. As DA receptors are GPCRs, basic knowledge of this superfamily of proteins is a critical starting point.

1.2 G Protein-Coupled Receptors

1.2.1 - GPCR Classification

Following the identification of many receptor subtypes during the late 1980's and early 1990's a number of classification systems for GPCRs were developed based on the ligand or sequence similarities. The most common originally divided GPCRs found in multi-cellular organisms into families A through F. Other names also developed to identify these groupings: the rhodopsin-like receptors, secretin receptors, metabotropic glutamate/pheromone receptors, fungal mating pheromone receptors, cAMP receptors, and frizzled/smoothened receptors (Kolakowski, 1994, Kristiansen, 2004).

The GRAFs system was developed following the availability of genome-wide data. This classification method divides GPCRs into five families based on their chromosomal positions and sequence fingerprints. The Glutamate (family C), Rhodopsin (family A), Adhesion (family D), Frizzled/Smoothened/Taste2 (family E), and Secretin (family B) families are proposed to have arisen from a common ancestor over evolution (Fredriksson et al., 2003). The GRAFS system is now the most commonly used classification system for GPCRs, however, these families are often still referred to by a letter association (given above in brackets).

In order to facilitate comparison of homologous amino acid residues between GPCR species, a universal numbering system is required. The Ballesteros-Weinstein numbering system is virtually the only such system currently in use (Ballesteros and Weinstein, 1995). Each transmembrane domain (TM) is identified by a whole number (TM1-7). Within each TM, the most conserved residue is designated .50 and acts as a reference point for identifying the other residues in that region: i.e. the most conserved residue in TM4 = 4.50, that of TM3 = 3.50. Residues in the N-terminal direction from 4.50 are assigned descending numbers (i.e. 4.49, 4.48); whereas residues in the C-terminal direction are assigned ascending numbers (i.e. 4.51, 4.52). As there are some residues in each TM that are well conserved among the vast majority of GPCRs, the user is able to compare similar regions between receptors that are quite divergent in primary sequence without referring to the sequence location specific to each receptor.

1.2.2 – Receptor Structure

GPCRs are involved in the facilitation of virtually all physiological processes in the body and hence, have been the subject of intense study over the past 30 years. Despite this focus, detailed structural characterization of GPCRs has only come to light in the last decade with the success of crystallography work. GPCRs are a superfamily of transmembrane receptors that all share a similar design (**Figure 4**). They consist of an extracellular amino-terminal domain (NT), seven α-helical transmembrane domains (TMs), three extracellular loops (ELs), three intracellular loops (ILs) and an intracellular carboxy-terminal tail (CT) (Kristiansen, 2004, Luttrell, 2008).

The TMs sit angled in the plasma membrane and are arranged in a counter-clockwise fashion, creating a twisted barrel shape. As will be discussed more below, changes in the conformation of the TM regions are thought to facilitate receptor activation. This barrel arrangement creates a vestibule in the centre of the receptor in which, for many receptors, ligands can bind and effect change in the activation state of the receptor. The primary binding site of the natural ligand for a receptor is termed the orthosteric ligand binding site. Interactions between residues of the TM domains and the ligand allow for specificity as to which ligands can bind. This specificity is impressive as many GPCR receptors share a high degree of homologous residues within these domains and ligand structure can be quite diverse. Mutagenesis studies have been employed to highlight specific residues of the orthosteric binding pocket that are critical to the distinct affinities of each particular receptor for its ligands (Kristiansen, 2004).

Contribution to ligand binding is also made by the NT of some receptors, such as those binding peptide hormones like FSH. This receptor domain is the most variable between GPCRs. It can act as the orthosteric binding site for some receptors, usually through a "hand-clasp" mechanism. It may also act as a cap to stabilize the interaction of a ligand with the receptor as observed in the binding of retinal to rhodopsin and proposed for the amine binding GPCRs, like the DA receptors (Park et al., 2008b). Another interesting feature observed in Class 3 GPCRs (i.e. metabotropic glutamate receptor) is the venus flytrap ligand binding mechanism, in which the two lobes of the NT act as a trap for ligands, with the closed trap conformation corresponding to receptor activation (Park et al., 2008b). The NT also acts a platform for post-translational modifications, such as

glycosylation, which can affect the export of the receptor to the cell surface and stabilize its conformation (Luttrell, 2008).

The ELs may also contain glycosylation sites and have been shown to be involved in receptor folding and trafficking, as well as ligand binding in certain receptor types. EL2 especially has been implicated in the binding of ligands to small molecule binding GPCRs (Park et al., 2008b). In most GPCRs, a disulphide bridge between EL1 and EL2 exists, which likely acts to stabilize the receptor by enabling proper folding. There is notable divergence in the function of EL2 between rhodopsin and more typical receptors, such as β_2AR. Rhodopsin EL2 acts to guard the bound ligand retinal, while β_2ARs binding pocket is made accessible by the restraint of EL2 by the disulphide bridge and a helical segment (Luttrell, 2008).

The intracellular domains are responsible for transmitting the signal from the receptor to the intracellular signalling proteins. The ILs are the critical interface with the G protein and other proteins that modulate GPCR function. There is greater variation in the ILs, and for the most part, these domains are disordered; an important feature that facilitates interaction with a variety of other proteins. The architecture of the ILs depends on the G protein family that a particular receptor interacts with. For example, receptors interacting with inhibitory G proteins have large IL3s, while those interacting with stimulatory G proteins possess shorter IL3s (Kristiansen, 2004).

The CT is also variable in length and sequence between receptor families and subtypes and is also involved the coupling of the receptor to its G protein as well as other

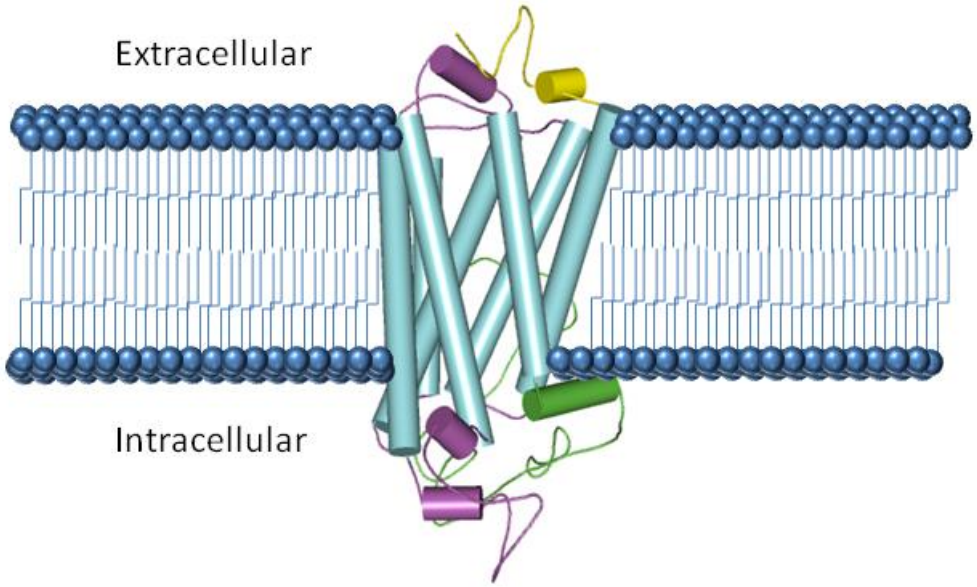


Figure 4. Typical structure of a GPCR based on the β_2 adrenergic receptor structure. The N-terminal region (yellow) and three extracellular loops (magenta) are located in the extracellular space and contain important elements for receptor stability. Transmembrane domains 1-7 (cyan) sit within the plasma membrane of the cell and form the orthosteric ligand binding pocket. The three intracellular loops (magenta) and C-terminal tail region (green) are located within in the cytoplasm and are involved in G protein coupling and regulation events. Helix 8 is formed within the C-terminal tail and is a common feature among many GPCRs. Other intracellular and extracellular helices may form and are receptor specific.

protein/protein interactions. A notable feature of the CT is a cysteine residue that acts as palmitoylation site, anchoring the CT to the plasma membrane and forming a fourth intracellular loop. This fourth loop contains an α-helical structure that is known as helix 8 and has been shown to participate in important interactions with proteins and lipids (Huynh et al., 2009).

1.2.3 – Receptor Activation

In the last few years, a tremendous effort has been made towards determining the mechanisms of activation of GPCRs. It has been assumed that distinct conformational changes in the TM regions must take place to produce changes at the cytoplasmic surface, facilitating an active state which recognizes the G protein. It is thought this is likely manifested through the disruption of interhelical interactions, which leads to the receptor assuming a different, more energetically favourable state (Wess et al., 2008). Rhodopsin activation has been extensively characterized by biochemical and biophysical studies. In contrast to this work, the crystal structure of rhodopsin in an intermediately activated state displayed only minor changes. However, this may have been an artifact of the process of crystallization, or simply an indication of multiple activation states, as the active opsin crystal structure displays similar structural changes to those predicted by other approaches (Salom et al., 2006, Park et al., 2008a, Scheerer et al., 2008, Wess et al., 2008). An overall summary of major predicted structural changes occurring with activation is as follows. Upon agonist binding, it has long been thought that a rotamer rearrangement of the Trp 6.48 (number represents Ballesteros-Weinstein general GPCR numbering) residue in the highly conserved CWXP motif (where X = any residue)occurs, which forms the basis for the so-called “toggle switch” mechanism (Ballesteros and Weinstein, 1995). The rotamer

rearrangement disrupts interactions involving these residues and causes the extracellular portions of the TMs to move inward. This in turn causes TM6 rotates clockwise outward away from the bundle core of TM1-4 and TM7, but closer to TM5 which also moves outward (**Figure 5A**). The movement of TM6 is amplified by the characteristic bend at Pro 6.50, the most conserved residue in TM6 among GPCRs (Shi et al., 2002, Schwartz et al., 2006). Recently, this model has come into question as it was shown using a constitutively active rhodopsin receptor that agonist-induced activation does not involve a rotamer rearrangement. Instead, motion of the β-ionone ring of retinal (agonist) below the CWXP motif releases Trp6.48 allowing for rotation of TM6 and rearrangement of a hydrogen bonding network facilitated through ordered water molecules that connects to the important NPXXY motif and "ionic lock" on the cytoplasmic side of the receptor (**Figure 5B**) (Standfuss et al., 2011). The NPXXY motif is a highly conserved sequence at the cytoplasmic end of TM7. Pro 7.50 of the motif imparts a kink in TM7 that likely allows for the Tyr 7.53 to interact with water molecules and stabilize the inactive state. This interaction is weak and may easily toggle during activation (Rosenbaum et al., 2009). The "ionic lock" is a strong interaction between Glu 3.49, Arg 3.50 of the highly conserved (E) DRY motif, and Glu 6.30 towards the cytoplasmic ends of TM3 and TM6. Upon activation this interaction is broken liberating the intracellular components and allowing for movement. These same residues may also form new interactions which stabilize an active state. The end result of these changes is the formation of an intracellular cavity that allows G proteins access to important residues in the cytoplasmic extensions of TM5 and TM6 (Ambrosio et al., 2011; Huang and Tesmer, 2011; Rosenbaum et al., 2009).

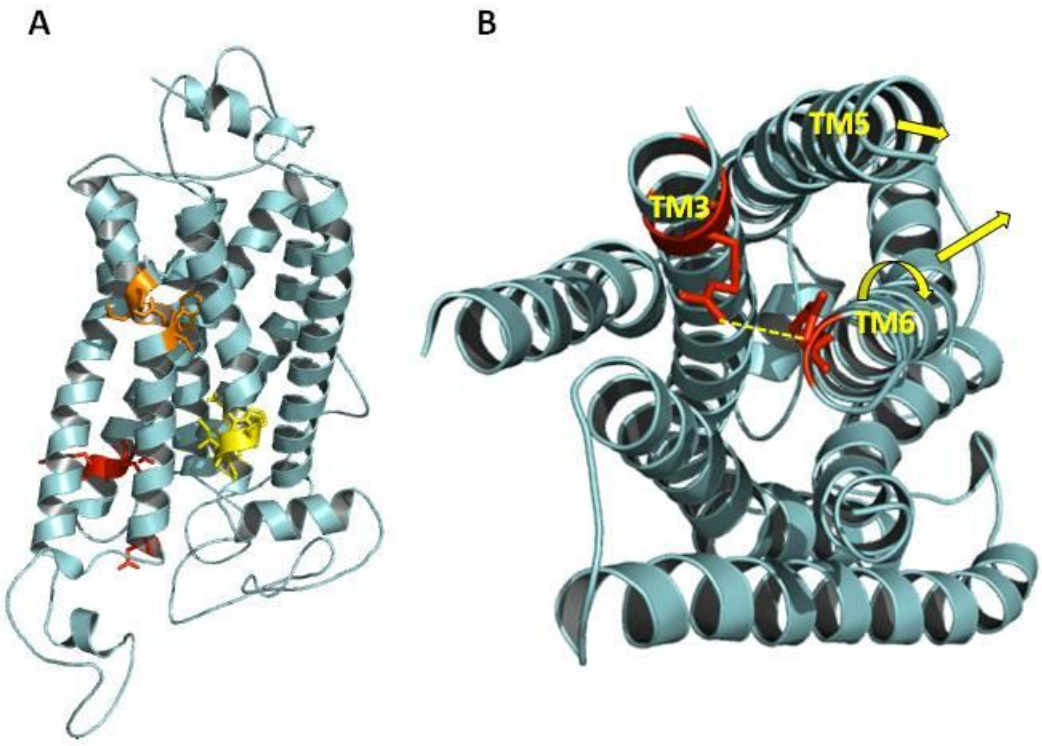


Figure 5. Predicted major changes in GPCR conformation upon activation. Model of the β2 adrenergic receptor (A) Side view of the receptor. Agonist binding induces movements in TM6 which propagates to the intracellular regions, residues of the CWXP motif of the "toggle switch" shown in purple, NPXXY shown in blue and E/DRY of the "ionic lock" shown in red. (B) Cytoplasmic view of the TM regions. Upon activation TM6 moves outwards and rotates thereby breaking the interaction with TM3 known as the ionic lock (residues 3.50 and 6.30; highlighted in red). TM5 extends into the cytoplasm. The cytoplasmic face of the receptor becomes primed for G protein coupling/activation.

Recently, the crystal structure of β_2AR bound to G_s was successfully solved (Rasmussen et al., 2011). This study revealed distinct changes in the bound G_s and interactions between its $G\alpha_s$Ras domain and cytoplasmic face of the receptor. Crystallization of the complex was facilitated by reducing receptor flexibility through replacement of the NT with a T4 lysozyme and stabilizing the receptor/G_s interaction with an antibody. This left the cytoplasmic domains of β_2AR intact to interact with G_s. A stretch of 26 amino acids within the IL3 displays a disordered conformation, while the IL2 assumes an α-helix structure, from the extended loop it exists as in the inactive conformation. Furthermore, these data reiterated the outward movement of TM6, in this case by 14 Å, with TM5 also moving outward and extending further into the cytoplasm by seven residues. The highly conserved NPXXY and ionic lock motifs were also confirmed to have very similar orientations to previous reports. This work bolsters previous hypotheses and is an important step in obtaining a more comprehensive understanding of GPCR activation (Rasmussen et al., 2011).

Investigations into the movements and kinetics of receptor domains upon agonist binding have provided further insight that complements that of the static state information gathered from crystallography. Disulphide cross-linking and resonance energy transfer (RET) strategies have reiterated predicted movements in the cytoplasmic extensions of the TMs and helix 8, corresponding to receptor activation upon the binding of an agonist (Wess et al., 2008). Interestingly, these studies have further provided evidence for a mechanism of partial agonism (Ambrosio et al., 2011). In the α_{2A} AR fluorescence resonance energy transfer (FRET) studies have shown that movements of TM5 and TM6 upon receptor activation differ in timing and extent depending on the pharmacological

profile of the agonist binding. Whereas full agonists induce the extensive movement of TM6 and relatively smaller movement of TM5, partial agonists are only able to induce movement in TM6 (Zurn et al., 2009). Furthermore, differences in the kinetics of receptor activation induced movements differ depending on the strength of the agonist (Ambrosio et al., 2011). These works support the existence of multiple activation states of the receptors and provide a molecular basis for the proposed ligand biasing of a receptor for particular subsets of G proteins based on the structure of the G protein binding pocket that they induce (Eason and Liggett, 1995; Nikolaev et al., 2006). Caveats involving signal specificity exist for both disulphide cross-linking and RET studies, however, these approaches used in concert with functional, crystallography and other biophysical methods, are complementary to each other and provide a more comprehensive analysis of GPCR activation (Wess et al., 2008).

1.2.4 – Heterotrimeric G Proteins and Associated Effector Proteins

The "middle men" of GPCR signalling, the heterotrimeric guanine nucleotide binding proteins (G proteins) have evolved in concert with GPCRs. It is the G protein that transmits the signal from the receptor to the primary effector proteins, typically an ion channel or enzyme like AC. G proteins are GTPases consisting of three tightly interacting subunits, the Gα and Gβγ dimer. When bound to a guanosine diphosphate (GDP) molecule, the Gα and Gβγ subunits form a heterotrimeric complex facilitated through noncovalent bonds (Luttrell, 2008). Coupling to the GPCR allows the G protein to eject the GDP which is replaced with a guanosine triphosphate (GTP), therein liberating the Gα and Gβγ subunits from each other and enabling them both to activate their effectors. GTP hydrolysis inactivates Gα, which subsequently reforms a complex with Gβγ. GTPase

activity of Gα is enhanced by proteins known as regulators of G protein signalling (RGS), thereby accelerating the hydrolysis of GTP (Wettschureck and Offermanns, 2005).

There are 16 mammalian isoforms of Gα, some with splice variants. These proteins weigh 39-52 kDa and can be grouped into four families based on sequence: $G\alpha_{s/olf}$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12}$. These families are either stimulatory or inhibitory, and act on different effector systems, therein bestowing great diversity to the signalling capabilities of GPCRs. Additional complexity is provided by the signalling capacity of the Gβγ subunits. There are 5 Gβ and 12 Gγ subunits, allowing for a large number of Gβγ dimer combinations and therefore almost 1000 possible heterotrimeric combinations with Gα (Wettschureck and Offermanns, 2005, Luttrell, 2008).

The Gα consists of a Ras-like GTPase domain and an α-helical domain, the interface of the two segments forms the binding domain for GDP/GTP. The Ras-like domain interacts with both the GPCR and the Gβγ subunit, while the α-helical segment acts like a hinge, closing on the bound GPD/GTP (Rasmussen et al., 2011). Upon hydrolyzation of GTP, regions of the Ras-like domain become disordered and the N and C terminal regions become structured, facilitating a loss of binding sites for effector proteins and a gain of Gβγ binding sites. Thus, the heterotrimeric complex is able to reassemble (Luttrell, 2008).

Just as GPCRs initiate the signalling cycle of the various heterotrimeric G proteins, activated Gα and Gβγ dimers induce the activation of their effector proteins. In mammals, the nine membrane-bound isoforms of AC are classified into four groups based on their sequence and regulation profiles, while the one soluble isoform acts alone as bicarbonate

sensor (Patel et al., 2001). The overall structure of the membrane bound ACs consists of an intracellular amino-terminal tail, two subunits containing 6-transmembrane spanning domains (M1 and M2) that are linked by a large cytoplasmic loop (C1) and followed by a carboxy-terminal tail (C2). The interaction of C1 and C2 induces cAMP production and is stabilized by the cAMP inducer forskolin. $G\alpha_s$ stimulates the activation of all of the membrane-bound ACs. $G\alpha_i$ inhibits directly six of the 10 AC isoforms, while these and the other may be regulated by various $G\beta\gamma$ dimers, second messenger-dependent kinases and Ca^{2+} (Patel et al., 2001, Wettschureck and Offermanns, 2005). $G\alpha_q$ on the other hand, is coupled to the phospholipase C (PLC), an enzyme that catalyzes the hydrolysis of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) (Wettschureck and Offermanns, 2005). While DAG acts in co-operation with phophatidylserine and Ca^{2+} to activate PKC, IP_3 activates its receptors on the endoplasmic reticulum (ER) resulting in intracellular calcium release. Both the activation of PKC and release of Ca^{2+} can have widespread effects on the cellular machinery and GPCRs themselves.

1.2.5 – Pharmacological Principles of GPCRs

The basic cycle of a typical GPCR can thus be depicted as such: The resting receptor is bound to the heterotrimeric G protein complex which binds a GDP molecule. Ligand binding to the receptor activates it such that it allows for the exchange of GDP for GTP on the bound G protein. GTP binding liberates the $G\alpha$ and $G\beta\gamma$ subunits allowing them to activate their effector proteins. GTP is hydrolyzed and the receptor/$G\alpha\beta\gamma$ complex reforms (**Figure 6**).

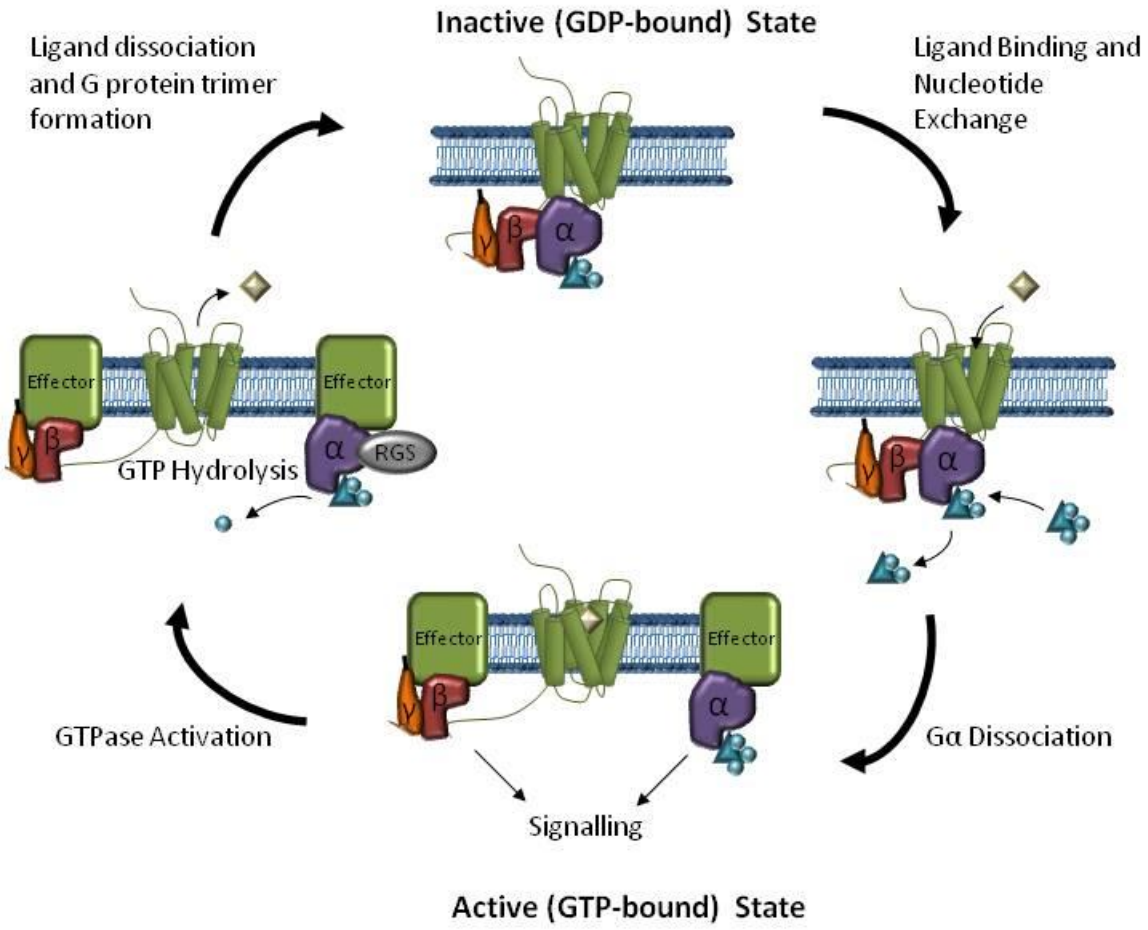


Figure 6. Basic cycle of GPCR signalling. The R state receptor is bound to the GDP-bound heterotrimeric complex. Upon ligand binding, conformational changes move the receptor into the R* state which facilitates exchange of GDP for GTP on the Gα subunit. GTP binding mediates Gα dissociation from Gβγ, both of which are then able to activate effector proteins. The inherent GTPase activity of Gα is enhanced through interaction with RGS proteins which leads to GTP hydrolysis into GDP. The GDP bound Gα then reassembles with Gβγ and the vacant receptor.

GPCRs exist in a dynamic equilibrium between an inactive, or R state, and an active or R* state (**Figure 7**). In a sense, they can be considered as rheostats that can be set to signal at any intermediary levels between two extremes. Full agonists are ligands which stabilize the receptor in its fully active R* state, whereas partial agonists induce the receptor to signal at some submaximal level. The term antagonist is given to a ligand which binds the receptor but has no effect upon its ability to activate its G protein (neutral ligands). GPCRs do not all require agonist binding to activate their G protein, some possess an inherent basal level of G protein activation. Agonist-independent, or constitutive activity, can be abrogated by ligands that stabilize the R state and are termed inverse agonists. Ligands all bind the receptor via different contact points and therefore, each possesses their own selectivity and characteristics at each receptor subtype.

1.2.6 – Regulation, Trafficking and Oligomerization of GPCRs

GPCR signalling was long presented as a simple two state model. We now know that it is an extremely complex process, with the existence of multiple receptor activation states and multiple G protein species coupling receptors to various effector systems. As the GPCR field has begun to mature, it has become apparent that these signalling systems are vastly more complicated. Additional factors that have come to light include receptor trafficking, regulation and oligomerization.

To avoid toxicity due to over stimulation of receptors, cells have developed methods to dampen signals and maintain an optimal range of response. This is accomplished in three major ways: homologous and heterologous desensitization, sequestration and recycling, and long term regulation of receptors. Heterologous

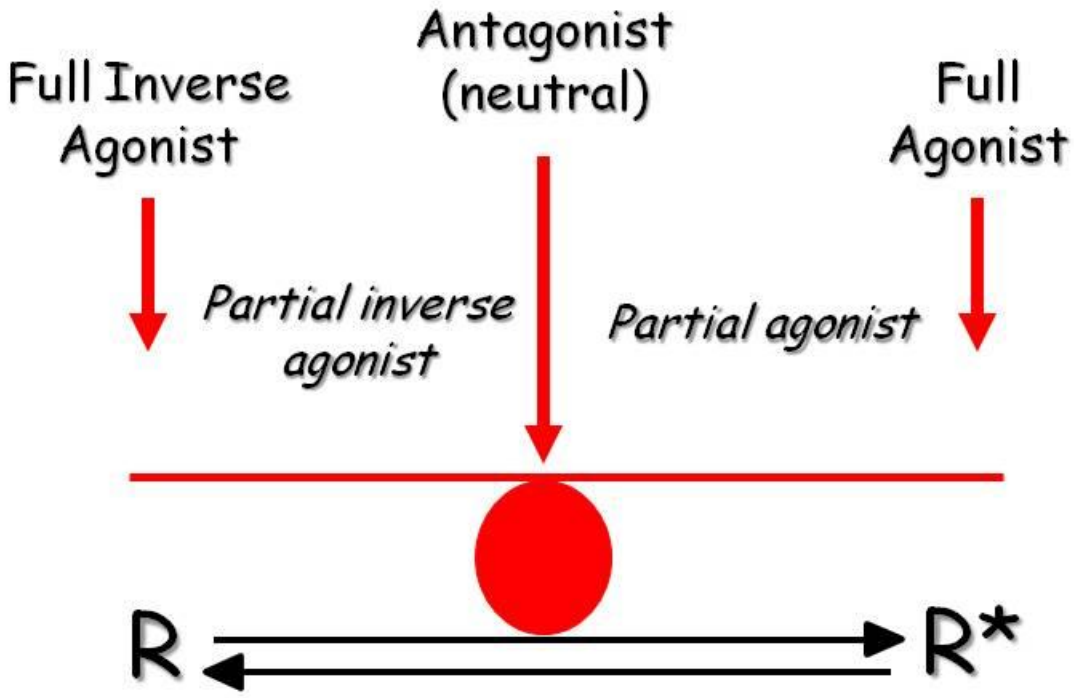


Figure 7. The dynamic nature of GPCRs. GPCRs exist in a dynamic equilibrium between the inactive (R) state and the active (R*) state. Full agonists stabilize the receptor population in R*, whereas full inverse agonist stabilize R. Partial agonists and partial inverse agonists stabilize intermediate activation states allowing for submaximal effects. Antagonists are neutral ligands that bind but have no effect on the receptor activation state.

desensitization occurs following the activation of second messenger-dependent protein kinases, such as PKA and PKC. These kinases phosphorylate residues within the ILs and CT of receptors which directly impairs G protein coupling. This process does not require the receptor to be bound to an agonist and can therefore occur following the activation of other receptors in the environment (Luttrell, 2008). Homologous desensitization occurs following agonist binding of the receptor. The change in receptor conformation induced by the agonist unveils phosphorylation sites on the IL3 and CT of the receptor, allowing G protein-coupled receptor kinases to phosphorylate serine and threonine residues. This increases the affinity of the receptor for arrestins, which then block G-protein interactions. β-arrestins (those found outside the visual system) also interact with clathrin, thereby targeting receptors for endocytosis (Luttrell, 2008). GPCRs undergo sequestration into clathrin-coated pits over a longer timescale than desensitization. Subsequent to endocytosis, receptors are either degraded or undergo rapid recycling to the plasma membrane. Long term regulation involves decreasing the density of receptors in the plasma membrane through both endocytosis and gene regulation, however, this process is not well understood (Luttrell, 2008).

In order to optimize the localization, specificity and efficiency of GPCR signalling, receptors interact with themselves and other modulatory proteins within the cell. More than a decade of study has established oligomerization of GPCRs with both themselves (homodimer) or other receptors (heterodimer), as an important functional consideration which remains at the forefront of current GPCR research. Many studies have shown that receptor oligomerization can ensure proper protein folding and delivery to the plasma membrane, as evidenced by the dominant negative effect on expression that some mutant

receptors exert over their wild-type partners (Luttrell, 2008). Furthermore, oligomerization can affect the ligand binding, signal transduction and receptor trafficking, thereby establishing distinct pharmacological profiles (George et al., 2002). Moreover, heterodimers have been shown to diversify signalling by facilitating completely new interactions with different G proteins and effectors, endowing the oligomers with physiological niches distinct from their constituent receptors alone (Milligan, 2009, Ng et al., 2010). Interactions with scaffolding and trafficking proteins, such as those containing PDZ domains, Homers and AKAPs, often facilitate the formation of signalosomes and the creation of microdomains within the cell. These environments cluster together the signalling components and increase the efficiency of signalling within the cell (Kristiansen, 2004, Luttrell, 2008).

1.3 D1-class Receptors

1.3.1 – Gene Structure, Knock Outs, and Expression Patterns

D1R was the first DA receptor to be discovered when it was shown that DA stimulated AC in rat caudate nucleus homogenates (Kebabian et al., 1972). The early nineties brought the cloning and characterization of D1R as well as the discovery of a second stimulatory DA receptor the D5R (originally named D1B in rat) (Dearry et al., 1990, Zhou et al., 1990, Sunahara et al., 1991, Tiberi et al., 1991). The gene structure of both D1R and D5R are similar in that neither contains introns and therefore, unlike the D2-class receptors, splice variants are not possible. However, two pseudogenes exist for D5R which encode non-functional proteins (Grandy et al., 1991). Polymorphisms exist in both

the D1-class receptors. D1R gene (drd1) polymorphisms are not associated with any change in amino acids as they are located in the 5' untranslated region or result in a silent mutation, but have been associated with essential hypertension and alcohol dependence (Sato et al., 2000, Kim et al., 2007, Batel et al., 2008, Le Foll et al., 2009). Polymorphisms of the D5R gene (drd5) have been linked to the age of onset of ADHD. However, despite the fact that 6 out of the 10 identified polymorphisms result in amino acid changes, there is no evidence implicating these genetic anomalies in the pathogenesis of the diseases (Wong et al., 2000, Lasky-Su et al., 2007, Zeng et al., 2008, Beaulieu and Gainetdinov, 2011)

Knock-out (KO) and knock down approaches have been employed to decipher the subtype specific physiological roles played by the D1-class receptors. Antisense mRNA interference has the advantage of utilizing mice that have developed normally and temporarily disabling a gene of interest. This approach has uncovered interesting behavioural phenotypes in D1-class knock downs, wherein rotational movement induced by SKF38393 in unilaterally lesioned rats was inhibited by D1 antisense oligonucleotides and potentiated by those of D5R. However, these results have been questioned as the levels of receptor protein appeared to be unaltered with the gene knock down (Dziewczapolski et al., 1998).

Complete ablation of D1R and D5R in mice has been achieved through homologous recombination. D1R KO results in mice that develop normally for the most part, but display failure to thrive that can be rescued with easier access to palatable food. This suggests a defect in fine motor movement and further supports the results of motor control tests in D1R KOs that indicate D1R is important for maintenance of proper motor control. Inconsistencies again have been present in these studies as D1R KO mice displayed

opposing locomotor phenotypes. These discrepancies may be attributed to the genetic background of the mice or experimental differences (Holmes et al., 2004). D1R KO mice also display deficits in behavioural and cognitive tasks that invoke the hippocampus and prefrontal cortex and in long term potentiation. Hypertension is also observed in D1 KO mice, which likely develops from a lack of receptors in the kidneys and vasculature and therefore a deficit in natriuresis and vasodilation, although a central mechanism involving interactions with NMDA receptors and sympathetic input to the kidney has yet to be ruled out (Yang et al., 2004, Zeng et al., 2008).

Unlike the D1R KO models, D5R KO mice display normal growth patterns. These mice also develop hypertension but this is develops centrally, as a complex interaction between D5R, oxytocin, vasopressin, non-NMDA glutamate and adrenergic receptors becomes unbalanced (Hollon et al., 2002, Zeng et al., 2008). Furthermore, D5 KO mice have increased systemic oxidative stress produced in the brain and kidneys, another significant contributor to hypertension (Zeng et al., 2008). Gastric ulcers are also prevalent in these KO mice. Studies have demonstrated motor phenotypes to be negligible in the D5R KO which is in keeping with its relatively low levels in the striatum. Hyperactivity in response to D1-class agonists however is attenuated to the same level as in D1R KO mice (Holmes et al., 2001). It has been suggested that D1R and D5R, when co-expressed in the same striatal neurons, exert synergistic control over motor function (Holmes et al., 2004). Surprisingly, impaired learning is not apparent in hippocampal behavioural tests of D5R KO mice, despite the fact that hippocampal expression of D5R exceeds that of D1R. Studies have shown a decrease in acetylcholine release in the hippocampus of D5R KO mice (Laplante et al., 2004). This corresponds to earlier work showing a link between

acetylcholine release and D5R activation and suggests a modulatory role for D5R in the hippocampus (Holmes et al., 2004).

KO models for D1R and D5R have been used to assess comparatively any functional differences between the subtypes in cocaine locomotor response, induction of seizures, hypertension, spatial learning, hearing and acoustic injury, sexual behaviour and skeletal muscle mass and force, however clear-cut distinctions between the receptors are not always apparent (O'Sullivan et al., 2004, Yang et al., 2004, Kudwa et al., 2005, Karlsson et al., 2008, O'Sullivan et al., 2008, Asico et al., 2011, Maison et al., 2012). Caveats of KO approaches such as incomplete interference or developmental compensation can hinder confident interpretation of results.

In order to confidently assess KO results and to develop further hypotheses regarding the physiological and pathological roles that D1-class receptors play, extensive characterization of the patterns of their gene activation is required. D1R is the most widely and highly expressed in the brain of the DA receptors. D1R mRNA is found in the caudate, nucleus accumbens and olfactory tubercle (Dearry et al., 1990). Immunostaining shows the D1R is densely expressed in the striatum, nucleus accumbens, substantia nigra, olfactory bulb, amygdala and frontal cortex and found at lower levels in the hippocampus, thalamus, hypothalamus and retina (Beaulieu and Gainetdinov, 2011). The areas of D1R protein and mRNA expression do not always overlap, suggesting that the receptor may be present in projections (Missale et al., 1998).

In contrast to D1R, D5R expression is less dense and restricted to fewer regions. D5R mRNA is found in the hippocampus, lateral mammillary nucleus, parafascicular

nucleus, cortex and lateral thalamus, and in lesser amounts in the hippocampus, medial thalamus and substantia nigra (Tiberi et al., 1991, Meador-Woodruff et al., 1992, Missale et al., 1998). D5R protein is expressed in the hippocampus and dentate gyrus, prefrontal cortex, premotor cortex, entorhinal cortex, substantia nigra and hypothalamus and at low levels in the striatum (Missale et al., 1998, Ciliax et al., 2000, Beaulieu and Gainetdinov, 2011). In the periphery both D1-class receptors are expressed in the cardiovascular system, kidneys, gastrointestinal tract, cochlea and adrenal glands (Missale et al., 1998, Iversen and Iversen, 2007, Maison et al., 2012). The different expression patterns of D1-class receptors suggest their involvement in different physiological functions.

D1R and D5R also differ in their subcellular localization. Within the neuronal circuitry, D1-class receptors are located both pre and post-synaptically, with the latter being found most frequently. However, whereas D1R is found in the dendritic spines of neurons, the D5R are predominantly found in the dendritic shafts and necks of spines, suggesting distinct functions of the two receptors when co-expressed in the same cells (Missale et al., 1998, Kruusmagi et al., 2009).

1.3.2 – Regulation and Protein Interactions

Further evidence for subtype specific functions comes from differences observed in the regulation of the D1-class receptors. Work in our lab has demonstrated that phorbol-12-myristate-13-acetate (PMA) induces PKC desensitization and sensitization in D1R and D5R, respectively (Jackson et al., 2005). This opposite effect, unique to the D1-class receptors, was shown to be mediated by the IL3 (Plouffe et al., 2012). Trafficking properties of the D1R and D5R also differ between each other. Endocytosis of D5R is

induced both homologously and heterologously, whereas D1R is only subject to homologous desensitization (Thompson and Whistler, 2011). This subtype specific element is also manifested through residues within the IL3.

Dopamine receptor-interacting proteins (DRIPs) include other receptors, scaffolding and trafficking proteins, and are integrally involved in mediating various aspects of D1-like signalling and may contribute to distinct functions. Heterodimerization of D1R and D2R has been demonstrated to occur in neurons and results in novel signalling through the G_q/PLCβ system. This interaction involves the CT of D1R and the IL3 of D2R (Ng et al., 2010). D1R has also been shown to dimerize with the adenosine A_1R, an interaction that antagonizes D1R-mediated cAMP production and demonstrates functional crosstalk between the two receptors (Wang et al., 2008). Interactions between D1-class receptors and ligand-gated ion channels are critical for mediating modulation of synaptic transmission in dopaminergic neurons. The CT of D5R interacts directly with the IL2 of $GABA_A$ receptor γ2 subunit. D5R activation results in decreased $GABA_A$-mediated inhibitory postsynaptic currents, while $GABA_A$ activation attenuates D5R-mediated cAMP production. Furthermore, these receptors colocalize in dendritic shafts of hippocampal neurons which suggests a functional difference between D1R and D5R (Wang et al., 2008). D1R's CT has been shown to interact with that of NR1 and NR2A subunits of the NMDA receptor. Whereas D1R activation suppresses NMDA-mediated currents and NMDA receptor-mediated cell death, NMDA receptor activation increases cell surface expression of D1R and therefore increases cAMP production (Pei et al., 2004). These proteins all coexist in the heads of dendritic spines where glutamatergic synapses are formed. The

relationships with ligand-gated ion channels underline the direct modulation of fast-synaptic transmission by the D1-class receptors.

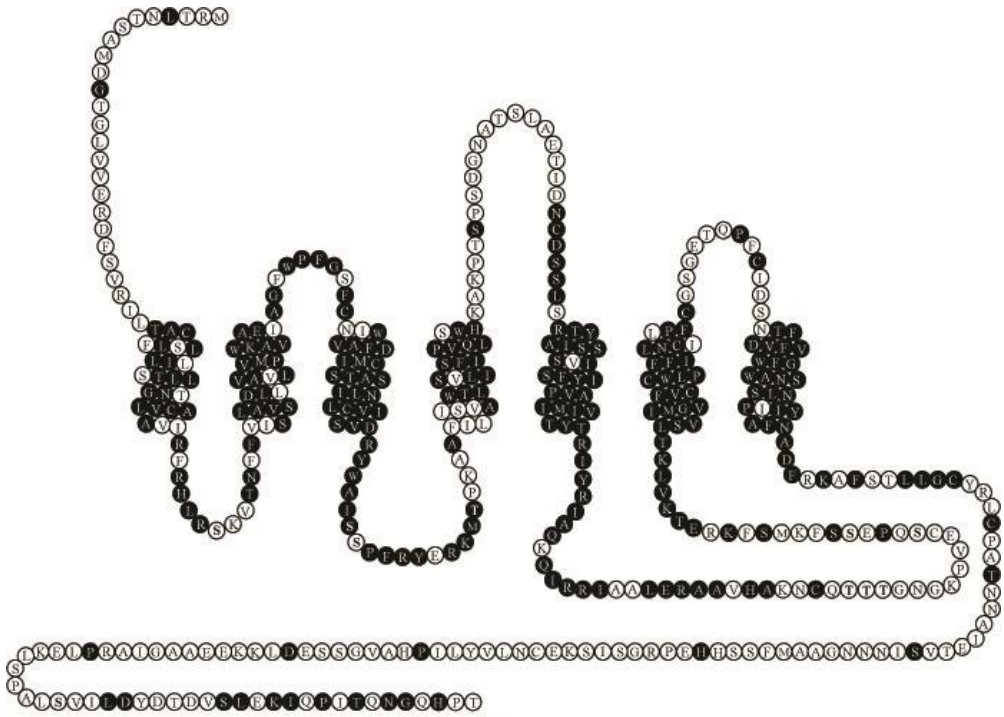
Further complexity arises with the interactions of D1-class receptors with cytoskeletal and trafficking proteins. Proper protein folding and cell surface expression is also dependent upon ER chaperone proteins such as calnexin and DRIP78. Cytoskeletal elements such as Neurofilament M and PSD-95 are among those proteins interacting with the D1-class receptors and the oligomers they reside within (Wang et al., 2008). These proteins facilitate receptor localization and cell surface expression, as well as modulating receptor interactions with other proteins. PSD-95 has been shown to constitutively reduce D1R signalling by uncoupling the interaction between D1R and NMDA receptor subunits, thereby reducing the insertion of D1R into the synapse and therefore the production of cAMP (Kim et al., 2002, Gu et al., 2007, Zhang et al., 2007, Zhang et al., 2009b). Interaction with PSD-95 and NMDA further dictates functional differences in diffusion and localization between the D1-class receptors, within striatal neurons (Kruusmagi et al., 2009). Although distinct functional roles of D1R and D5R seem likely based on their localization in tissues and cells, demonstrating this has proven challenging without subtype-specific pharmacological agents. In order to develop the selectivity required to better define these roles, a detailed knowledge of D1-class receptor structure is required.

1.3.3 – Structural Comparison and the Ligand Binding Pocket

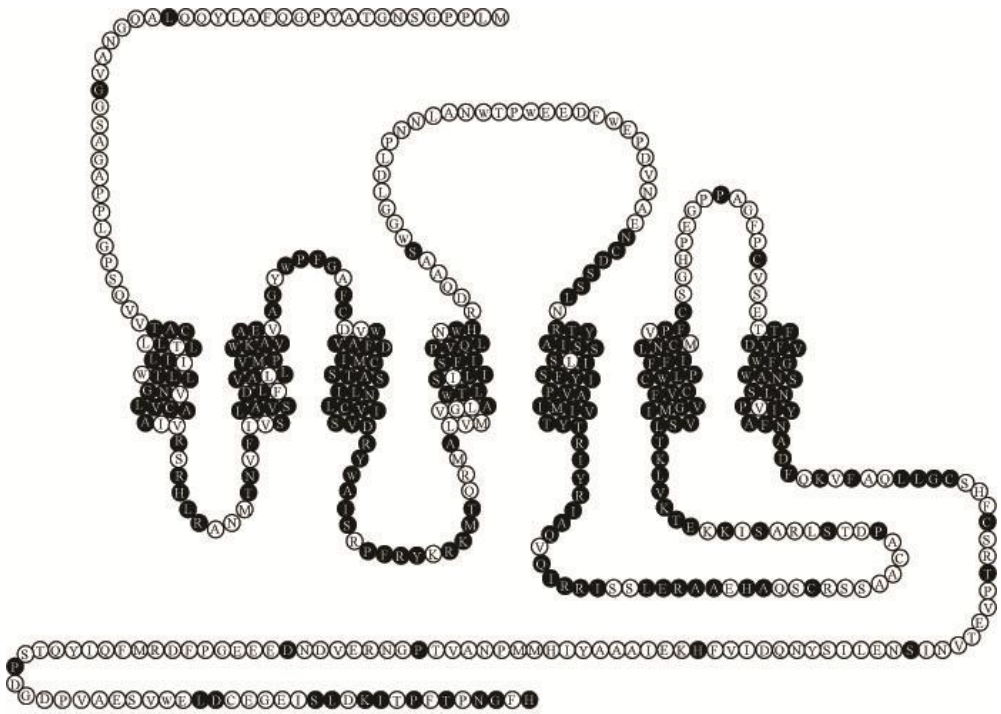
The first characteristics of the D1-class receptors to be identified were their abilities to bind DA. Based on mutagenesis studies in the β-ARs it had previously been established that an aspartic residue in TM3 and serine residues in TM5 were conserved among all

bioamine receptors and critical to catecholamine binding (Fong and Strader, 1994). Further work in D1R confirmed the importance of these residues and others within TM 1, 2, 3, 5, 6 and 7 in binding DA (Pollock et al., 1992, Tomic et al., 1993, Cho et al., 1996, D'Aoust and Tiberi, 2010). The D1-class receptors share over 80% homology in the TM regions and the lack of ligands distinguishing the two subtypes D1R and D5R can likely be attributed to this similarity (**Figure 8**). It is therefore also surprising that D5R displays an approximately 10-fold higher affinity for DA than D1R. Higher affinity at D5R than D1R is true of all agonists, while antagonists display higher affinity at D1R (Sunahara et al., 1991, Tiberi et al., 1991).

Previous studies in our lab employing chimera approaches have demonstrated that ligand affinity and selectivity between D1R and D5R is dependent upon intracellular and extracellular regions of the receptors as well. Swapping the terminal receptor locus (TRL), constituting the TM6, EL3, TM7 and CT regions, increased the DA affinity of the D1 (TRL^{D5}) and decreased it for D5 (TRL^{D5}). EL3 alone chimeras saw similar results though to a lesser extent (Iwasiow et al., 1999). Similarly, exchange of the CT alone of the D1-class receptors led to a striking, almost complete swap in DA affinity between D1R and D5R; a result also confirmed by another group (Demchyshyn et al., 2000, Jackson et al., 2000). This effect can be almost entirely attributed to the helix 8 region of the CT (Chaar et al., 2001, Tumova et al., 2004). The changes in ligand binding affinity that result from the exchange of regions outside of the TM domains suggest that the intracellular and extracellular regions confer conformational profiles within the orthosteric binding pocket of D1-class receptors that are distinct from one another.



human D1R



human D5R

Figure 8. Topology of the human D1-like receptors. Conserved residues are shown in black (filled) and divergent residues are shown in white (circles).

The extracellular and intracellular regions of the D1-like receptors contain much of the amino acid diversity between the subtypes. Although EL1 is fairly similar between the D1-class receptors, NT and EL2 and EL3 are very much different. The NT is almost exclusively divergent between the D1R and D5R with the exception of two residues, one of which is an N-linked glycosylation site that plays a role in receptor surface expression but not ligand binding in D5R and not D1R (Karpa et al., 1999). ECL2 and ECL3 are also dissimilar between the D1R and D5R, however they contain two conserved cysteine residues that potentially forms a salt bridge that is thought to be key to receptor stability(Missale et al., 1998). The CT and IL3 also contain many divergent residues and as these are intracellular regions, they are likely implicated in the subtype specific differences in activation observed between the receptors (Charpentier et al., 1996, Missale et al., 1998, Iwasiow et al., 1999, Demchyshyn et al., 2000, Jackson et al., 2000).

1.3.4 - Subtype Specific Signalling Characteristics

The differences in the binding profiles of the D1-class receptors correspond with differences in activation as well. Agonists induce activation more potently at D5R than D1R; however, maximal agonist stimulation induces a greater response in D1R than D5R. The most distinguishing feature between the D1-class receptor is the increased agonist-independent activity of D5R over D1R (Tiberi and Caron, 1994, Missale et al., 1998, Beaulieu and Gainetdinov, 2011). The correlation between increased agonist affinity and increased basal activity fit with what is known as the "extended ternary complex model" and is the hallmark feature usually associated with constitutively active mutant (CAM) GPCRs (Ren et al., 1993, Samama et al., 1993). In this line of thinking, D5R can be considered as a naturally occurring CAM of D1R (Tiberi and Caron, 1994). This feature

may be physiologically important, as the basal activity of D5R has been suggested to support the tonic burst firing of neurons of the subthalamic nucleus and the normal release of atrial natriuretic factor from the hypothalamus (Lee et al., 1999, Baufreton et al., 2005).

In the past, CAMs had been generated by mutating residues in the IL3 region adjacent to TM6, now considered its cytoplasmic extension (Kjelsberg et al., 1992, Ren et al., 1993, Samama et al., 1993). This region is well conserved in biogenic amine receptors, however, there is divergence of two residues at the border of the TM6 cytoplasmic extension between D1R and D5R (**Figure 9**) (Cotecchia et al., 1990, Charpentier et al., 1996). The basal activity of D5R is reduced by the exchange of one of these residues, Phe264 in D1R and Ile288 in D5R, whereas in D1R it is induced (Charpentier et al., 1996). This partial swap is the basal activity was also accompanied by partial recapitulation of the other aspects of the D1-class receptor phenotypes (i.e. agonist affinity and potency). This suggested that other determinants outside of the TM6 cytoplasmic extension were involved in bestowing D1R and D5R their phenotypes (Charpentier et al., 1996).

Following this line of thought, our lab has demonstrated that a number of other regions of the D1-class receptors contribute to their distinct attributes. Basal activation and agonist induced characteristics were found to be interchangeable between chimeric receptors which harbour a swapped TRL cassette, whereas maximal agonist-induced activation was unchanged. Swap of the EL3 alone however, resulted in the complete exchange of DA-induced maximal activation characteristics of D1R and D5R (Iwasiow et al., 1999). Exchange of both EL3 and CT switched the basal activity but not the DA-induced maximal stimulation of AC, whereas, the swap of the CT alone resulted in a reversal of basal activity between D1R and D5R, while negatively affecting the DA

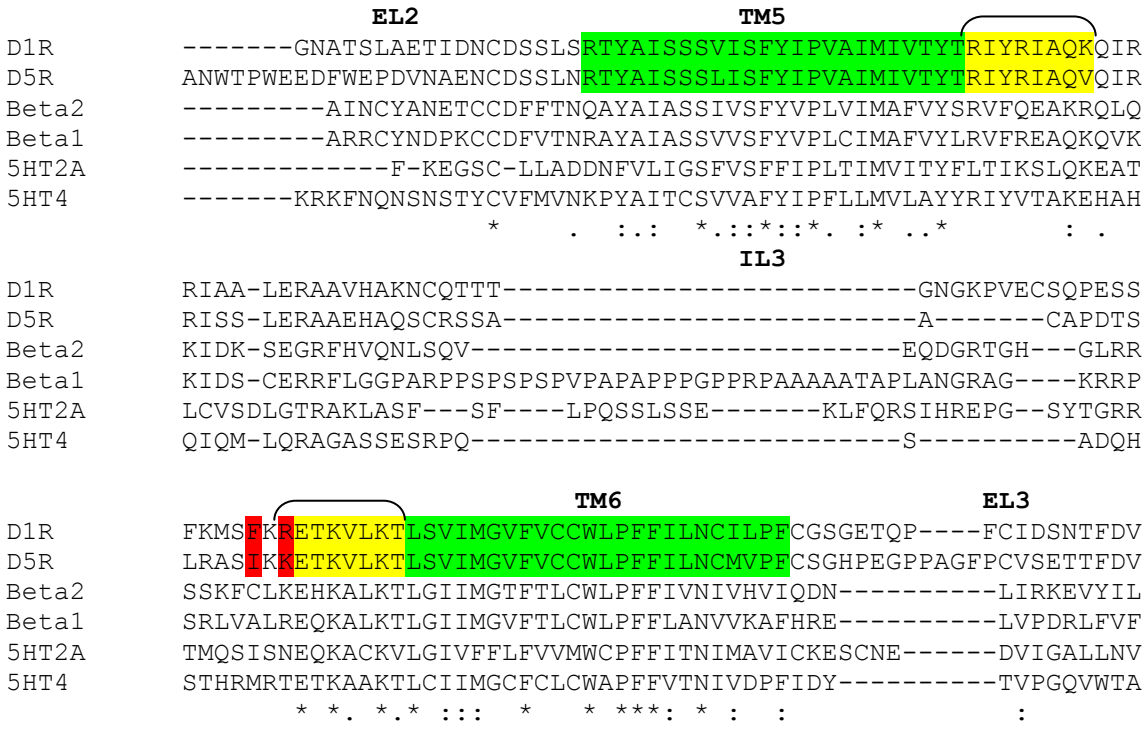


Figure 9. Sequence alignment of TM5, IL3 and TM6 of the D1-class receptors (D1R, D5R) with β_2 adrenergic receptor (Beta2), β_1 adrenergic receptor (Beta1), $5HT_{2A}$ receptor (5HT2A) and $5HT_4$ receptor (5HT4). The cytoplasmic extensions of TM5 and TM6 are highlighted in yellow and delimited by brackets. Two divergent regions at the border of the TM6 cytoplasmic extension are highlighted in red. Extracellular loops 2 and 3 (EL2, EL3) are flanking TM5 and TM6, respectively. Sequence alignment performed using the Clustal Omega (www.clustal.org). TM regions highlighted in green. * denotes fully conserved residue, : indicates strongly similar residues, . indicates weakly similar residues.

potency of both (Jackson et al., 2000, Tumova et al., 2003). Investigation into the specific role of helix 8, located within in CT, showed that it is a critical region in the control of subtype specific constitutive activity and affects DA-induced cAMP synthesis (Tumova et al., 2004). These chimeric studies highlight the complex interaction between

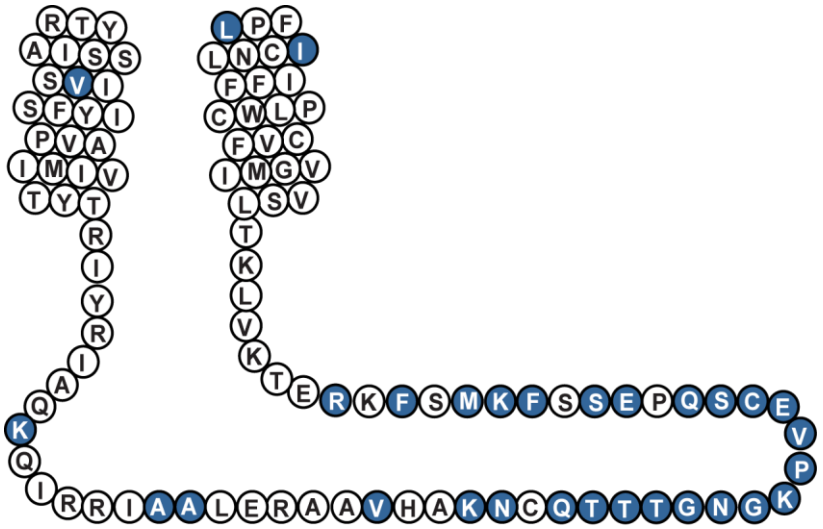
the intracellular, TM and extracellular regions of the D1-class receptors that leads to their distinct basic signalling properties.

As mentioned above, the IL3 contains high levels of diversity between the D1-class receptors and has also been associated with the opposite regulation observed between them. The IL3 of D1R, including the cytoplasmic extensions of TM5 and TM6, contains 57 amino acids, whereas that of D5R contains 50. The structural differences between the two subtypes, when combined with previous implications in divergence of coupling and regulation, pose the IL3 of the D1-class receptors as a prime target for exploiting subtype specific characteristics (**Figure 10**).

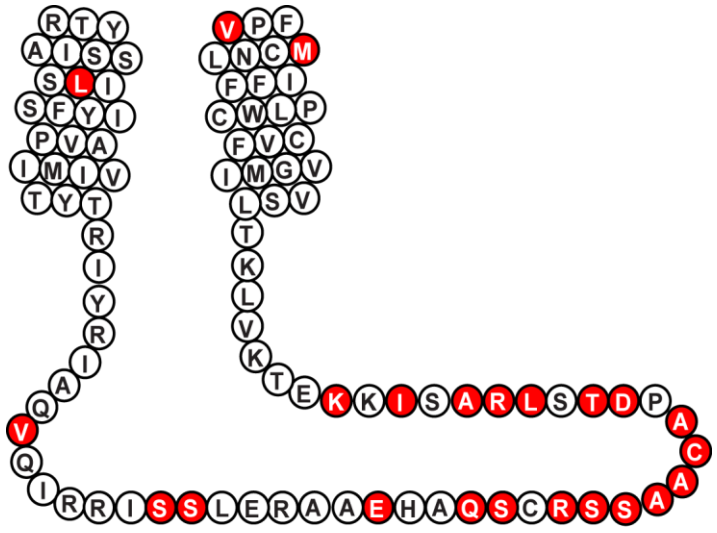
1.4 The Third Intracellular Loop

1.4.1 – The IL3 in Structural Studies

The IL3 is the intracellular domain linking TM5 and TM6. It has long been identified as a critical domain for G-protein coupling and GPCR activation, interactions with regulation and trafficking proteins. Despite these important roles there remains a paucity of information regarding its structure. As there is broad variability in the length and sequence of this domain, its contribution remains somewhat receptor specific. This



D1R



D5R

Figure 10. Schematic of the TM5, IL3 and TM6 of the D1-class receptors. White circles represent residues conserved between D1R and D5R. Coloured circles represent divergent residues.

variability also induces flexibility in the receptor and for a long time thwarted crystallography of GPCRs (Deupi and Kobilka, 2007). In order to facilitate solving the crystal structure, extensive modification of the IL3 has been necessary. Successful cystallization has been achieved by stabilizing receptors through the replacement of the IL3 with a T4 lysozyme, deletion and residue replacement or the use of specifically raised antigen binding fragments of antibodies (FABs) against the IL3 (Cherezov et al., 2007, Rasmussen et al., 2007, Warne et al., 2008, Haga et al., 2012, Kruse et al., 2012).

Although these studies have provided a wealth of information regarding the remainder of the receptors, they have not provided support to some of the widely accepted theories of receptor activation. In particular, the crystallization of receptors lacking a complete IL3 has shown the residues involved in the "ionic lock" lack the proximity to interact (Warne et al., 2008). Recently, three studies have successfully solved the crystal structure of the adenosine 2A receptor ($A_{2A}AR$) with an intact IL3 (Dore et al., 2011, Lebon et al., 2011, Hino et al., 2012). These receptors have all displayed the ionic lock, suggesting that an intact IL3 is required for its maintenance in the inactive state receptor (Hino et al., 2012). Interestingly, examination of crystals obtained while solving the turkey β_1 adrenergic (β_1AR) structure (thermostabilized through IL3 modifications) determined that the IL3 exists in two distinct conformations in the inactive state. One receptor possesses a bent TM6 and the interactions required of the ionic lock, while the other has a straight TM6 and no ionic lock (Moukhametzianov et al., 2011). This may suggest that one inactive receptor conformation may possess the ability to signal constitutively, while the other does not.

NMR and circular dichroism spectroscopy assessment of the IL3 peptides of the $5HT1_A$ receptor, turkey β-adrenergic erythrocyte receptor and human $AT1_A$ receptors indicated that the proteins contain both α-helical and disordered regions, usually with the helix region residing towards the C-terminal and N-terminal sides of the peptide (Jung et al., 1995, Franzoni et al., 1999, Chen et al., 2011). Rigid helical structures were also demonstrated to exist in a V2 vasopressin receptor IL3 circular peptide and in the central and TM5 and TM6 adjacent regions of the IL3 of the human CB_1R (Ulfers et al., 2002, Bellot et al., 2009). The structural features seem to vary between receptor species, but a common feature that is shared by all is the location of a helical structure juxtaposed to either TM5 or TM6 or both.

1.4.2 – The Cytoplasmic Extensions of TM5 and TM6

Classically, the juxtamembrane regions at the N-terminal and C-terminal sides of the IL3 are well conserved between GPCRs and have been attributed as the major but not sole G protein interacting regions (**Figure 9**) (Strader et al., 1987, Kobilka et al., 1988, Voss et al., 1993, Charpentier et al., 1996). This notion arose from mutagenesis studies in a number of GPCR species from all classes which repeatedly demonstrated either one of the IL3 juxtamembrane regions to be critical to G protein coupling and activation (Strader et al., 1987, Eason and Liggett, 1995, Takhar et al., 1996, Couvineau et al., 2003, Kuniyeda et al., 2007). Furthermore, these regions have also been attributed to G protein specificity and the constitutive signalling ability of GPCRs (Strader et al., 1987, Kobilka et al., 1988, Cotecchia et al., 1990, Kjelsberg et al., 1992, Ren et al., 1993, Samama et al., 1993, Charpentier et al., 1996). Over the course of time, these mutagenesis and functional studies have been further supported by structural, kinetic and homology modelling work (Franzoni

et al., 1999, Ulfers et al., 2002, Zurn et al., 2009, Chen et al., 2011, Umanah et al., 2011). These studies have emphasized the importance of the rigid α-helical structure of the cytoplasmic extensions of TM5 and TM6. As discussed above, the movement of TM5 and TM6 appears to be the principle rearrangement of the cytoplasmic face of the receptor that facilitates G protein coupling. It has often been proposed that the cytoplasmic extension of TM5 is responsible for G protein coupling, while that of TM6 is responsible for G protein specificity and activation (Franzoni et al., 1999). Although extensive study of these regions has determined them to be critical, there remains significant diversity in their specific contribution to G protein coupling between receptor species.

1.4.3 - The Central Region of the IL3

Literature addressing the role of the IL3 in receptor activation is sparse in comparison to that of the TM5 and TM6 juxtamembrane regions. There is a growing body of work implicating the central IL3 as participating in interactions critical to receptor regulation, oligomerization and other protein interactions (Borroto-Escuela et al., 2010, Chen et al., 2011, Kluetzman et al., 2011, Umanah et al., 2011). The paucity of studies focusing on the central IL3 in the context of receptor activation however, likely arises from the variety of results that have been achieved by those who have chosen to focus on this area, depending on the receptor investigated. Many mutagenesis studies have reported negligible effects of mutations within the central IL3 (Kameyama et al., 1994, Pellegrini et al., 1996, Peverelli et al., 2009). For example, deletion of 196 amino acids within the central IL3 in the G_q-coupled muscarinic M3 receptor proved to have no effect on ligand binding and coupling (Maggio et al., 1996). However, a number of studies have provided

evidence that the central IL3 does indeed significantly affect G protein coupling and ligand binding.

Chimeric receptors swapping the central IL3 of G_q-coupled α_1AR for the homologous region in G_s-coupled β_2AR, and deletions within the central IL3 of the G_q-coupled NK_1receptor both led to decreased phophotidylinositol production, while agonist affinity was maintained at wild-type levels (Cotecchia et al., 1990, Nielsen et al., 1998). Deletion of the central IL3 in the glucagon receptor (G_s-coupled) also resulted in proteins that were poorly coupled to the G protein and displayed comparable glucagon binding (Chicchi et al., 1997). Interestingly, with deletion mutations introduced into the G_i-coupled μ-opioid receptor, differential effects on coupling were observed depending on the agonists applied to the system and the position of the deletion within the central IL3. Certain mutations induced increased potency and coupling efficiency for some agonists, whereas others displayed decreases (Chaipatikul et al., 2003). FRET and NMR studies of GPCR activation may further aid in the interpretation of these mutational studies data.

Fluorophores inserted into the central portion of the IL3 of α_{2A} AR have demonstrated movement of the central IL3 during receptor activation. This corresponds to the agonist-induced movement of TM5 and TM6, the extent of which differs with the pharmacological profile of the ligand, i.e. decreases with activation of the receptor by partial agonists (Zurn et al., 2009). NMR characterization of the rat angiotensin $AT1_A$ receptor suggests that a cis, trans-isomerization around a proline residue in the central IL3 may act as a switch, propagating changes in TM6 induced by agonist binding into conformational changes of the helices at the cytoplasmic face of the receptor (Franzoni et al., 1999). This body of work suggests the possibility that the central IL3 plays a critical

role in facilitating the co-ordinated movement of TM5 and TM6 during receptor activation. However, the importance of this domain may extend beyond simply enabling activation to exerting modulation to control the extent of the coupling induced.

In the NPY receptor it has been shown that the central IL3 acts to stabilize the receptor in the inactive conformation. The ability to signal constitutively was assessed and the lack of constitutive activity in the wild-type receptor was attributed to the central IL3 and interactions affecting the ionic lock (Chee et al., 2008). Similarly, naturally occurring point mutation P230L in the melanocortin 4 receptor results in a constitutively active mutant, suggesting this region acts to maintain optimal levels of basal activation (Kim et al., 2008). This implicates the central IL3 as possibly participating in the control of signalling and maintenance of optimal levels of receptor signalling for its function. Furthermore, differences in the activation profiles of the β_1 AR and β_2 AR, as well as isoforms of the histamine H_3 receptor have been attributed to the central IL3 region (Green and Liggett, 1994, Bongers et al., 2007). Overall, these studies suggest that the central IL3 should be considered a critical piece of the receptor activation machinery and likely plays an important role in the differences observed between many receptor subtypes.

1.5 Rationale and Hypothesis

One of the most vexing issues in the field of DA receptors has been the inability to pharmacologically isolate the D1-class receptor subtypes. Although D1-class receptors have been implicated in many normal physiological and pathological processes in the body, the specific contribution of D1R or D5R has sometimes been vague (Missale et al., 1998, Beaulieu and Gainetdinov, 2011). The distinct constitutive activity of D5R has been

suggested to be an important functional feature; however, it has only thus far been implicated in a couple of *in vivo* processes (Lee et al., 1999, Baufreton et al., 2005). Constitutive activity in other GPCRs has proven to be an important part of their physiological function and has been implicated in the pathophysiology of many diseases (Costa and Cotecchia, 2005, Tao, 2008). The development of selective ligands for the D1-class receptors would therefore amount to a great advance in the study of dopaminergic signalling, making possible the further characterization of the specific physiological and pathological roles of both receptors.

The subtype specific characteristics of D1-class signalling are most likely to result from the divergent residues of the IL3 or CT (Missale et al., 1998). In fact, subtype specific differences in D1-class regulation have been attributed to residues within the IL3 (Thompson and Whistler, 2011, Plouffe et al., 2012). Moreover, the cytosolic extension of TM6 of the D1-class receptors have previously been demonstrated to each harbour a divergent residue which imparts the constitutive activity properties specific to each receptor (Charpentier et al., 1996). These residues are not sufficient to fully explain the phenotypes of D1R and D5R and therefore other regions either outside or within the IL3 must also be involved.

The central IL3 of D1-class receptors consists of a mostly conserved N-terminal half and a mostly divergent C-terminal half (**Figure 10**). On the basis of primary structure alone, this layout seemed convenient for elucidating the function of the two halves in each receptor. Unlike the cytoplasmic extensions of TM5 and 6, the primary structure of this domain is for the most part quite varied in structure between GPCR subtypes; so elucidating a general functional role for it may not be possible (**Figure 9**). However, it

appeared as though it may be critical to G protein-coupling domain, by supporting the proper conformational changes to the intracellular face that allow for interaction between the cytoplasmic extensions of TM5 and 6 and the G protein (Green and Liggett, 1994, Zurn et al., 2009). Furthermore, the difficulty in obtaining the crystal structure of the intact IL3 in GPCRs emphasizes the importance of characterizing this region functionally as a complement to structural data, in order to recognize its potential contribution to signalling.

A deletion approach had previously been adopted in our lab in the process of identifying the specific residues involved in the opposite PKC-dependent effects attributed to the IL3 (Jackson et al., 2005, Plouffe et al., 2012). These mutants took advantage of the natural boundaries of the central IL3, with the mostly conserved N-terminal side of the IL3 being removed (Q224-T245 of D1R and Q255-S275 of D5R). It was rationalized that deletion of the N-terminal half would highlight its function and any subtype-specific characteristics that may reside in the remaining residues of the C-terminal side. The reciprocal approach would then be taken by deleting the C-terminal side of the central IL3 (G246-K265 of D1R and A276-K289 of D5R).

The N-terminal side of the central IL3 was hypothesized to be vital to the G protein coupling of the receptor, as it contains mostly conserved residues. In contrast, the C-terminal side contains mostly divergent residues. It was thus also hypothesized that this region may impart some of the subtype specific characteristics to the D1-class receptors, such as the high agonist affinity and constitutive activity of D5R. Overall, the central IL3 was thought to contribute significantly to receptor activation and this study was therefore carried out in order to highlight the function of a region that has often been underappreciated.

Manuscript

Two structurally distinct domains of the third intracellular loop of human D1-class dopaminergic receptors regulate the transition between inactive and active states

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Abstract

Dopamine D1-class G protein-coupled receptors (D1R and D5R) play critical physiological roles in the brain. Despite noticeable differences in ligand binding and G protein coupling of D1R and D5R, the development of subtype-specific ligands has been difficult. Divergence in the intracellular domains, particularly the central region of the third intracellular loop (IL3), may explain subtype-specific features. Deletion mutations of the mostly conserved N-terminal moiety or divergent C-terminal moiety of the central region of IL3 of D1-class receptors (D1ΔN and D5ΔN; D1ΔC and D5ΔC) were used to highlight the role of these IL3 moieties in transfected HEK293 cells. Saturation binding studies with the D1-class selective antagonist [^{3}H]-SCH23390 showed that deletions did not prohibit receptor expression and [^{3}H]-SCH23390 affinity was not drastically altered. We found that affinity of agonists were significantly increased and reduced at D1ΔN and D5ΔN, respectively. However, D1ΔC and D5ΔC displayed significantly increased affinity for agonists. Whole cell cAMP assays showed ablation of constitutive activity of D1ΔN and D5ΔN and a severe blunting in dopamine-induced cAMP formation. In opposition, the constitutive and agonist-dependent activity of D1ΔC and D5ΔC was robustly increased. 3D homology models of wild-type and mutant receptors predicted striking differences between IL3 of D1R and D5R. Models of deletion mutants suggest that the central IL3 may contain conformational switches regulating the transition between inactive and active states of D1-class receptors. This work complements recent structural studies of GPCRs and implicates the central region of IL3 as being critical to the subtype-specific activation of D1-class dopaminergic signalling.

Keywords: G protein-coupled receptors, Dopamine, D1-class Receptors, Third Intracellular Loop, Deletion, Homology Modelling

Introduction

Dopamine (DA) is a catecholamine neurotransmitter that participates in the control of important physiological processes such as voluntary movement, learning and memory, attention and reward (Missale et al., 1998, Iversen and Iversen, 2007, Cools and D'Esposito, 2011, Young et al., 2011). DA receptors are seven-transmembrane G protein-coupled receptors (GPCRs) that are grouped as either D1-class (D1R and D5R) or D2-class (D2R short, D2R long, D3R and D4R) based on their ability to stimulate (D1-class) or inhibit (D2-class) the production of cAMP by adenylyl cyclase (AC) through the coupling to heterotrimeric $G_{s/olf}$ and $G_{i/o}$ proteins, respectively (Beaulieu and Gainetdinov, 2011). The D1-class receptors are differentially expressed in the basal ganglia, olfactory bulb, amygdala, hippocampus and frontal cortex (Levey et al., 1993, Bergson et al., 1995, Missale et al., 1998, Ciliax et al., 2000). In agreement with this brain distribution, impairment in D1-class signalling is implicated in many debilitating diseases displaying motor and cognitive dysfunction such as Parkinson's disease, Huntington's disease and schizophrenia as well as in drug addiction (Zhang et al., 2009a, Beaulieu and Gainetdinov, 2011). The D1-class receptors therefore represent important drug targets (Mailman et al., 2001, Lewis et al., 2006, Giorgioni et al., 2008, Zhang et al., 2009a).

Our knowledge of the exact contribution of D1R and D5R in normal and pathophysiological conditions is hampered by the lack of ligands which can fully discriminate between the two subtypes. The difficulty of developing D1-class subtype-specific drugs is likely explained by the over 80% primary sequence identity shared between D1R and D5R transmembrane (TM) domains, which contains the orthosteric ligand binding sites (Jarvie and Caron, 1993). Interestingly, despite sharing high degree of TM identity, D1R and D5R display significant differences in their ligand binding and G protein coupling properties (Sunahara et al., 1990, Tiberi et al., 1991, Tiberi and Caron, 1994). As the intracellular domains of GPCRs are responsible for G protein coupling and protein-protein interactions, and as the first and second intracellular loops are well conserved between the D1-class receptor subtypes, the third intracellular loop (IL3) and the cytoplasmic tail (CT) have been implicated in bestowing functional phenotype differences to D1R and D5R (Charpentier et al., 1996, Iwasiow et al., 1999, Demchyshyn et al., 2000,

Jackson et al., 2000). However, the structural basis underlying the function of IL3, the prime region involved in G protein coupling and activation of D1-class receptors is not well understood.

Studies into the role of the IL3 of GPCRs have primarily focused upon two stretches of residues forming the cytoplasmic extensions of TM5 and TM6, as these regions have been shown to be critical to G protein coupling and activation sites (Strader et al., 1987, Eason and Liggett, 1995, Helmreich and Hofmann, 1996, Takhar et al., 1996, Gether et al., 1997, Ghanouni et al., 2001, Couvineau et al., 2003, Yao et al., 2006, Kuniyeda et al., 2007, Bockenhauer et al., 2011). However, earlier work showed that the cytoplasmic extensions of TM5 and TM6 in themselves are not sufficient to activate G proteins and their primary and secondary structures alone cannot account for G protein binding (Strader et al., 1987, Voss et al., 1993). The IL3 amino acid stretch joining the cytoplasmic extensions of TM5 and TM6 (referred herein as the central region of IL3) has received considerably less attention and thus its role in GPCRs remains unclear. Although a number studies have demonstrated this domain plays a minimal role in signalling (Kameyama et al., 1994, Maggio et al., 1996, Pellegrini et al., 1996, Takhar et al., 1996, Peverelli et al., 2009), others have shown that the central region does indeed exert effects on G protein coupling and stability of the receptor for particular conformations (Chicchi et al., 1997, Nielsen et al., 1998, Chaipatikul et al., 2003, Chee et al., 2008). By acting as the linker region between the G protein binding sites, it has been suggested that the central region of IL3 may act to modulate the function of the cytoplasmic extensions (Green and Liggett, 1994, Franzoni et al., 1999, Zurn et al., 2009). This hypothesis may help to resolve lack of a general consensus of the role of the central region of IL3 as this region greatly varies in length and sequence homology between GPCRs and therefore may provide different degrees of modulation.

The typical variability of the IL3 and other intracellular regions has provided an obstacle in the crystallization of GPCRs as it impedes the required stability of the protein (Kobilka and Deupi, 2007). To overcome this, these studies are often forced to exclude or greatly modify the intracellular regions (Cherezov et al., 2007, Rasmussen et al., 2007, Warne et al., 2008, Chien et al., 2010, Haga et al., 2012, Kruse et al., 2012, Manglik et al.,

2012). To complement the astounding contribution these studies have provided to the field, in depth analyses of the functional role of the intracellular regions are required. With this in mind, we took a truncation approach to elucidate the role the IL3 central region of the D1-class receptors may play. We rationalized that as the central region of the human D1-class receptors is naturally divided into a mostly conserved half (Q224-T245 in D1R and Q255-S275 in D5R) and mostly divergent half (G246-K265 in D1R and A276-K289 in D5R), removal of each half respectively would affect receptor functionality, therein highlighting a specific role for the IL3 central region moieties. Furthermore, this approach may also underline any subtype-specific characteristics the remaining divergent residues may hold. Our study suggests distinct structural features and functional roles for the two moieties of the central region of IL3 of D1-class receptors that extends beyond receptor stabilization, conferring conformational control which differentially affects ligand binding and is critical to DA-independent (constitutive) and dependent receptor activation and G protein coupling.

Materials and methods

Materials

Phosphate buffer saline (PBS) and minimal essential media (MEM) were obtained from Wisent (Saint-Bruno, QC, Canada). Fetal Bovine Serum (FBS) was from PAA laboratories (Etobicoke, ON, Canada). Gentamycin and trypsin-EDTA were from Invitrogen (Burlington, ON, Canada). [^{3}H]-SCH23390 was purchased from Perkin Elmer (Boston, MA, USA). [^{14}C]-cAMP was produced by Moravek Biochemicals and Radiochemicals (Brea, CA, USA), and [^{3}H]-adenine by Perkin Elmer (Boston, MA, USA). Biosafe II scintillation cocktail was from Research Products International Corp (Mt. Prospect, IL, USA). Ascorbic acid, dopamine hydrochloride, *cis*-flupenthixol , thioridazine, (+)-butaclamol and 3-isobutyl-1-methylxanthine (IBMX) were procured from Sigma-Aldrich (Oakville, ON, Canada) and dihydrexidine hydrochloride from Tocris Bioscience (Ellisville, MO, USA). Expand High Fidelity Taq DNA polymerase was from Roche (Laval, QC, Canada). Restriction enzymes used in generating DNA constructs were acquired from Fermentas Life Sciences (Burlington, ON, Canada) and New England Biolabs (Pickering, ON, Canada).

Construction of IL3 deletion mutants of human D1R and D5R

Mutant human D1R and D5R constructs were designed using a PCR overlap extension strategy wherein the N-terminal (Q224-T245 in D1R; Q255-S275 in D5R) and C-terminal (G246-K265 in D1R; A276-K289 in D5R) moieties of the IL3 central region was deleted (**Fig. 1**). These IL3 deletion mutant constructs will herein be referred to as D1ΔN, D1ΔC, D5ΔN and D5ΔC. The human D1R and D5R subcloned in the mammalian expression vector pCMV5 were used as PCR templates. Two PCR products, A and B, were amplified separately using P1-P2 and P3-P4 primer pairs, respectively (**Table 1**). An overlapping region between A and B allowed the amplification of the final PCR product using primers P5 and P6. PCR reactions were carried out with Expand High Fidelity Taq DNA polymerase in an Eppendorf thermal Mastercycler using the following conditions: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 3 min), 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for

Table 1. Sequences of oligonucleotide primers for the construction of IL3 truncated D1R and D5R using a polymerase chain reaction-based overlapping approach.

Constructs	Oligonucleotide primers (5'-3')
D1ΔN	**P1: TTCATCCCAGTGCAGCTC**
	P2: AGGCTTTCCATTACCTTTCTGCGCAATCCTGTAGATCCTGGTGTA
	P3: ATTGCGCAGAAAGGTAATGGAAAGCCTGTCGAATGTTCTCAACCG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
D5ΔN	**P1: TACGGTGGGAGG**
	P2: GTCGGGTGCGCAGGCTGCCACCTGGGCGATGCGGTAGATGCGCGTGTA
	P3: ATCGCCCAGGTGGCAGCCTGCGCACCCGACACCAGCCTGCGCGCT
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA
D1ΔC	**P1: TTCATCCCAGTGCAGCTC**
	P2: GACTTTAGTCTCTCTTGTGGTGGTCTGGCAATTCTTGGC
	P3: CAGACCACCACAAGAGAGACTAAAGTCCTGAAGACTCTG
	P4: TTAGGACAAGGCTGGTGG
	P5: ACTGTGACTCCAGCC
	P6: GGCCAGGAGAGGCA
D5ΔC	**P1: TACGGTGGGAGG**
	P2: CAGGGTCTTGAGGACCTTGGTCTCCTTGCTGCTCCGGCAGCTCTGCGCGTGCTC
	P3: TGCCGGAGCAGCAAGGAGACCAAGGTCCTCAAGACCCTGTCGGTGATCATG
	P4: TCATGTGGATGTAGGCAG
	P5: ACCTGGCCAACTGGA
	P6: TGTTCACCGTCTCCA

8 min. Overlap PCR reactions were done with equimolar amount of purified P1-P2 and P3-P4 product with the following conditions: 1 cycle (94°C for 3 min, 50°C for 1 min, 72°C for 10 min). At the end of the cycle, P5 and P6 primers were added to mixture and final product generated using the following conditions: 25 cycles (94°C for 45 s, 50°C for 1 min, 72°C for 1 min) completed by an anneal extension step at 72°C for 8 min. PCR products were separated on 1% agarose gel, excised and purified on QIAEX resin (Qiagen). Purified PCR products were digested with *Hind*III and *Xba*I (D1ΔN), *Box*I and *Xba*I (D1ΔC), *Bsm*I and *Eag*I (D5ΔN), or *Box*I (D5ΔC), and purified DNA bands ligated in-frame with linearized wild type human D1R (*Hind*III-*Xba*I) or D5R (*Bsm*I-*Eag*I) pCMV5 DNA construct sequences to generate IL3 truncated D1R and D5R mutants. The integrity of mutants in pCMV5 was confirmed by automated fluorescent DNA sequencing done at the StemCore Laboratories of the Ottawa Genomics Innovation Center.

Cell culture and transfection

Human embryonic kidney 293 cells (HEK293) were cultured in a 5% CO_2 atmosphere at 37℃ in MEM with Earle's salts supplemented with 10% (v/v) heat inactivated FBS and 40 µg/ml gentamycin sulfate. Cells were seeded in 100-mm dishes (~2.2×10^6 cells/dish) and transiently transfected with 5 µg/dish of DNA using a calcium-phosphate method previously described (Plouffe et al., 2010). Empty pCMV5 vector was used to bring the total amount of DNA transfected to 5 µg when less than 5 µg/dish of receptor plasmid was required to obtain similar and moderate expression levels (1-3 pmol/mg membrane proteins) of wild type and mutant receptors (studies using whole cell cAMP assays described below). Cells were incubated with DNA-calcium phosphate precipitate for 18-24 h prior to reseeding in assay dishes as described in the following sections.

Preparation of cell membranes and radioligand binding assays

Transfected cells were washed with PBS, trypsinized and reseeded in 150-mm dishes. They were grown for an additional 48 h, then washed with PBS, scraped in cold lysis buffer (10 mM Tris–HCl, pH 7.4; 5 mM EDTA, pH 8.0) and centrifuged for 20 min at 40,000×*g* and 4˚C. Pellets were washed in lysis buffer and homogenized using a Brinkman

Polytron (velocity of 17,000 rpm; 15 s), then centrifuged again for 20 min (40,000×*g*; 4˚ C). These pellets were homogenized in lysis buffer with a portion being used immediately for saturation studies and the rest aliquoted, frozen using liquid nitrogen, and stored at -80℃ until used in competition studies. Membrane samples, either fresh or frozen (thawed) were diluted in resuspension buffer (1 M Tris-HCl, pH 7.4; 120 mM NaCl) and mixed (thawed membrane preparations only) using Polytron as described above. Saturation studies were performed using increasing concentrations of the D1-like radioligand [^{3}H]-SCH23390 (0.01-10 nM) and 100 µL of fresh membrane preparation in binding buffer (final in assays: 50 mM Tris–HCl, pH 7.4; 120 mM NaCl; 5 mM KCl; 4 mM $MgCl_2$; 1.5 mM $CaCl_2$; 1 mM EDTA, pH 8.0) for a total volume of 500 µL. Non-specific binding was assessed by the presence of 10 µM *cis*-flupenthixol. Competition studies were performed using a single concentration of [^{3}H]-SCH23390 (approximately the K_d value measured in saturation studies), thawed frozen membrane preparation and increasing concentrations of cold ligand (dopamine (DA), dihydrexidine (DHX), (+)-butaclamol, (BUTA), *cis*-flupenthixol (FLUP), SKF83566 (SKF), thioridazine (THIO)). In studies using DA, 0.1 mM ascorbic acid (AA) was used as a vehicle (to prevent DA oxidation). Incubation of binding reactions for 90 minutes was terminated by rapid filtration through glass fibre filters (GF/C, Whatman). Filters were washed 3 times with cold washing buffer (50 mM Tris-HCl, pH 7.4 and 100 mM NaCl) and radioactivity measured through liquid scintillation counting. Protein concentration was evaluated with a Bio-Rad assay kit with bovine serum albumin as standard.

Whole cell cAMP assays

For whole cell cAMP assays, transfected cells were reseeded in 6-well (constitutive activity) or 12-well (dose-response curves) plates as well as in one 100-mm dish (for assessment of receptor expression using binding assays). AC activation (cAMP formation) in intact cells incubated with or without drugs were assessed for 30 min in the presence of cAMP phosphodiesterase inhibitor IBMX (1 mM) as previously described (Plouffe et al., 2010). Briefly, the amount [^{3}H]-cAMP was assessed from perchloric acid-lysed samples (containing ~3.3 nCi of [^{14}C]-cAMP) that were neutralized using KOH (4.2 M). Samples were purified over Dowex and alumina resin columns and the amount of relative AC

activity was calculated as the amount of intracellular [^{3}H]-cAMP formed (CA) over the total amount of intracellular [^{3}H]-adenine (total uptake or TU), expressed CA/TU × 1000. Receptor expression values were determined by applying a saturating concentration of [^{3}H]-SCH23390 (~7-10 nM) in the absence or presence of 10 µM FLUP (to delineate non-specific binding).

3D homology modelling of wild type and mutant human D1R and D5R

Amino acid sequences of the wild-type human D1R, wild-type D5R, D1ΔN, D1ΔC, D5ΔN and D5ΔC receptors were individually entered into the Phyre2 protein recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) on April 20, 2012. Phyre2 generates a sequence alignment by using repeated PSI-Blast iterations against over 10,000,000 protein amino acid sequences to identify close and remote sequence homologues of the query protein. This alignment is then converted into a Hidden Markov Model (HMM). The HMM is scanned against the fold library (library of ~65,000 HMMs generated from the sequences of protein with known structures) generating an alignment of the query HMM with those of known structures. 3D models are generated based on the highest scoring alignments, with missing and inserted regions being repaired using a loop library and reconstruction procedure. This procedure generates highly accurate models (root mean square deviation of 2-4 Å) with as little as 15% sequence identity. On average, over 70% of residues of the wild-type and mutant receptors (D1R, 73%; D5R 67%; D1ΔN 72%; D1ΔC, 71%; D5ΔN, 81%; D5ΔC, 65%) were modelled with > 90% confidence. The remainder (mostly situated in CT region), were modelled *ab initio*. Each model was built using 6-7 of the following template structures: D3R in complex with eticlopride (PDB: 3PBL) (Chien et al., 2010), human histamine H1 receptor in complex with doxepin (PDB: 3RZE) (Shimamura et al., 2011), β_2-adrenergic receptor T4 lysozyme fusion protein (PDB: 2RH1) (Cherezov et al., 2007), M2 muscarinic receptor bound to antagonist (PDB: 3UON) (Haga et al., 2012), irreversible agonist-β_2-adrenergic receptor complex (PDB: 3PDS) (Rosenbaum et al., 2011), β_2-adrenergic receptor-Gs protein complex (PDB: 3SN6) (Rasmussen et al., 2011), human A2A adenosine receptor bound to zm241385 (PDB: 3EML) (Jaakola et al., 2008), Substance P in DMPC/CHAPS isotropic bicelles as a ligand for NK1 (PDB: 2KSA) (Gayen et al., 2011).

Statistics

Equilibrium dissociation constant (K_d, nM) and maximal binding capacity (B_{max}, pmol/mg protein) of [^{3}H]SCH23390 in saturation studies, and inhibitory dissociation constant of cold drugs ($K_{i,}$ nM) in competition studies were derived from analysis of binding isotherms using nonlinear curve-fitting program from GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Geometric means of K_d and K_i are expressed with the 95% upper and lower confidence intervals. Arithmetic means of B_{max} are expressed ± S.E. Relative expression and affinity and constitutive activation values were calculated as the arithmetic mean ± S.E. Using a four-parameter logistic equation from GraphPad Prism the raw data for individual dose-response curves of DA were analyzed to obtain the DA concentration producing 50% of the maximum response (EC_{50} or potency) and DA-induced maximal activation of AC (E_{max}). Raw data were then normalized as a percent of the best-fitted E_{max} obtained with the wild type receptor. Normalized data were averaged and the curves again analyzed using a simultaneous fitting with a shared slope parameter from GraphPad Prism, generating best-fitted E_{max} ± S.E. and EC_{50} with 95% confidence intervals. Significant differences between best-fitted values of wild type and mutant receptors were determined using curve fitting with unconstrained and constrained parameters. One sample *t* test and one-way ANOVA with Newman-Keuls and Dunnett's post hoc tests were performed using GraphPad prism with a critical level of probability of $\alpha = 0.05$.

Results

Primary amino acid sequence alignment of TM5, IL3 and TM6 of the human D1-class receptors (D1R and D5R) with several biogenic amine GPCRs shows a low degree of identity in the central region of IL3, which links TM5 and TM6 cytoplasmic extensions (***Supplemental Information***). Meanwhile, a comparison of the central region of IL3 of D1R with that of D5R suggests the presence of two moieties as described in *Materials and Methods*. The N-terminal moiety is highly conserved between D1R and D5R (~60%) and made of 22 and 21 residues, respectively. The C-terminal moiety is much less conserved (~25%) and differs in length with 20 residues in D1R and 14 in D5R (***Supplemental Information***). To explore the functional role of these two IL3 moieties, we generated two deletion mutants each for D1R and D5R (**Fig. 1**).

Functional Expression of the Deletion Mutant Receptors

To assess the functional expression of the mutant proteins (**Table 2**), saturation studies with ΔN and ΔC mutants were performed in two independent series of experiments using the classical D1-class specific ligand [^{3}H]-SCH23390. The dissociation constant (K_d) and maximal binding capacity (B_{max}) of [^{3}H]-SCH23390 in membrane preparations from transfected HEK293 cells maximally expressing receptors were determined using saturation studies. Wild-type and mutant receptors were found to interact with one homogenous class of binding sites (***Supplemental Information)***. K_d was significantly higher than the wild-type for both D1ΔN and D5ΔN, however, this loss in affinity for [^{3}H]-SCH23390 was not robust (**Table 2**). The K_d of D1ΔC and D5ΔC remained unchanged relative to their respective wild-type receptors (**Table 2**). B_{max} values of D1ΔN, D1ΔC and D5ΔC were 10, 4 and 6 times lower than their corresponding wild-type, respectively. In contrast, B_{max} of D5ΔN remained approximately the same as D5R (**Table 2**). Overall, K_d values from saturation studies indicate that deletion of the N- or C-terminal moiety of IL3 did not have an aberrant effect on the overall ligand binding conformation of mutant receptors.

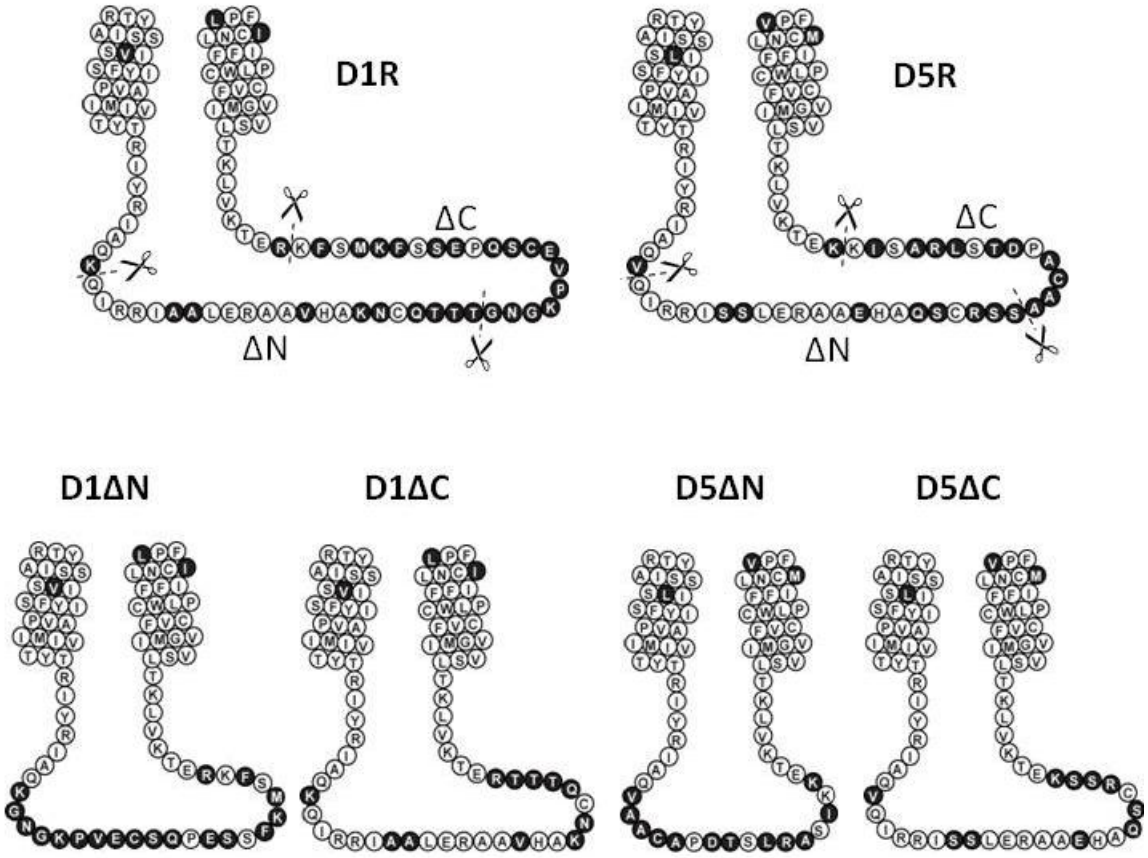

Figure 1. Schematic representation of deletion mutations. D1R, D5R, D1ΔN, D5ΔN, D1ΔC and D5ΔC are depicted with open circles representing identical residues and shaded circles representing residues distinct between the two subtypes. Deletion regions of IL3 are delimited by scissors in the wild-type receptors.

Table 2. [^{3}H]-SCH23390 affinity (K_d) and maximal binding capacity (B_{max}) for wild-type and mutant receptors

Receptor	K_d (nM)	Relative K_d (Fold of WT)	B_{max} (pmol/mg protein)	Relative B_{max} (Fold of WT)
D1R (n=15)	0.53 (0.45-0.61)	1	8.78 (6.31-11.2)	1
D1ΔN (n=15)	0.99 (0.55-1.40)	1.80* (1.11-2.48)	1.65 (1.05-2.25)	0.19*** (0.13-0.25)
D5R (n=15)	0.79 (0.68—0.90)	1	9.98 (5.78-14.2)	1
D5ΔN (n=15)	1.05 (0.92-1.17)	1.41* (1.10-1.73)	8.33 (5.53-11.1)	0.95 (0.77-1.30)
Receptor	**K_d (nM)**	**Relative K_d (Fold of WT)**	**B_{max} (pmol/mg protein)**	**Relative B_{max} (Fold of WT)**
D1R (n=4)	0.57 (0.40-0.73)	1	15.7 (6.02-25.3)	1
D1ΔC (n=4)	0.67 (0.55-0.79)	1.20 (0.99-1.40)	4.17 (3.05-5.29)	0.30** (0.11-0.48)
D5R (n=4)	0.89 (0.56-1.23)	1	16.2 (9.00-23.5)	1
D5ΔC (n=4)	1.06 (0.59-1.52)	1.19 (0.73-1.65)	2.62 (1.08-4.16)	0.16*** (0.10-0.22)

B_{max} (pmol/mg protein) displayed as the arithmetic mean of 4 to 15 experiments with upper and lower 95% confidence interval in brackets. K_d (nM) displayed as the geometric mean of 4 to 15 experiments with upper and lower 95% confidence interval in brackets. Experiments for D1ΔN and D5ΔN performed independently from those of D1ΔC and D5ΔC. Relative values were normalized as the fold-change of their respective wild-type (value set to 1). All relative values compared to 1 using a one sample *t* test. *p < 0.05, **p < 0.01, ***p < 0.001

N- and C-Terminal Moieties of IL3 Induce Opposing Subtype-Specific Ligand Affinities

Competition binding studies for ΔN and ΔC mutants using [^{3}H]-SCH23390 were performed in two separate series of experiments with dopamine and the full agonist dihydrexidine (***Supplemental Information* & Table 3**). In addition, the impact of deletions on the binding of the partial agonist SKF83566 and inverse agonists (+)-butaclamol thioridazine and *cis*-flupenthixol was also measured (**Table 3**). All displacement curves were best-fitted to a homogenous population of binding sites (***Supplemental Information***). Surprisingly, our studies showed an approximately 3.5-fold increase in dopamine and dihydrexidine affinities of D1ΔN when compared to D1R but an approximately 8-fold decrease in affinity of D5ΔN for DA and DHX relative to D5R (**Table 3, Fig. 2A**). D5R distinctively demonstrates higher affinity for dopamine and other agonists than D1R (Sunahara et al., 1990, Tiberi et al., 1991, Tiberi and Caron, 1994, D'Aoust and Tiberi, 2010). This selectivity for dopamine is essentially swapped between D1ΔN and D5ΔN, albeit reduced. On the other hand, the selectivity of dihydrexidine for D5R over D1R is completely lost (**Fig. 2A**). D1ΔC and D5ΔC both displayed larger increases in affinity for dopamine and dihydrexidine with D1ΔC increasing approximately 25-fold and D5ΔC increasing approximately 11.5-fold. The agonist selectivity of D5R over D1R in these mutants is still apparent, though reduced (**Fig. 2B**). In contrast to full agonists, both D1ΔN and D1ΔC displayed small losses in affinity for the partial agonist SKF83566 (~2-fold and 1.5-fold, respectively). Affinities of D5ΔN and D5ΔC for the partial agonist were unchanged. D1ΔN also displayed an approximate 1.5-fold decrease in affinity for both (+)-butaclamol and thioridazine, however *cis*-flupenthixol remained unaffected. The IL3 deletions had no effect on D5ΔN, D1ΔC, D5ΔC affinities for inverse agonists (**Table 3, Fig. 2**). Collectively, our findings suggest that the N- and C-terminal moieties of IL3 only modulate the affinity of full agonists, and do so in a distinctive manner in D1R and D5R.

Agonist-Independent Activity of Deletion Mutant Receptors

Similar to constitutively active mutant (CAM) GPCRs relative to their wild type forms, D5R displays a higher level of activation in the absence of agonists (constitutive

Table 3. Ligand affinity (K_i) values from competition studies with [^{3}H]-SCH23390

Receptor	Dopamine (n=10)	DHX (n=10)	Butaclamol (n=7)	Thioridazine (n=7)	Flupenthixol (n=6)	SKF83566 (n=6)
D1R	8283 (6888-9960)	537 (468-617)	10.8 (7.60-15.3)	83.9 (56.3-125)	8.89 (7.90-10.1)	1.71 (1.20-2.50)
D1ΔN	2128 (1227-3692)	209 (147-296)	15.8 (9.20-27.2)	130 (74.7-227)	10.5 (6.70-16.2)	3.56 (2.30-5.50)
D5R	980.1 (838.8-1145)	57.0 (48.7-66.8)	52.8 (36.3-76.8)	229 (202-329)	17.0 (13.2-22.0)	2.94 (2.10-4.20)
D5ΔN	8428 (7722-9197)	374 (304-461)	53.8 (37.5-77.2)	258 (202-329)	14.8 (10.7-20.4)	2.98 (2.40-3.80)

Receptor	Dopamine (n=4)	DHX (n=4)	Butaclamol (n=4)	Thioridazine (n=4)	Flupenthixol (n=4)	SKF83566 (n=4)
D1R	8518 (6960-10423)	577 (463-718)	6.03 (5.30-6.80)	78.6 (59.7-103)	10.4 (6.40-16.8)	2.37 (1.20-4.90)
D1ΔC	272 (108-687)	27.6 (22.5-33.8)	6.80 (4.90-9.40)	103 (73.4-145)	9.96 (5.30-18.9)	3.59 (1.90-6.90)
D5R	813 (586-1126)	71.1 (51.7-97.9)	33.6 (13.0-86.8)	273 (116-642)	14.5 (9.40-22.4)	3.55 (2.70-4.70)
D5ΔC	87.7 (64.7-119)	5.05 (3.50-7.40)	26.5 (12.9-54.5)	254 (107-606)	15.0 (7.80-28.9)	3.73 (2.20-6.40)

K_i (nM) displayed as the geometric means of 4 to 10 experiments with upper and lower 95% confidence interval in brackets. Experiments for D1ΔN and D5ΔN performed independently from those of D1ΔC and D5ΔC.

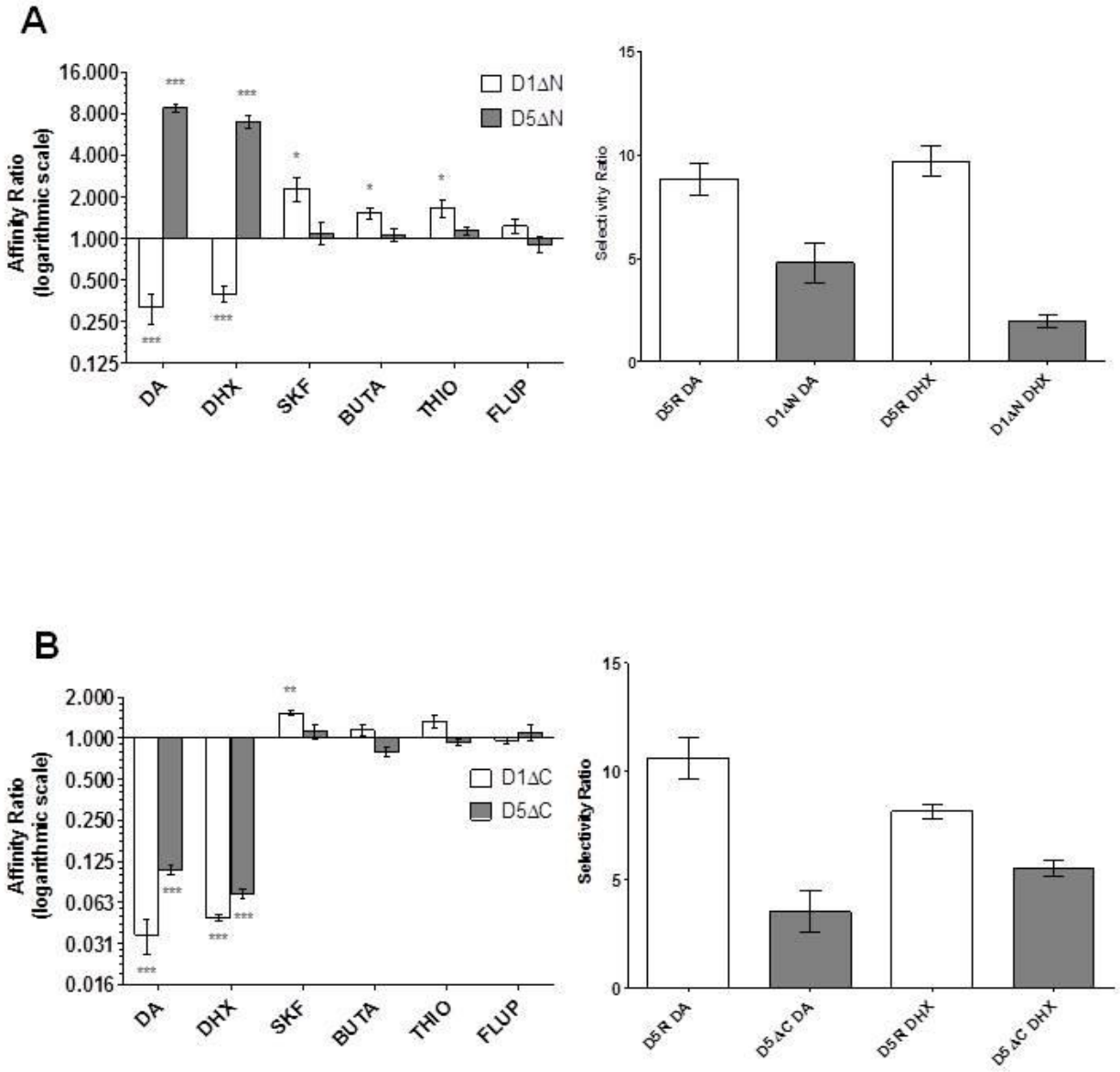

Figure 2. Relative ligand affinities (K_i) and agonist selectivity of deletion mutant receptors. K_i values from competition studies for agonists (dopamine, DA; dihydrexidine, DHX), a partial agonist (SKF83566, SKF) and inverse agonists (butaclamol, BUTA; thioridazine, THIO; and flupenthixol, FLUP) (see Table 3) in N-terminal (A) and C-terminal (B) deletion mutants. Relative values for deletion mutants are expressed as the geometric mean ± S.E. of 4-15 experiments normalized as the fold-change of their respective wild-type and displayed on a log2 scale. All relative values compared to 1 using a one sample *t* test. *p < 0.05, **p < 0.01, ***p < 0.001. Selectivity ratios are calculated in wild-type and C-terminal mutants as the ratio of K_i values for D1 over the corresponding D5 value. In the N-terminal mutants selectivity ratios are calculated as the ratio of K_i values for D5ΔN over D1ΔN.

or basal activity) when compared to D1R (Tiberi and Caron, 1994, Plouffe et al., 2010). To investigate the effect of IL3 deletions on agonist-independent activation of D1R and D5R, whole cell cAMP studies were performed and basal cAMP levels assessed, comparing the mutants to their respective wild-type receptor. Results showed that the constitutive activation of both D1ΔN and D5ΔN was ablated to the level of mock-transfected cells in comparison with D1R and D5R (**Fig. 3**). The opposite was observed upon deletion of the C-terminal moiety of IL3. D1ΔC displayed a drastically increased basal activity, approximately 10-fold (**Fig. 3**). The constitutive activity of D5ΔC was also increased approximately 2-fold (**Fig. 3**).

Dopamine-Induced cAMP Formation of Deletion Mutant Receptors

In addition to the increased constitutive activity, cells expressing D5R display higher dopamine potency and lower level of maximal activation of AC (E_{max}) than cells transfected with D1R (Tiberi and Caron, 1994, Plouffe et al., 2010). Dose-response experiments were performed with dopamine, comparing the D1R and D5R mutants to their respective wild-type receptor to assess the contribution of N- and C-terminal moieties on dopamine-induced activation properties of D1-class receptors. A robust decrease in the E_{max} and a rightward EC_{50} shift in the curve were observed for D5ΔN. D1ΔN displayed a less severe decrease in dopamine-mediated coupling response efficiency, however, this was still largely dampened compared to that of D1R (**Fig. 4**). Although reduced, agonist-dependent activity remained in the N-terminal deletion mutants. The opposite was true of D1ΔC and D5ΔC, in which a strong increase in E_{max} and a corresponding leftward shift in EC_{50} were observed (**Fig. 4**). This demonstrates that these receptors exhibit enhanced signalling capacity and efficiency, especially D1ΔC in which these characteristics are particularly pronounced. Similar E_{max} results were reiterated with another full agonist, dihydrexidine (**Fig. 5**). Overall, these findings imply an important role of the N- and C-terminal moeities of IL3 in facilitating and restraining agonist-induced activation of D1-class receptors, respectively.

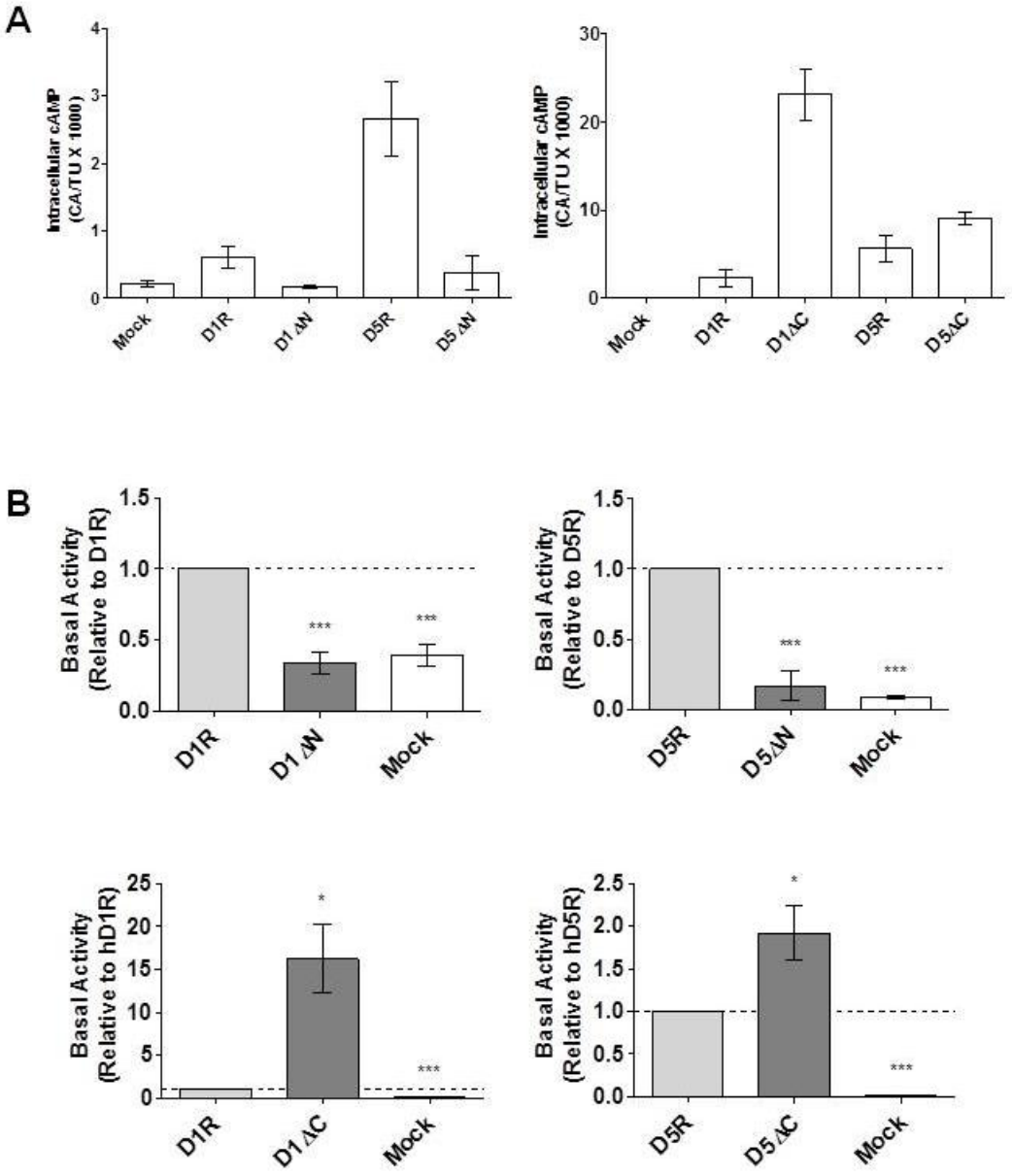

Figure 3. Constitutive adenylyl cyclase activation in HEK293 cells expressing wild-type and deletion mutant receptors. Constitutive activation of adenylyl cyclase was determined in single wells of 6-well plates using a whole cell cAMP assay. Mock denotes condition in which cells were transfected with empty DNA vector. (A) Basal intracellular cAMP values for cells treated with ascorbic acid + H_2O are displayed as the arithmetic mean ± S.E. of 4 experiments performed in triplicate determinations. (B) Relative values for deletion mutant receptor constitutive activity are displayed as the arithmetic mean of control condition normalized as the fold-change of their respective wild-type (value set to 1). Relative values compared to 1 using a one sample *t* test. *p < 0.05, **p < 0.01, ***p < 0.001; B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are D1R: 3.27, hD1ΔN : 2.54, D5R: 3.62, hD5ΔN : 2.13 ; D1R: 2.75, hD1ΔC: 4.09, D5R: 2.32, hD5ΔC: 3.24

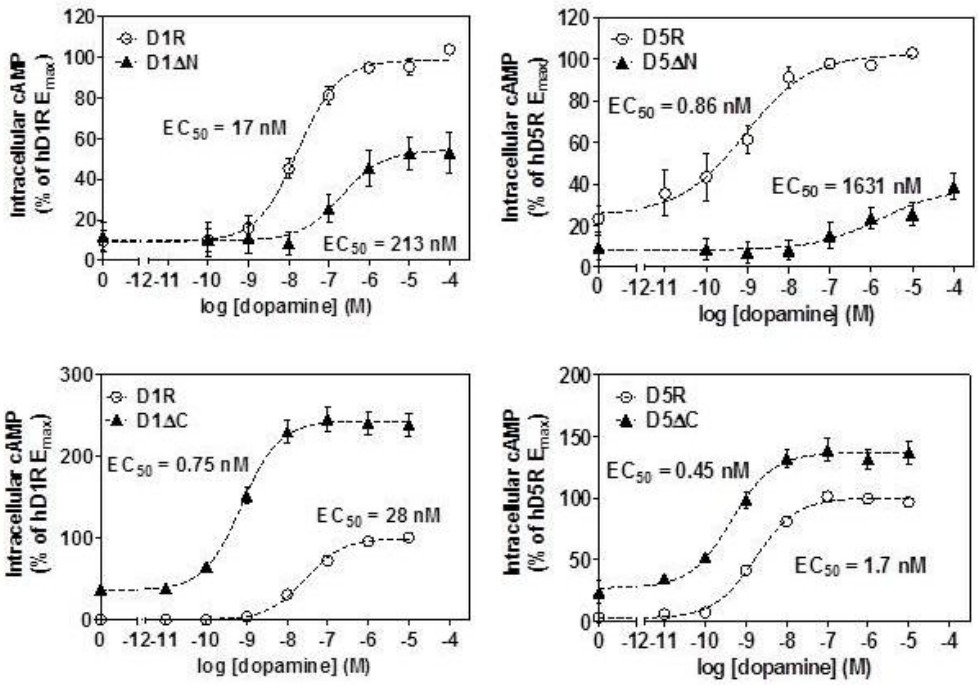

Figure 4. Dopamine induced adenylyl cyclase activity in HEK293 cells expressing wild-type and deletion mutant receptors. Dose-response curves were determined with transfected HEK293 cells seeded in 12-well plates in the presence of increasing concentrations of dopamine (DA) (0 to 10^{-4} M). Each point represents the arithmetic mean of 7 experiments performed in triplicate determinations and plotted as a function of log [DA]. Simultaneous nonlinear curve fitting was performed using GraphPad Prism; curves were normalized relative to 100% of the wild-type E_{max}, then refit to determine the EC_{50} and relative E_{max} of the mutant response. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are D1R: 0.92, hD1ΔN: 0.80, D5R: 1.25, hD5ΔN: 1.00; D1R: 2.02, hD1ΔC: 3.59, D5R : 2.22, hD5ΔC : 2.73.

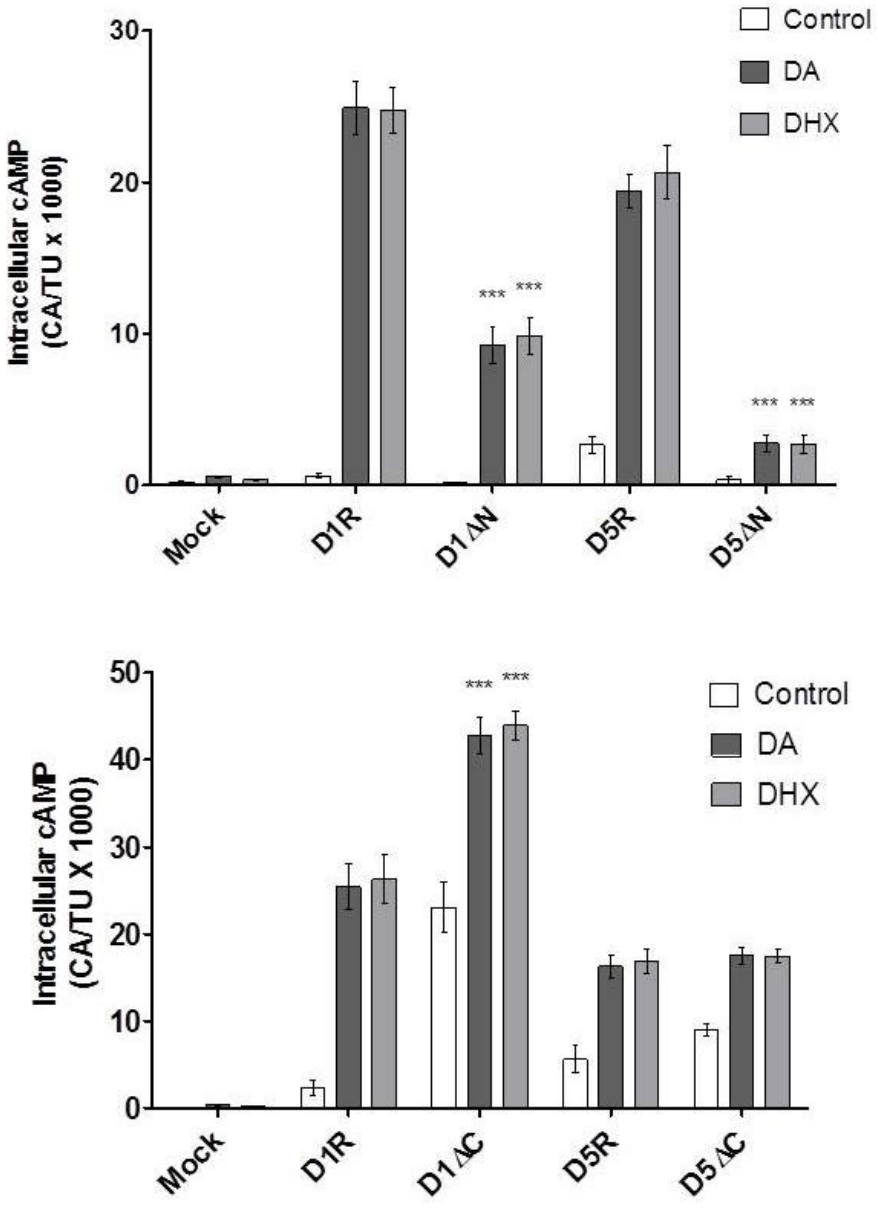

Figure 5. Maximum AC activation (E_{max}) induced with full agonists DA and DHX in HEK293 cells expressing wild-type and deletion mutant receptors. Maximal AC activation was determined in 6-well plates using a whole cell cAMP assay. Wild type receptor expression level was matched to that of N-terminal (A) and C-terminal (B) mutants. Intracellular cAMP values for cells treated with ascorbic acid + H_2O (control), or a saturating concentration of dopamine (DA) or dihydrexidine (DHX) displayed as the arithmetic mean ± S.E. of 4 experiments performed in triplicate determinations. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are D1R: 3.27, hD1ΔN: 2.54, D5R: 3.62, hD5ΔN: 2.13; D1R 2.75, hD1ΔC: 4.08, D5R:

2.32, hD5ΔC: 3.24. Values compared to respective basal condition using a one way ANOVA with a Neuman-Keuls post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Partial Agonism and Inverse Agonism at Deletion Mutant Receptors

To determine whether the N- and C-terminal moieties regulate intrinsic activity and efficacy of other synthetic ligands, AC activation was assessed in the presence of partial agonists (SCH23390 and SKF83566) and inverse agonists ((+)-butaclamol, thioridazine and *cis*-flupenthixol). SCH23390 is considered to be the typical D1-class antagonist. However, studies have shown that SCH23390 displays weak partial agonism at D1R sites (Tiberi and Caron, 1994, Sugamori et al., 1998, Martin et al., 2001, D'Aoust and Tiberi, 2010). In agreement with the partial agonism at CAM receptors, SCH23390 has higher intrinsic activity at D5R (Tiberi and Caron, 1994, D'Aoust and Tiberi, 2010). The D1ΔN and D5ΔN showed no significant AC activation compared to their respective wild-types upon incubation with partial agonists (**Fig. 6**). Similar to agonist-induced responses by dopamine and dihydrexidine, the C-terminal deletion mutants exhibited enhanced AC activation by partial agonists when compared to their wild type counterparts. However, this enhancement was even more robust than that of the full agonist-induced response, increasing ~8-fold in D1ΔC and ~2-fold in D5ΔC (**Fig. 6**).

No effect on AC activation was observed when the inverse agonists (+)-butaclamol, thioridazine and *cis*-flupenthixol were applied to cells expressing D1ΔN or D5ΔN, instead suggesting the behavior of pure antagonists (neutral ligands). Moreover, all the drugs remained strong inverse agonists when tested at D1ΔC and D5ΔC relative to their wild-type receptors, with thioridazine being slightly less effective than the others (**Fig. 7**).

Collectively, results obtained with partial agonists and inverse agonists suggest a critical role of the N- and C-terminal moieties of IL3 in controlling the ligand efficacy.

3D Homology Modelling of Wild-Type and Deletion Mutant Receptors

Sequences for the wild-type and mutant receptors were submitted to the Phyre2 (protein homology/analogy recognition engine) server and 3D homology models were generated (Kelley and Sternberg, 2009). Approximately 70% of residues in all the receptors were modelled at >90% confidence, with the vast majority of *ab initio* modelling

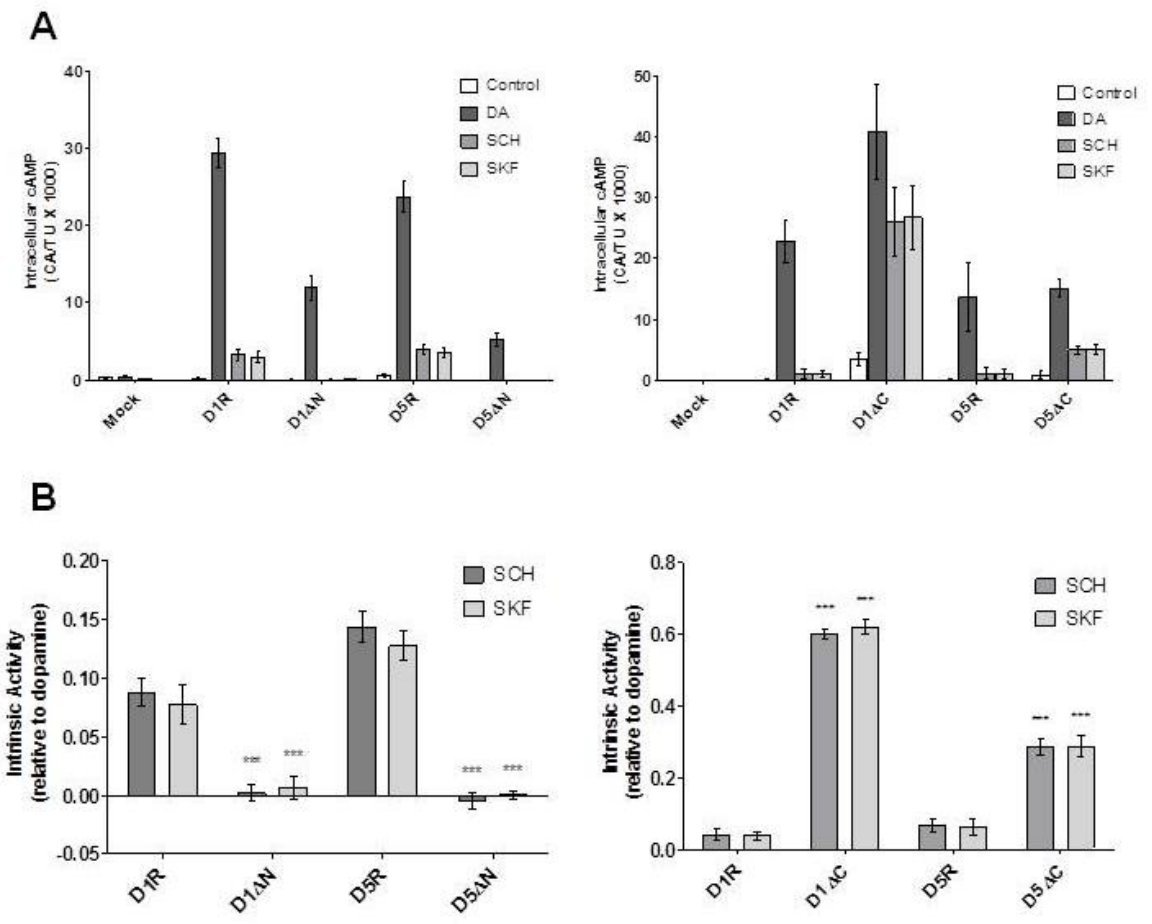

Figure 6. Partial agonist induced adenylyl cyclase activity in HEK293 cells expressing wild-type and deletion mutant receptors. AC activation was determined using a whole cell cAMP assay. Wild type receptor expression level was matched to that of respective mutants. Partial agonists SCH23390 and SKF83566 were tested using 12-well plates. (A) Intracellular cAMP values are the arithmetic mean ± S.E. of 4 experiments performed in triplicate determinations. (B) Intrinsic activity values are displayed as the arithmetic mean corrected for basal activity and normalized relative to the stimulation induced by dopamine. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are D1R: 5.58, hD1ΔN: 2.49, D5R: 4.80, hD5ΔN: 4.24; D1R: 5.16, hD1ΔC: 5.39, D5R: 5.60, $hD1_{\Delta A276\text{-}K289}$: 2.49. Values compared to dopamine stimulation of respective receptor condition using a one way ANOVA with Newman-Keuls post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

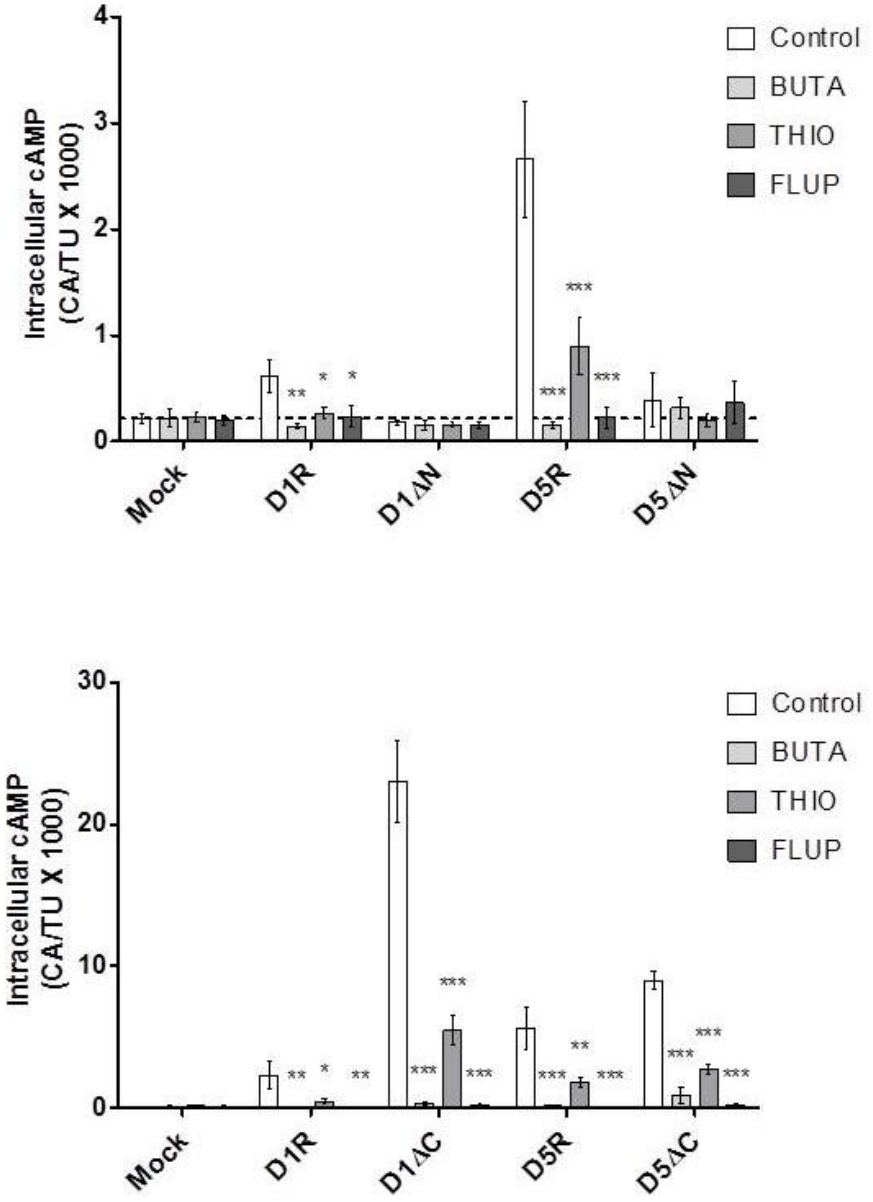

Figure 7. Inverse agonist induced adenylyl cyclase activity in HEK 293 cells expressing wild-type and deletion mutant receptors. AC activation was determined using a whole cell cAMP assay. Wild type receptor expression level was matched to that of their respective mutants. Inverse agonists (+) butaclamol (BUTA), thioridazine (THIO) and *cis*-flupenthixol (FLUP) were tested using 6-well plates. Values are the arithmetic mean ± S.E. of 4 experiments performed in triplicate determinations. B_{max} values in pmol/mg membrane protein for [^{3}H]-SCH23390 (expressed as arithmetic means) are D1R: 3.27, hD1ΔN: 2.54, D5R: 3.62, hD5ΔN: 2.13; D1R 2.75, hD1ΔC: 4.08, D5R: 2.32, hD5ΔC: 3.24. Values compared to respective basal condition using a one way ANOVA with a Dunnett's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

occurring in the carboxy-tail domains of the receptors. The template structures used to generate our models were for the most part from receptors bound to antagonists or inverse agonists (see *Materials and Methods*). Therefore, our models may be considered as being represented in an inactive state. The wild-type D1R and D5R were predicted to have an overall similar structure, with the most striking differences occurring in the IL3 (**Fig. 8A**). Both receptors displayed an α-helix similarly located within the N-terminal moiety, however, this helix was longer in D5R. Following the α-helix, the remainder of the N-terminal moiety and the C-terminal moiety of D5 form an open loop extending up and outwards before connecting to TM6. In contrast, at the junction of N-terminal and C-terminal moieties the D1R IL3 folds back upon itself, forming a more tangled structure that is predicted to facilitate many polar interactions between the side chains of its own residues (**Fig. 8B, C**).

Similar to the wild-type models, the ΔC mutant receptors were predicted to possess a central α-helix in the N-terminal moiety. This α-helix in D1ΔC was shifted towards TM6 compared to that of D1R; the entire loop displaying a more open structure, oriented up and outwards (**Fig. 8B**). The loop of D5ΔC was predicted to assume an open orientation similar to D5R, however its α-helix was shifted even more outwards comparatively (**Fig. 8C**). On the other hand, both of the ΔN mutants lacked any helical structure in the IL3 (**Fig. 8B, C**). The loops of these receptors were withdrawn upwards towards the plasma membrane compared to their respective wild-type receptors. D5ΔN was the only mutant predicted to show any major differences in the TM regions, with TM5 being shorter by a turn and TM6 extending slightly longer into the cytoplasm (**Fig. 8C**).

Together the predicted structures of the wild-type and mutant receptors suggest that the presence of an α-helix in the N-terminal moiety that may be required for robust activation of the receptor. Furthermore, the tangled loop observed in D1R correlates with reduced constitutive activation, whereas D5R, D1ΔC and D5ΔC possess both an α-helix and an open loop structure and display robust basal activity. Thus, the C-terminal moiety may control on the extent of signalling by increasing the energy threshold required for the loop to assume an open active conformation through interactions within the loop itself, other receptor regions and the G protein.

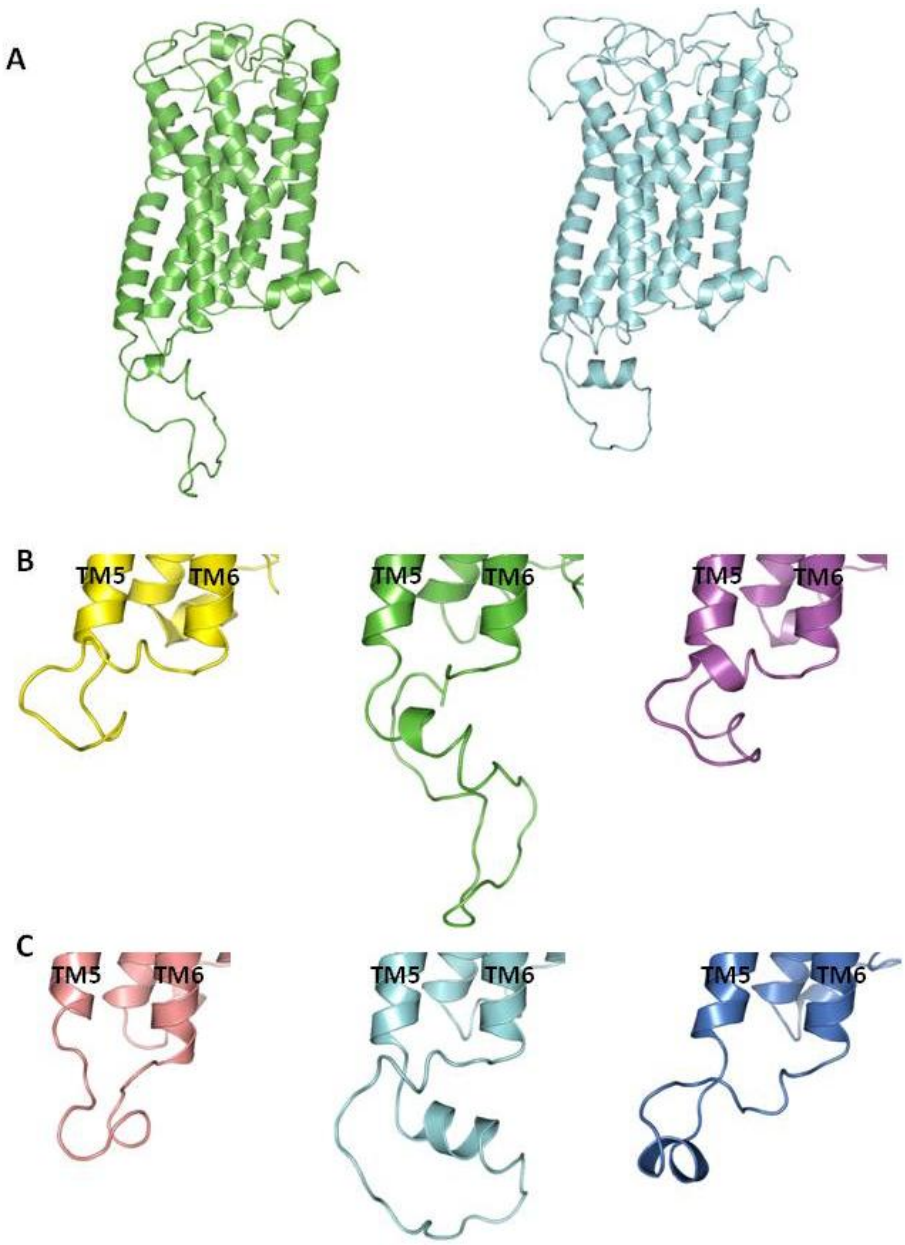

Figure 8. 3D homology modelling of the human D1-class receptors and Deletion Mutants. (A) The predicted structures of D1R (green) and D5R (cyan) are display similar overall conformation, with major differences in the IL3 region. (B) Images focusing on the IL3 of D1ΔN (yellow), D1R (green) and D1ΔC (purple). An α-helix is present in D1R and D1ΔC but not D1ΔN. D1ΔC also displays a more open structure than D1R which folds back upon itself. (C) Images focusing on the IL3 of D5ΔN (pink), D5R (cyan) and D5ΔC (blue). An α-helix is present in D5R and D5ΔC but not D5ΔN. All three loops display an open structure. D5ΔN possesses a shorter TM5 cytoplasmic extension and a longer TM6 cytoplasmic extension. The cytoplasmic tail has been excluded from this image to simplify interpretation. 3D models were predicted using the Phyre2 Structural Informatics Group prediction tools (Kelley and Sternberg, 2009) and images rendered using The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC.(Schrodinger, 2010).

Discussion

Crystallography has yielded a plethora of GPCR structural information, vastly advancing our knowledge of their activation states. To this end, modification or exclusion of the IL3 has often been required, as this domain induces flexibility and instability in the proteins (Kobilka and Deupi, 2007). Here we have shown that the central region of the IL3 is a controlling factor in maintaining all active states of the D1-class receptors. Whereas, removal of the mostly conserved N-terminal region drastically shifted the receptor population into a predominantly inactive state, removal of the divergent C-terminal side allowed the receptor populations to more easily attain an active state. Our results suggest the presence of two structural moieties in the central region of IL3 serving as potential conformational switches for the transition between the inactive and active states, and the extent of G protein coupling.

Deletions within the central region of IL3 of other GPCR species have led to changes in G protein coupling without directly modifying the G protein interacting domains (Nielsen et al., 1998). The β_1 adrenergic receptor has a proline-rich, 24 residue sequence not present in the IL3 of β_2 receptor. Similar to the C-terminal moiety of IL3 of D1-class receptors, this region imparts decreased agonist affinity and G protein coupling (Green and Liggett, 1994). A "natural deletion" of 80 amino acids in the central region of IL3 of the spliced variant histamine H3 receptor results in increased basal activation of the shorter form and a corresponding increase in the potency and affinity for agonists (Bongers et al., 2007). These investigations into naturally occurring isoforms of GPCRs displaying deletions within the central region of IL3 support our findings and suggest that the role of the central region of IL3 may have evolved to give each receptor subtype the characteristics required of their distinct activation process and physiological function. These observations, combined with our data and the support of recent biophysical studies, provide a potential explanation for divergence in D1-class receptor signalling.

The "ionic lock", a putative interaction involving the E/DRY motif at the cytoplasmic ends of TM6 and TM3, is thought to maintain the receptor in an inactive state. However, solved crystal structures of inactive receptors with modified IL3s has failed to detect the required proximity of the interacting residues of the lock (Cherezov et al., 2007,

Warne et al., 2008). This was reiterated in the homology models of wild-type and deletion mutants as they are based strongly upon inactive receptor structures. Recently, two conformational states of the IL3 have been reported from the solved crystal structure of the modified turkey β1 adrenergic receptor, a thermostabilized inverse agonist bound-receptor. One conformation contains a bent TM6 cytoplasmic extension and an intact "ionic lock". The second contains a straight TM6 cytoplasmic extension which is too distant for the ionic lock interactions to form (Moukhametzianov et al., 2011). An intact ionic lock may completely silence the receptor, while an inactive receptor without the ionic lock may permit constitutive signalling. Three groups have recently reported solved crystal structures of the adenosine A2A receptor with the IL3 intact (Dore et al., 2011, Lebon et al., 2011, Hino et al., 2012). Comparison to the crystal structure of previously solved adenosine A2A receptor structures in which the IL3 was replaced by a T4 lysozyme showed significant differences in the cytoplasmic extensions of TM5 and TM6. Furthermore, the intact IL3 appears to be involved in the maintenance of an ionic lock interaction which may in turn be critical for the formation of the helical structure within the cytoplasmic extensions and/or IL3 (Hino et al., 2012).

Disulphide cross-linking in the IL3 of the yeast Ste2p receptor has revealed changes in the cytoplasmic extensions of TM5 and 6 upon agonist binding. This work also suggested that the central region of IL3 harbours a 3_{10} helix which is maintained during activation and likely involved in protein interactions (Umanah et al., 2011). NMR studies in the cannabinoid 1 receptor also confirmed the presence of an α-helix in the central region of IL3 (Ulfers et al., 2002). Our homology models predicted the presence of a centrally located α-helix in the N-terminal moiety of the central region of IL3 of D1R and D5R, which was also present in 3D models of D1ΔC and D5ΔC (**Fig. 8**). The N-terminal moiety mutants, which demonstrate repressed signalling, lack a distinct helical structure in deleted IL3 (**Fig. 8**); suggesting that α-helix in the IL3 may hold a significant function in the constitutive and agonist-dependent activation of D1R and D5R.

NMR characterization of the structure of the rat angiotensin II $AT1_A$ receptor IL3 peptides suggests that cis/trans-isomerisation of P232 located in the central region of IL3 may represent a "switch" that could propagate conformational changes upon agonist

binding (Franzoni et al., 1999). Activation of this switch could either stabilize helical structures or expose residues required for G protein coupling in the TM5 cytoplasmic extension. FRET fluorophores introduced into the TM5 cytoplasmic extension, IL3 central region and TM6 cytoplasmic extension of the α2A-adrenergic receptor have demonstrated that while movement of TM6 cytoplasmic extension occurs for all activation states of the receptor, TM5 cytoplasmic extension movement only occurs during the fully activated state (Zurn et al., 2009). Correspondingly, the active crystal structure of β_2AR-G_s complex predicts movement of TM6 and the extension of TM5 into the cytoplasm by 7 residues (Rasmussen et al., 2011).

Interestingly, the N-terminal moiety of IL3 central region contains an α-helical structure in D1R and D5R (**Fig. 8**) which may be incorporated into TM5 as it extends into the cytoplasm during activation. The α-helix of D5R is almost twice longer than that of D1R, suggesting the length and activating function of the cytoplasmic extension of TM5 may be controlled by the IL3 α-helix and may underlie differences in the extent of constitutive activity in D1-class receptors. Location of the α-helix may also play a part. The α-helix observed in the model of D1ΔC is shifted towards TM6 and corresponds not only with increased constitutive and full agonist activity, but a robust increase in the intrinsic activity of partial agonists to level of full agonists. Furthermore, removal of this α-helix in the N-terminal mutants may impair the extension of TM5 upon receptor activation, culminating in achievement of only a partially active state despite full stimulation. Conversely, the C-terminal moiety of the IL3 central region displays a disordered conformation that may normally allow the receptor to assume an inactive conformation or spectrum of partially active conformations. By removing this portion any restraint that was imposed in maintaining only partial activation may also have been removed leading to the increased signalling observed. This restraint may occur through intermolecular interactions within the IL3 that impede movement within the loop and/or access of the G protein to its interacting domains on the receptor and contribute to the basal activation phenotypes of the D1-class receptors.

Collectively, based on these predictions our data speculatively suggest that the central IL3 of the D1-class receptors acts as a molecular switch affecting the stabilization

of the α-helical structures present in the extensions of TM5 and TM6 and therein modulating the formation of active conformations and interactions with the ionic lock and the G protein. The N-terminal moiety of IL3 central region appears to behave as a critical conformational switch promoting constitutive and agonist-induced activation, and G protein coupling, whereas the C-terminal moiety seems to play a role as a conformational switch that constrains the N-terminal moiety switch in an "*off*" mode.

Removal of the similar N-terminal moieties of the IL3 central region also appears to have resulted in a differential unmasking or concealment of key residues that participate in ligand binding. Upon deletion, the remaining IL3 of D1ΔN contains a newly formed stretch of hydrophilic residues and shows increased agonist affinity, whereas D5ΔN contains a stretch of hydrophobic residues in the same region and decreases in agonist affinity. Hydrophobic interactions with elements of the TM domains could be involved in liberating or masking of key residues of the ligand binding pocket. In fact, the orientation of residues known to be involved in the orthosteric binding pocket of D1R display differing orientations in the homology models of D5R and all the mutant receptors. These observations further highlight the central region of IL3 as a target for the pharmacological distinction between D1R and D5R; more specifically suggesting that the C-terminal moiety of IL3 central region may harbour residues capable of eliciting subtype-specific effects.

The central region of IL3 of D1-class receptors may therefore be a candidate target for diffusible compounds acting on the intracellular side of the receptor. This approach has been utilized in the development of a small molecules and pepducins, and has been successful in modulating GPCR signalling *in vivo* (Dowal et al., 2011, O'Callaghan et al., 2012). Adopting a similar strategy for the development of compounds for treatment of D1-class implicated conditions, such as L-DOPA-induced dyskinesia or schizophrenia, may be warranted. Constitutive signalling is a potentially important functional distinction D5R and has been suggested to be implicated in the release of atrial natriuretic factor and regulate the burst-firing of subthalamic neurons (Lee et al., 1999, Baufreton et al., 2005). Further investigation into the physiological and pathophysiological contributions of D5R constitutive signalling would benefit immensely from pharmacological selectivity among the D1-class receptors.

The primary structure of the central region of IL3 is quite varied between GPCR species; therefore a general role of this cytoplasmic domain in GPCR function may be difficult to conclude. However, by highlighting the functional significance of the IL3 central region in D1-class receptor signalling, these data emphasize its contribution to the physiologically distinct subtypes that have evolved. Furthermore, this study lends credence to the notion that intracellular receptor structures may have direct or indirect effects on the ligand binding pocket of GPCRs. It demonstrates that the central region of the IL3, a region often assumed to be physiologically insignificant, displays a strong influence on G protein coupling and plays a critical role in basal activity and agonist-induced receptor activation. These data implicate the central region of IL3 as a potentially relevant drug target and may be interpreted in conjunction with structural studies to provide a more complete understanding of GPCR constitutive and agonist-induced signalling.

Supplemental Information

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                    EL2                          TM5
D1R         -------GNATSLAETIDNCDSSLSRTYAISSSVISFYIPVAIMIVTYTRIYRIAQKQIR  226
D5R         ANWTPWEEDFWEPDVNAENCDSSLNRTYAISSSLISFYIPVAIMIVTYTRIYRIAQVQIR  257
β2AR        ---------AINCYANETCCDFFTNQAYAIASSIVSFYVPLVIMAFVYSRVFQEAKRQLQ  231
β1AR        ---------ARRCYNDPKCCDFVTNRAYAIASSVVSFYVPLCIMAFVYLRVFREAQKQVK  256
5HT2AR      -------------F-KEGSC-LLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEAT  263
5HT4R       -------KRKFNQNSNSTYCVFMVNKPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHAH  224
                           *    .  :.:  *.::*::*. :* ..*       : .
                          Central IL3
D1R         RIAA-LERAAVHAKNCQTTT-------------------------GNGKPVECSQPESS  259
D5R         RISS-LERAAEHAQSCRSSA-------------------------A-------CAPDTS  283
β2AR        KIDK-SEGRFHVQNLSQV---------------------------EQDGRTGH---GLRR  260
β1AR        KIDS-CERRFLGGPARPPSPSPSPVPAPAPPPGPPRPAAAAATAPLANGRAG----KRRP  311
5HT2AR      LCVSDLGTRAKLASF---SF----LPQSSLSSE-------KLFQRSIHREPG--SYTGRR  310
5HT4R       QIQM-LQRAGASSESRPQ---------------------------S----------ADQH  246

                                TM6                          EL3
D1R         FKMSFKRETKVLKTLSVIMGVFVCCWLPFFILNCILPFCGSGETQP----FCIDSNTFDV  315
D5R         LRASIKKETKVLKTLSVIMGVFVCCWLPFFILNCMVPFCSGHPEGPPAGFPCVSETTFDV  343
β2AR        SSKFCLKEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQDN----------LIRKEVYIL  310
β1AR        SRLVALREQKALKTLGIIMGVFTLCWLPFFLANVVKAFHRE----------LVPDRLFVF  361
5HT2AR      TMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNE------DVIGALLNV  364
5HT4R       STHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDY----------TVPGQVWTA  296
                   * *. *.* :::  *   * ***: * :  :             :
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Figure S1. Sequence alignment of TM5, IL3 and TM6 of biogenic amine receptors. Sequences for D1R, D5R, β_2 adrenergic receptor (β2AR), β_1 adrenergic receptor (β1AR), serotonin 2A receptor (5HT2A) and serotonin 4 receptor (%HT4) was performed using Clustal Omega (www.clustal.org). TM regions are highlighted in black, the cytoplasmic extensions of TM5 and TM6 are highlighted in yellow. * indicates fully conserved amino acids, : indicates highly conserved amino acids and . indicates weakly conserved amino acids.

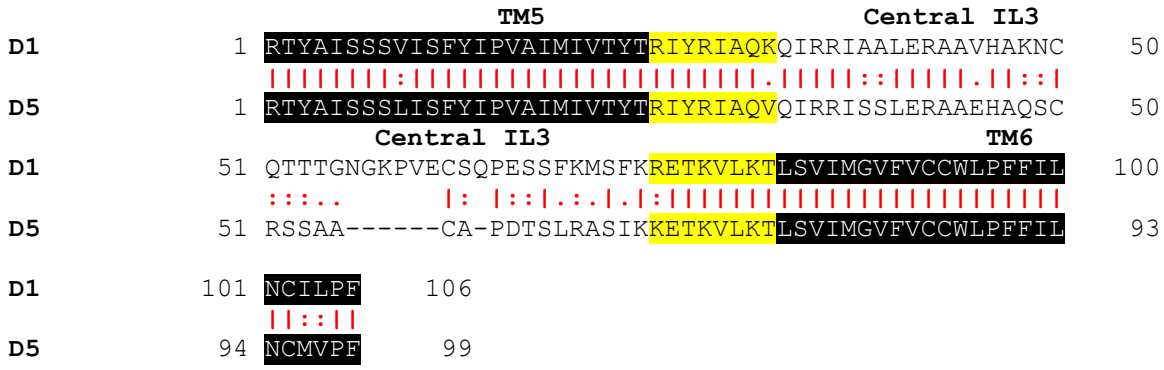

Figure S2. Sequence Alignment of TM5, IL3 and TM6 of D1-class receptors. TM5 and TM6 are highlighted in black, cytoplasmic extensions of TM5 and TM6 are highlighted in yellow. The N-terminal moiety of the central IL3 extends from Q224-T245 in D1R and Q255-S275 in D5R. The C-terminal moiety extends from G246-K265 in D1R and A276-K289 in D5R. Conserved amino acids are indicated by |, highly conserved amino acids are indicated by :, weakly conserved amino acids are indicated by .

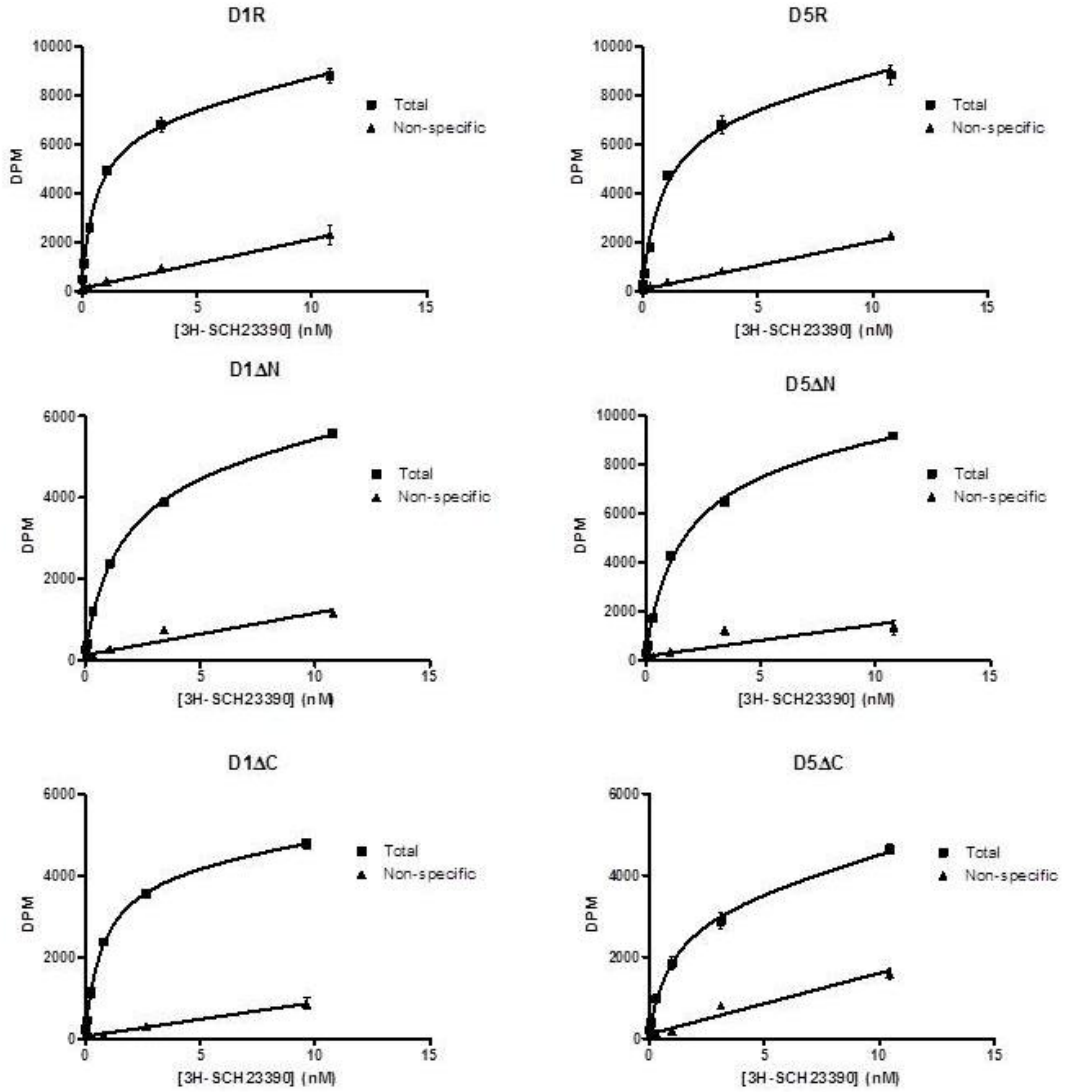

Figure S3. Saturation binding experiment with [^{3}H]-SCH23390. Examples of binding curves of HEK293 cells transfected with wild-type and mutant receptors demonstrate the interaction of [^{3}H]-SCH23390 with one homogenous binding site.

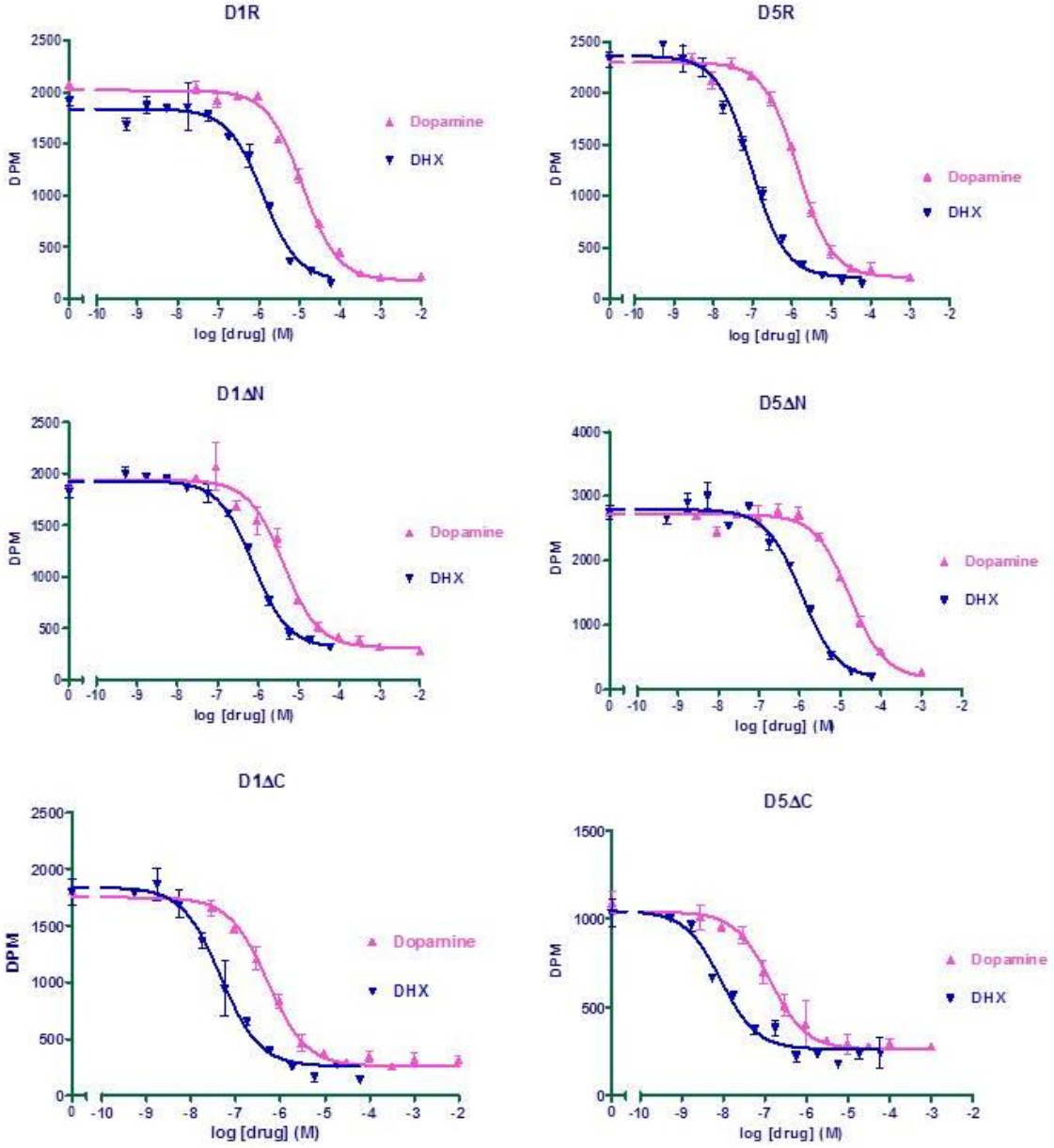

Figure S4. DA and DHX competition experiments with [^{3}H]-SCH23390. Examples of displacement curves from competition experiments demonstrating the interaction of drugs with one homogenous binding site.

General Discussion

This study has provided functional data that ascribe a role for the central region of the IL3 of D1-class receptors in facilitating and controlling the extent of G protein coupling. The N-terminal portion is required for constitutive activity and robust agonist-mediated AC activation, whereas the C-terminal portion imparts restraint upon both agonist-independent and dependent activation. 3D homology modelling has assisted in relating these observations to structural studies and formulating the hypothesis that the central IL3 acts as a molecular switch that is an integral piece of the receptor activation machinery. These homology models have raised additional issues that require further studies that are beyond the scope of this Master's thesis; 1) the role of the helix domain of the central IL3 region and receptor dimerization 2) the distribution of basic amino acid residues (Arg, Lys, His) in the IL3. Additionally, issues of receptor expression (B_{max}) obtained with the deletion mutants will be addressed with reference to pharmacological chaperones.

3.1 – A Potential Role of the Predicted Central IL3 Helix in Receptor Dimerization

As discussed in the manuscript, the results from the 3D homology modelling of the D1-class receptors also predicted a central helix in the IL3. Both D1R and D5R possessed the central helix of equal length; however, the helix of D5R is shifted outwards relative to D1R, representing the largest difference between the two receptors. The central IL3 helix was also found in a homology model generated of β_2AR, suggesting it may be an important feature in bioamine receptors. Furthermore, structural studies in other GPCR systems have also identified central IL3 helices (Franzoni et al., 1999, Umanah et al., 2011). In the yeast Ste2p receptor, evidence suggests the central IL3 helix facilitates homodimerization, an important possibility to consider when interpreting the predicted D1-class central helices as

both D1R and D5R have previously been shown to signal as a homodimers (Ng et al., 1994, George et al., 1998, Kong et al., 2006, So et al., 2009, Umanah et al., 2011). GPCR dimerization can significantly alter the function of receptors (Terrillon and Bouvier, 2004, Milligan, 2009) The decreased size of the disordered portion of the central IL3 in both D5R and the C-terminal mutants compared to D1R may allow for less steric hindrance and easier access of other proteins to this location. Thus, as these receptors maintain the central helix (unlike the N-terminal mutants) they may facilitate more efficient dimerization, which may explain the increased agonist-independent signalling in these receptors.

To assess the influence of dimerization on D1-class signalling, co-immunoprecipitation experiments in concert with RET studies of cells co-transfected with epitope tagged mutant and wild-type receptors may be employed in the future. If a particular mutant is found to maintain a beneficial phenotype (i.e. enhanced signalling of D1ΔC), when co-expressed with wild-type receptors, it may warrant development of a viral delivery system that would allow for the eventual assessment of the mutant receptor in an *in vivo* setting. In the case of D1ΔC, use of a frontal cortex specific promoter to drive expression may prove to be a valuable approach to attempt to treat the negative symptoms of schizophrenia.

In opposition to the notion that α-helices are required for G protein activation, peptide fragments of the CB_1R C-terminal juxtamembrane region demonstrated, by use of various detergent conditions, that the existence an α-helix conformation is not required for the peptide to activate the G protein (Mukhopadhyay et al., 1999). Instead, this study presents alternative notion; the BBXXB motif, or a variation of it, (where B represents a

basic amino acid and X represents any other amino acid) as the critical element involved in G protein coupling/activation (Mukhopadhyay et al., 1999).

3.2 – Basic Residues of the IL3

The combined literature describing the importance of the BBXXB motif reveals that there is broad variation between receptor species. The BBXXB motif appears predominantly at the cytoplasmic extension of TM6, however, it has also been reported to reside in more central regions of the IL3, as well as in the TM5 cytoplasmic extension (Pauwels et al., 1999, Kohen et al., 2001, Couvineau et al., 2003, Kuniyeda et al., 2007, Peverelli et al., 2009). Furthermore, reversed or slightly modified (ie. BBXB) motifs have also been reported to be involved in G protein activation (Ulloa-Aguirre et al., 2007). D1R contains a reversed BBXXB motif in the juxtamembrane regions at both the N-terminal (KQIRR) and C-terminal sides (KTERK) of the IL3, whereas D5R contains a reversed BBXXB at the C-terminal side (KTEKK) and a XXXBB at the N-terminal side (VQIRR) (**Figure 11**). In many instances, a point mutation approach has been employed to resolve the contribution of each residue within the motif. Often this has led to an increase in the constitutive activation of the receptor (Kjelsberg et al., 1992, Ren et al., 1993, Egan et al., 1998, Pauwels et al., 1999), but in some instances has resulted in silencing of the receptor (Kohen et al., 2001, Ulloa-Aguirre et al., 2007) or alterations in other properties (Couvineau et al., 2003, Peverelli et al., 2009). Overall, no clear pattern becomes apparent among the relative position of basic residues within the motif, as mutational effects appear to be receptor specific. This suggests that it may not necessarily be a motif that is required, rather that the presence and distribution of basic residues throughout the IL3.

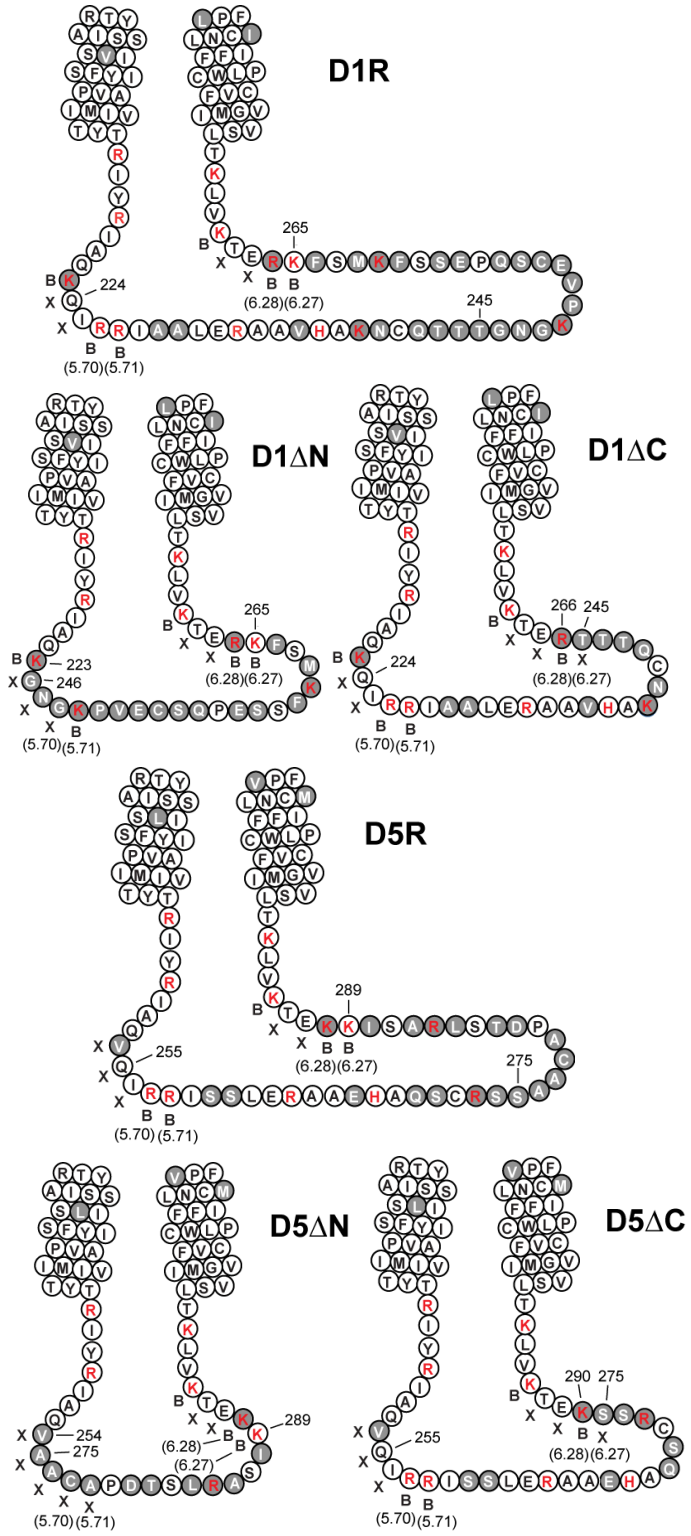

Figure 11. Schematic diagram comparing the BBXXB motifs of the wild-type and deletion mutants. Motifs are represented in outside the sequence. Basic residues are highlighted in red. Residues conserved between D1R and D5R are represented by white circles, divergent residues are depicted as grey circles.

In this study, the deletion mutations resulted in the alteration of motif patterns in both D1R and D5R (**Figure 11**). The arginine pair at position 5.70 and 5.71 (226, 227 in D1R and 257, 258 in D5R) may play a key role in G protein activation, as the poorly activating N-terminal mutants possess changes in these residues. D1ΔN maintains a basic residue at position 5.71 (R → K) and concomitantly displays higher DA-induced AC activation than D5ΔN, which lacks any basic residues at the same position and displays further reduction in DA-induced AC activation (**Figure 11, 12**). In contrast, both of the C-terminal mutants lose the last basic residue at position 6.28, which may explain the enhanced constitutive and agonist induced signalling of D1ΔC and D5ΔC (**Figure 11, 12**). Within the IL3 of D1R and D5R, additional basic residues are located outside of the motif regions and therefore are removed or reoriented with the deletion mutations.

Analysis of the homology models of the wild-type and mutant receptors bolsters the putative relationship between the distribution of basic residues within the IL3 and our functional data. The basic residues of the BXXBB motif align on the outer face of the helical extension of TM5 in the wild-type and C-terminal mutant receptors, with the R residues extending outwards towards the cytoplasm (**Figure 12**). In the N-terminal mutants, these residues are not present and the side chains of the substituted residues remain in close proximity to main chain of the loop (**Figure 12**). These mutants display less basic residues throughout the entire central IL3, which may be reflected by the lack of G protein coupling these mutants display. The removal of the cumbersome disordered region in the C-terminal mutants leaves many basic residues throughout the central IL3 and appears to allow for easier access of the G protein to the basic residues of the TM6 cytoplasmic extension; possibly mediating increased constitutive activity (**Figure 12**).

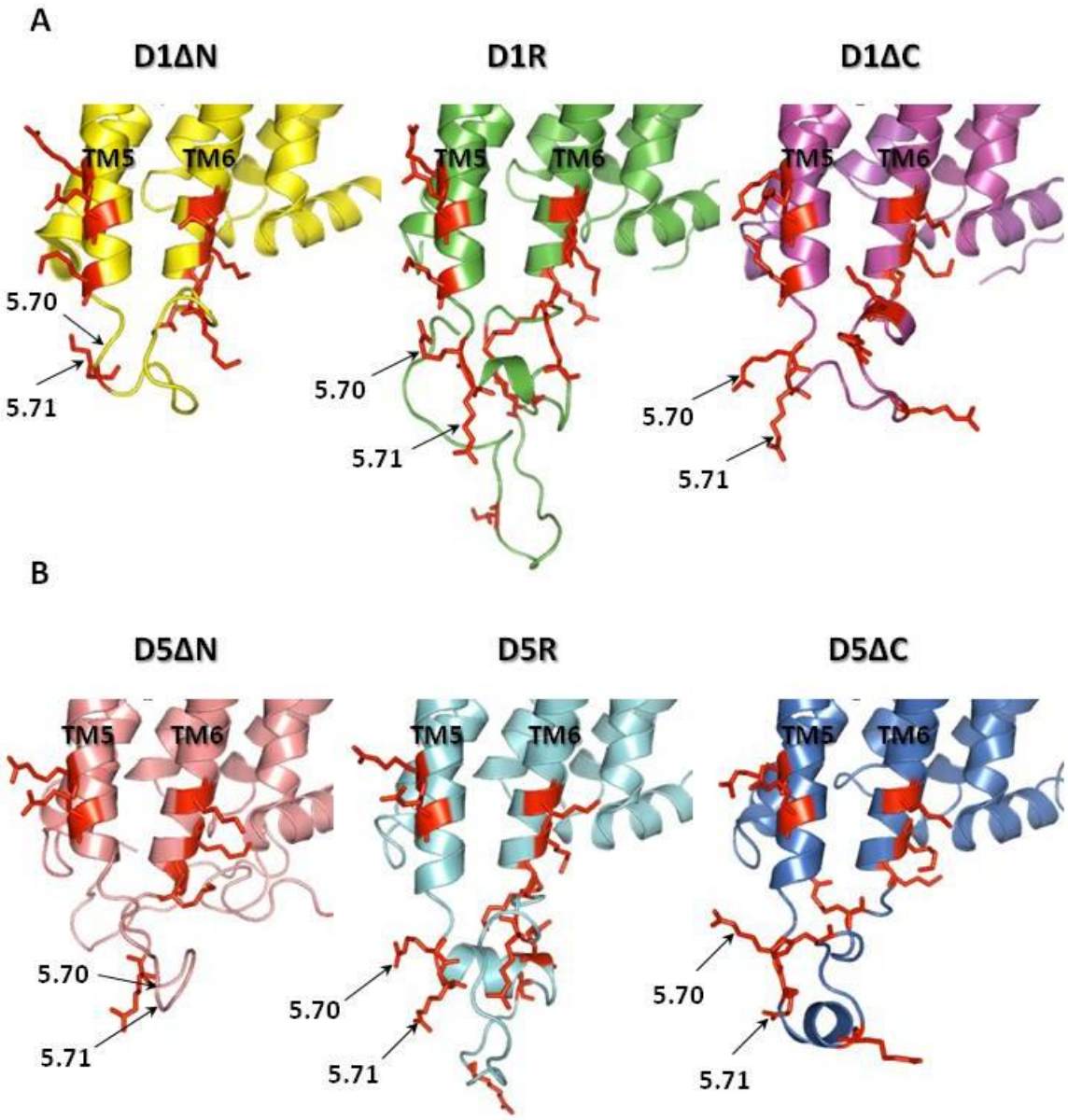

Figure 12. 3D homology models of wild-type and mutant D1-class receptors highlighting the distribution of basic residues throughout the IL3. Basic residues are shown in red. (A) D1ΔN is represented in yellow, D1R in green and D1ΔC in magenta. Arrows indicate the potentially important basic residues in positions 226 (5.70) and 227 (5.71). (B) D5ΔN is represented in pink, D5R in cyab and D5ΔC in blue. Arrows indicate the potentially important basic residues in positions 257 (5.70) and 258 (5.71). Models were predicted using Phyre2 and images generated using PyMol (Kelley and Sternberg, 2009, Schrodinger, 2010)

When considered with our functional data, the predicted orientations of the basic residues of the IL3 imply that the TM5 cytoplasmic extension residues are critical to the G protein binding site, while the TM6 cytoplasmic extension residues appear to control the basal activation and extent of agonist-induced activation. Furthermore, the presence of the BBXXB motif may not be the influencing factor mediating G protein interactions, rather, the distribution and accessibility of basic residues may facilitate them. Elucidating any effects that the length of the deletion may impart will be critical to demonstrating the role of these residues.

Work is currently underway in our lab to further narrow down the motifs/residues that are critical for the signalling effects observed in this study. The approach thus far has been to divide the deleted regions presented in this work each into two halves. To further rule out any spacing effects due to the length of the deletion, stretches of alanine residues will replace the residues of the newly deleted regions in a second set of mutants. The characterization of these mutants will hopefully help to identify any specific residues involved and allow for a minimal cassette of amino acids which can be targeted pharmacologically.

3.3 – B_{max}, Cell Surface Expression and Pharmacological Chaperone Rescue

The proper delivery of GPCR constructs to the plasma membrane has often been a challenge for the field. More often than not, artificially introducing a receptor construct into a heterologous cell system results in deficiencies in receptor cell surface expression (Dunham and Hall, 2009). To assess ligand binding properties, our lab utilizes a paradigm in which we maximally express each receptor by transfecting 5 µg of receptor DNA per dish; an amount of DNA that has previously been found to have minimal consequences on

cell survival, yet leads to maximal achievable expression in HEK293 cells. The cells are lysed and membranes prepared for binding assays using increasing concentrations of [^{3}H]-SCH23390, a drug that is specific for D1-class receptors. This assay quantifies both the affinity for the drug and the maximal binding capacity of [^{3}H]-SCH23390 (B_{max}), thus providing an index of functional receptor expression at the cell surface.

All of the deletion mutant constructs in this study displayed significantly reduced B_{max} levels compared to the wild-type, with the exception of D5Δ, using our transfection paradigm. It should be mentioned however, that the amount of expression achieved by the mutant receptors in HEK293 cells, albeit lower, was comparable to endogenous D1-class receptor expression in the striatum. Furthermore, in our cAMP assays, the level of DNA transfected into cells was titrated to match the B_{max} between receptors, allowing for accurate assessment of constitutive and agonist-dependent activation with similar receptor B_{max}.

It is well established that receptor expression can be "rescued" through the use of inverse agonists as "pharmacological chaperones" (Bernier et al., 2004, Conn and Ulloa-Aguirre, 2010). The notion being, that as these synthetic compounds are lipophilic, they cross the plasma membrane and act to stabilize the receptor in a conformation that is amenable to bypassing the cells quality control apparatus, allowing for export from the protein synthesis machinery (Morello et al., 2000). As a proof of principle, rescue experiments were performed with HEK293 cells transfected with either D1R, D5R, D1ΔN or D5ΔN using our transfection paradigm. These rescue studies allowed us to evaluate in parallel, one mutant with unchanged receptor expression and one with impaired expression, relative to their wild-type receptor. Specifically, we wanted to test whether D1N would

display up-regulation in B_{max}, an index of rescue. Cells were incubated with the inverse agonists, thioridazine (THIO) or flupenthixol (FLUP), for 24 hours and washed with PBS buffer prior to performing B_{max} assessment with a saturating concentration of [^{3}H]-SCH23390. Additionally, another series of experiments was performed to assess the effects on receptor B_{max} following long-term exposure to DA. In classical GPCR regulation, long-term exposure to agonists induces down-regulation of receptors, reflected by reduced B_{max}. While it remains to be established for D5R, such down-regulation has been demonstrated in D1R, both in endogenously expressing and transfected cell systems (Bates et al., 1991, Jiang and Sibley, 1999).

Partial rescue of D1ΔN was observed with both THIO and FLUP, while no major effects were observed in D1R, D5R or D5ΔN with these drugs (**Figure 13B**). The partial rescue D1ΔN with the inverse agonists only amounted to approximately 30 percent of the B_{max} of D1R (**Figure 13A**). With respect to long-term DA exposure, we saw a smaller decrease in B_{max} than was expected in D1R, and no change in that of D5R. The lack of effect in both receptors may be attributed to the high levels of expression masking the down-regulation. Surprisingly, we found that DA provoked an even more robust rescue than the inverse agonists, increasing greater than 3-fold over control levels (**Figure 13B**) to approximately 40 percent of D1R B_{max} (**Figure 13A**). Interestingly, despite the fact that D5ΔN had not displayed a significant decrease in expression, it also displayed a significant increase in binding following long-term treatment with DA (**Figure 13B**) surpassing the B_{max} of D5R (**Figure 13A**). This potentially suggests that D5ΔN is

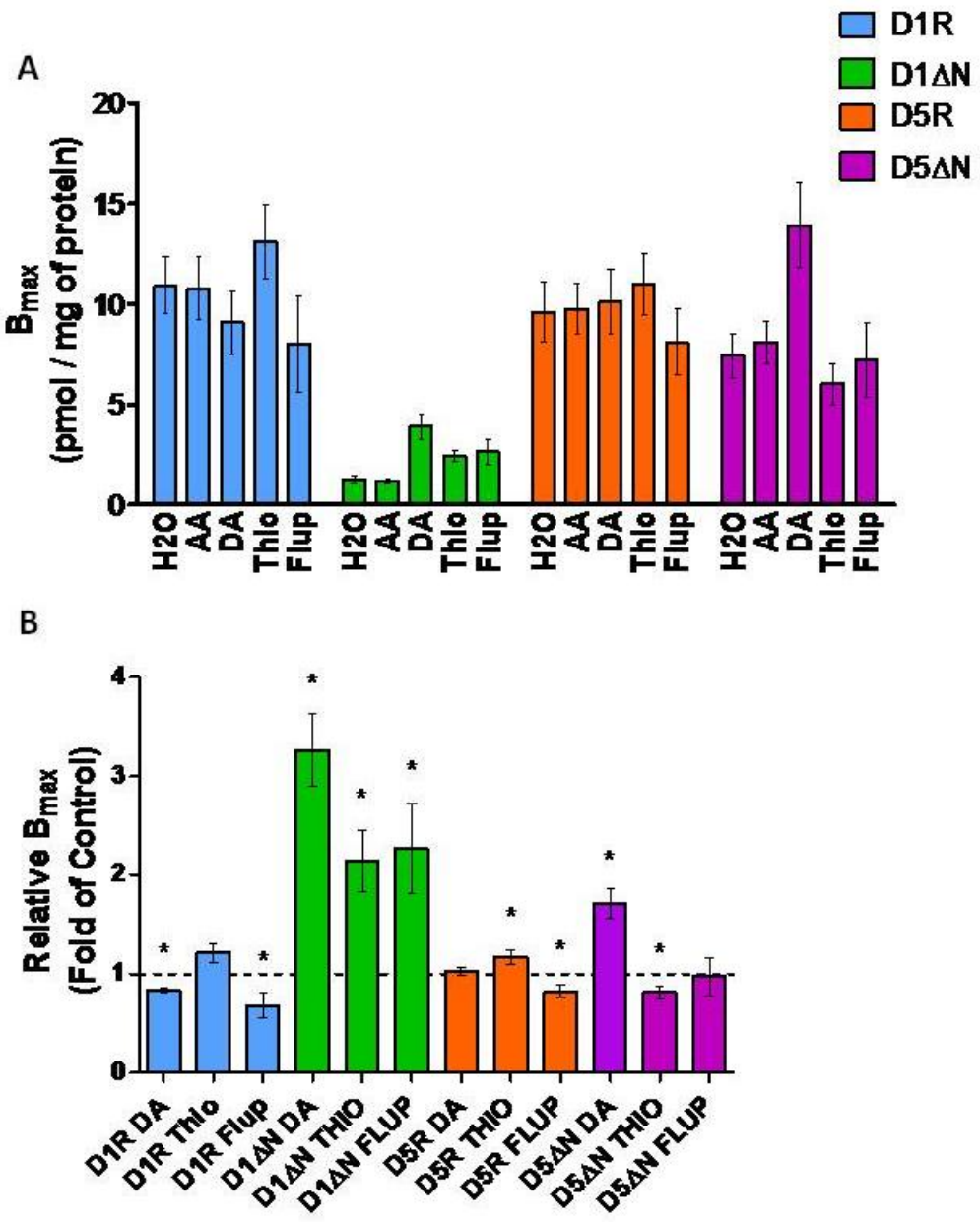

Figure 13. Long-term ligand treatment partially rescues receptor binding. Transfected HEK293 cells (2M cells/dish) were incubated with either H_2O (THIO/FLUP vehicle), ascorbic acid (AA, DA vehicle), DA (100 µM), THIO (1 µm) or FLUP (1 µm) for 24 hours, drugs cells were then washed with PBS and binding assays performed using a saturating concentration of [^{3}H]-SCH23390. (A) Mean B_{max} of [^{3}H]-SCH23390 from 8 experiments. (B) Mean B_{max} of drug treatments normalized to their respective control values and compared to 1 using a one sample t-test. * $p < 0.05$. AA, ascorbic acid; DA, dopamine; THIO, thioridazine; BUTA, butaclamol.

expressed at a higher level than we can detect with our binding assay due to some form of regulation.

The results of 24 hour incubation with the inverse agonists are in line with a pharmacological chaperone rescue. These preliminary data could be reiterated using an ELISA assay and followed up with immunofluorence confocal microscopy experiments using epitope tagged receptors and ER/Golgi markers to further elucidate the processing of the proteins. The results of long-term treatment with DA are perplexing as DA is unable to cross the plasma membrane in the absence of DAT (not endogenously expressed in HEK 293 cells). To reconcile these results alternative hypotheses are required.

A plausible explanation is that the deletions do not only induce the intracellular retention of the protein, but promote tonic internalization of cell surface receptors and direct sorting to degradation pathways (i.e. lysosomes). DA binding of an internalization prone receptor may induce a conformation that stabilizes the receptor at the plasma membrane, increasing its cell surface half-life and allowing for an increased B_{max} for [^{3}H]-SCH23390. Interestingly, cell surface retention time was shown to be decreased in α_{2A} adrenergic receptors containing deletions in the IL3, independent of any particular amino acid sequences (Edwards and Limbird, 1999). Stabilization at the cell surface may not be the sole mechanism responsible, but rather a cell sorting effect; wherein following DA binding, the fate of the internalized receptor is switched from degradation to a recycling pathway. Short-term homologous desensitization of D1-class receptors is mediated by β-arrestin, which has previously been shown to interact with the IL3 of the agonist-bound D1R (Kim et al., 2004). Deletion of the central IL3 may therefore interfere with β-arrestin

binding, but the contribution of this interaction to long-term regulation remains to be elucidated.

A second possibility is the reactive oxygen species generated by DA throughout the treatment may have permeated the plasma membrane, allowing DA to enter the cell and act as a pharmacological chaperone in a similar fashion to the inverse agonists (Pun et al., 2009). Alternatively, ROS itself may have direct effects on the protein folding, leading to up-regulation of B_{max}.

3.4 - Potential Mechanism of Pharmacological Rescue

It has been shown that the availability of a particular export or retention motif within a GPCR can affect the delivery of the protein to the cell surface. Often these motifs include LL, FF, or basic residues (Dong et al., 2007). D1R contains a $F(X_3)F(X_3)F(X_3)$ motif in the C-tail that interacts with the ER chaperone DRIP78 (Bermak et al., 2001). In the $GABA_B$ receptor, heterodimerization of the GB1 and GB2 subunits via their C-tail α-helices masks an RXR(R) retention motif and facilitates cell surface expression (Margeta-Mitrovic et al., 2000). An R rich retention motif is also present in the IL3 of the V_2 vasopressin receptor (Hermosilla and Schulein, 2001). Altering the central region of the IL3 of D1-class mutants may have enhanced an interaction with a retention sequence or masked an export motif, resulting in the observed reduced receptor expression. In turn, the inverse agonists or DA may have promoted a conformation in the D1ΔN that masked the putative retention sequence or restored a chaperone interaction, partially rescuing receptor expression. The result obtained with D5ΔN is surprising as it suggests that upon

interaction with DA (but not the inverse agonists), the mutant receptor is able to adopt a conformation that is more suitable for export to the cell surface than its wild-type.

Dimerization of GPCRs has also been proven to affect their cell surface expression (Terrillon and Bouvier, 2004). In fact, co-transfection of mutant and wild-type receptors has been shown to impede cell surface delivery of both receptors, even if the mutant receptor displays normal expression when transfected alone. Evidence from co-immunoprecipitation experiments has suggested that D1R signals as a homodimer *in vivo* (Ng et al., 1994, George et al., 1998). Kong et al. engineered a constitutively active D1R, unable to bind ligands and co-expressed it with D1R (Kong et al., 2006). Homodimers of the mutant receptor expressed normally, however heterodimers with D1R did not. Treatment with diffusible agonists rescued the lack of cell surface expression of the D1R/mutant heterodimer (Kong et al., 2006). Although it is unlikely that this is the sole explanation for the decreased cell surface expression observed in the deletion mutants of this study, it remains possible that homodimerization is a contributing factor in the rescue of their cell surface expression. This may resolve the increase in D5ΔN binding upon long-term treatment, as a DA-induced conformation may favour more effective dimerization and export of this receptor. Collectively, considering its possible effects on the signalling properties of the D1-like receptors discussed above, homodimerization may provide a link between the binding and functional phenotypes observed in the mutant receptors of this study.

3.5 – Concluding Remarks

The results of my research project have provided ample evidence implicating the central region of the IL3 as a critical determinant in D1-class receptor activation.

Furthermore, this region may hold key residues for developing selectivity among D1R and D5R. My hope is that this study will heighten awareness of the integral role that the IL3 plays in the activation of other GPCRs as well. As an accessible region of the receptor harbouring potential molecular switches, the IL3 may represent a perfect target for novel diffusible compounds (**Figure 14**). This approach has been utilized in the development of a small molecule, JF5, that acts on helix 8 of the PAR1 receptor to inhibit thrombus formation *in vivo* (Dowal et al., 2011). Furthermore, a number of pepducins, cell-penetrating lipidated peptides, have been successful *in vitro* and *in vivo* (O'Callaghan et al., 2012). These molecules can be designed as either agonists or antagonists, acting specifically on an intacellular domain of choice and displaying high degrees of selectivity. This study provides a framework on which to design such compounds; the N-terminal region serving as a target for antagonism and the C-terminal region as a target for agonism. Both hypodopaminergic conditions, as in the schizophrenic PFC, or hyperdopaminergia as observed in L-Dopa dyskinesia, represent possible therapeutic applications. Classically, allosteric compounds work as adjuvants to orthosteric ligands, however, based on the effects that the deletions had on the basal activity of the D1-class receptors, putative allosteric compounds may alone be therapeutically relevant. Characterization of the role that the central IL3 could play in other GPCRs may highlight additional systems in which other novel drugs can be developed. As academia collaborates more and more with industry, it is becoming more likely that biochemical characterizations, such as this study, can serve as the starting point to the development of new drugs.

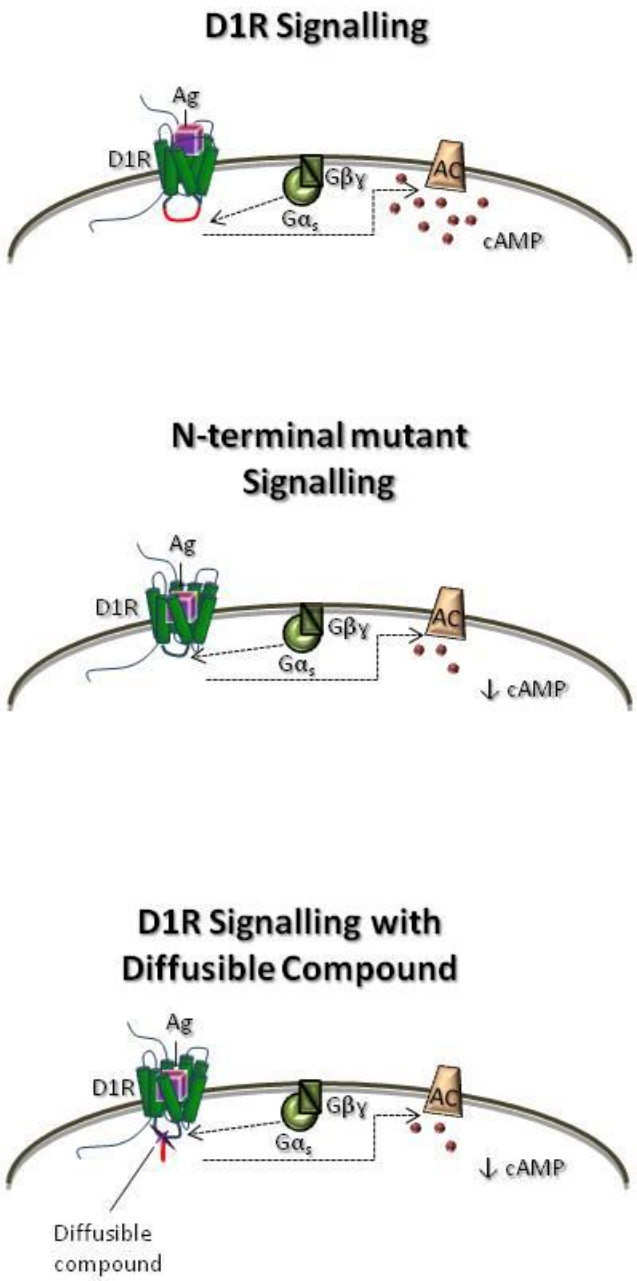

Figure 14. Schematic diagram depicting the potential action of a diffusible allosteric inactivating compound. Small diffusible molecules or pepducins could potentially be developed to mimic the action of the deletion mutants presented in this study. This figure depicts a compound, acting as an adjuvant to the natural ligand, crossing the plasma membrane and targeting the central IL3 to mimic the decrease in cAMP production of the N-terminal mutants. Such a compound may be therapeutically relevant for treatment of D1-class hyperactive states, such as L-Dopa dyskinesia.

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