Regulation of the Dopamine D3 Receptor by Adenylyl Cyclase 5

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Abstract

The D3 dopamine receptor (D3R) belongs to D2-class of dopamine receptors (DARs) and is involved in emotion, movement, and reward. D3R dysfunction has been reported in some neuropsychiatric disorders such as addiction, cognitive deficits, depression, schizophrenia, and Parkinson's disease. Genetic studies have shown two polymorphic variants of the D3R gene resulting from substitution of serine to glycine at position nine of the amino terminus. Isoform 5 of adenylyl cyclase (AC5) is one of the nine transmembrane bound ACs in the brain and myocardium. Previous studies in rats have shown that AC5 is expressed in the striatum, nucleus accumbens and olfactory tubercle and at lower levels in islands of Calleja, where the D3R is also expressed. Previous studies showed that although D2R and D4R inhibit ACs activity in different cell types, inhibition of ACs by D3R is weak and often undetectable. It has been shown that D3R selectively inhibits AC5 activity in human embryonic kidney 293 (HEK293) cells co-transfected with D3R and AC5. Co-expression of D3R and AC5 in brain regions which are major coordinators of normal and pathological movement, and the selective inhibition of AC5 activity by D3R raise the possibility of a functional link between AC5 and D3R in the modulation of signal transduction and trafficking. I hypothesized that AC5 plays a unique role in modulation of D3R trafficking and signaling pathways through interaction between D3R and AC5. Herein, I demonstrated an interaction between D3R and AC5 *in vivo* and *in vitro* using reciprocal co-immunoprecipitation/immunoblotting (co-IP/IB) assays. Interestingly, DA may facilitate the formation of protein complex between D3R and AC5 *in vitro*. Radio ligand binding assays revealed that heterodimerization of D3R polymorphic variants with AC5 does not change ligand binding affinity and expression of the D3R. Furthermore, taking advantages of GloSensor assays, selective inhibition of AC5 activity by D3Ser9 and D3Gly9 has been shown following activation by DA and quinpirole. Using ELISA studies showed that AC5 promotes cell surface expression and total expression of D3Ser9 and D3Gly9. Moreover,

ELISA results suggested that AC5 facilitates DA-induced D3Ser9 endocytosis in dynamin and β-arrestin 2 dependent process, while having no effect on D3Gly9 polymorphic variant. The results also revealed that AC5 attenuates heterologous (PKC-induced) internalization of D3Ser9, while it does not have any effect on D3Gly9 heterologous internalization. My results also displayed a complex formation between D3R, AC5 and, β-arrestin 2 under basal and DA stimulation conditions, which emphasize the role of β-arrestin 2 in D3R signal transduction. Overall, a new regulatory mechanism for D3R has been suggested. My results suggested that complex formation between both D3R polymorphic variants with AC5 can regulate signaling and trafficking properties of D3R without changing the binding affinity of the receptor. These data will be meaningful for understanding of diseases and developing treatment strategies.

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List of Abbreviations

2BP: 2-bromopalmitate

7-OH-DPAT: 7-hydroxydipropylaminotetralin

7TMRs: Seven-transmembrane receptors

A_{2A}R: Adenosine A_{2A} receptor

AA: Ascorbic acid

AADC: Aromatic amino acid decarboxylase

AC: Adenylyl cyclase

AD: Alzheimer's disease

AKAP79/150: A-kinase anchoring protein 79/150

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP-2: Adaptor protein

APP: Amyloid precursor protein

AT_{1A}: Angiotensin II type 1A

ATP: Adenosine triphosphate

BCS: Bovine calf serum

BDNF: Brain derived neurotrophic factors

BiFC: Bimolecular fluorescence complementation

B_{max}: Maximal binding capacity

BRET: Bioluminescence resonance energy transfer

BSA: Bovine serum albumin

CaMKII: Calcium/calmodulin-dependent PK II

cAMP: Adenosine 3′,5′- cyclic monophosphate

CCE: Capacitative calcium entry

CCPs: Clathrin-coated pits

CDK5: Cyclin-dependent kinase 5

CHC: Clathrin heavy chain

CHO: Chinese hamster ovary

Co-IP: Co-immunoprecipitation

COMT: Catechol-O-methyltransferase

CREB: cAMP response element-binding protein

CRFR1: Corticotropin releasing factor receptor 1

CT: Cytoplasmic tail

D1R: Dopamine D1 receptor

D2L: Dopamine D2 receptor-long

D2R: Dopamine D2 receptor

D2S: Dopamine D2 receptor-short

D3Gly9: Dopamine D3 receptor Gly9 polymorphic variant

D3R: Dopamine D3 receptor

D3Ser9: Dopamine D3 receptor Ser9 polymorphic varinat

D4R: Dopamine D4 receptor

D5R: Dopamine D5 receptor

DA: Dopamine

DAG: Diacylglycerol

DAR: Dopamine receptor

DARPP-32: cAMP-regulated phosphoprotein, 32kDa

DAT: DA transporters

DGKs: Diacylglycerol kinases

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediaminetetraacetic acid

eEF1Bβγ: Elongation factor-1Bβγ

EGFR: Epidermal growth factor receptor

EL: Extracellular loop

ELISA: Enzyme-linked immunosorbent assay

EMEM: Eagle's minimal essential medium

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

ERK1/2: Extracellular signal-regulated kinase 1 and 2

ET: Essential tremor

ETA: Endothelin type A

FBS: Fetal bovine serum

FDFM: Familial dyskinesia with facial myokymia

FEME: Fast endophilin-mediated endocytosis

FRET: Fluorescence resonance energy transfer

FSK: Forskolin

$GABA_AR$: γ-aminobutyric acid type A receptor

GAIP: G-alpha interacting protein

GASP-1: GPCR-associated sorting protein-1

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GIPC1: G-alpha interacting protein (GAIP) C terminus 1

GIRK: G protein-regulated inwardly rectifying K^+ channel

GIT1: GRK-interacting protein

GPCRs: G protein coupled receptors

GPR143: G protein-coupled receptor 143

GRK: GPCR kinase

GTP: Guanosine triphosphate

$G\alpha_{i/o}$: G protein αi/o subunit

$G\alpha_{s/olf}$: G protein αs/olf subunit

Gβγ: G protein βγ subunit complex

HBSS: Hanks' Balanced Salt Solution

HEK293: Human embryonic kidney 293

HIV: Human immunodeficiency virus

HRP: Horseradish peroxidase

IGF-1R: Insulin-like growth factor type 1 receptor

IL: Intracelullar loop

IL-1β: Interleukin-1β

IP3: Inositol trisphosphate

ISO: Isoproterenol

JNK: c-jun N-terminal kinase

JNK3: c-Jun N-terminal kinase 3

K_d : Equilibrium dissociation constant

K_i : Inhibitory constant

KO: Knockout

L-DOPA: L-3,4-dihydroxyphenylalanine

LID: L-DOPA-induced dyskinesia

LTB4: Leukotriene B4

MAO: Monoamine oxidase

MAPK: Mitogen-activated protein kinase

MEK: MAPK/ERK kinase

mGlu7R: Metabotropic glutamate 7 receptor

MOR: μ-opioid receptor

MSK1: Mitogen- and stress-activated protein kinase 1

MSNs: Striatal medium spiny neurons

MβCD: Methyl-β-cyclodextrin

NAc: Nucleus accumbens

nAChR: Nicotinic acetylcholine receptor

NAPA: Protein-NSF (N-ethylmaleimide-sensitive factor) attachment protein alpha

NFkB: Elements of the nuclear factor kB

NMDAR: N-methyl-D-aspartate receptor

NSF: N-ethylmaleimide-sensitive factor

NTSR1: Neurotensin receptor 1

OD: Optical density

OPD: o- phenylenediamine dihydrochloride

P21: Postnatal day 21

PA: Phosphatidic acid

PAM: Protein associated with Myc

PAR1: Protease-activated receptor type 1

PAR2: Protease-activated receptor

PBS: Phosphate-buffered saline

PD: Parkinson's disease

PDEs: Cyclic nucleotide phosphodiesterases

PGE2: Prostaglandin E2

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol-4,5-biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLA: Proximity ligation assay

PLA2: Phospholipase A2

PLC: Phospholipase C

PMA: Phorbol-12-myristate-13-acetate

PMSF: Phenylmethylsulfonyl fluoride

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

PP2B: Protein phosphatase 2B

PTX: Pertussis toxin

PVDF: Polyvinylidene difluoride

Quin: Quinpirole

RGS: Regulators of G protein signaling

RGS2: Regulators of the G protein signaling 2

Ric8a: RIC8 guanine nucleotide exchange factor A

RTK: Receptor tyrosine kinase

sAC: Soluble AC form

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SNP: Single nucleotide polymorphism

SNpc: The substantia nigra pars compacta

SP/NK1R: Substance-P/neurokinin-1 receptor

SSTR5: Somatostatin receptor 5

TH: Tyrosine hydroxylase

TM: Transmembrane regions

TNF-α: Tumor necrosis factor-α

TRH: Thyrotropin-releasing hormone

VEGFR: Vascular endothelial growth factor receptor

VMAT2: Vesicular monoamine transporter 2

VTA: Ventral tegmental area

WT: Wild type

α_1AR: α_1-adrenergic receptor

α_{1b} R: α_{1b} adrenergic receptor

β2AR: β2 adrenergic receptor

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Introduction

1. The Dopaminergic System

Dopamine (DA), also known as 3,4-dihydroxyphenethylamine serves as a predominant catecholamine neurotransmitter in the mammalian brain (Missale et al., 1998). DA is involved in cognition, emotions, food intake, locomotor activity, positive reinforcement, and endocrine regulation. In the periphery, DA also plays roles in renal function, vascular tone, cardiovascular functions, hormone secretion and gastrointestinal motility. Dysfunction of the DA system leads to several pathological conditions including Parkinson's disease (PD), schizophrenia, depression, drug and alcohol addiction, bipolar disease, Huntington's disease, Tourette's syndrome, and attention deficit/hyperactivity disorder (Beaulieu & Gainetdinov, 2011).

1.1. Dopamine Synthesis

DA is produced by presynaptic dopaminergic neurons of the brain from the amino acid tyrosine. Addition of a hydroxyl group to tyrosine by tyrosine hydroxylase (TH) converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). Subsequently, the aromatic L-amino acid decarboxylase (AADC) catalyzes decarboxylation of L-DOPA to DA (Fig. 1A). Once DA is synthesized, it is packaged into a synaptic vesicle by the monoamine transporter 2 (VMAT2) and stored until action potentials induce the release of DA into the synaptic cleft. Free DA in the synaptic cleft can bind to dopamine receptors (DARs) either on the postsynaptic neurons or on presynaptic sites (autoreceptors) which leads to activation of signaling pathways. DA activity in the synaptic cleft is terminated by reuptaking into presynaptic neurons through the DA transporters (DAT) or metabolising by either monoamine oxidase (MAO) in presynaptic terminal or catechol-O-methyltransferase (COMT) found in post-synaptic membrane (Fig. 1B) (Muñoz et al., 2012).

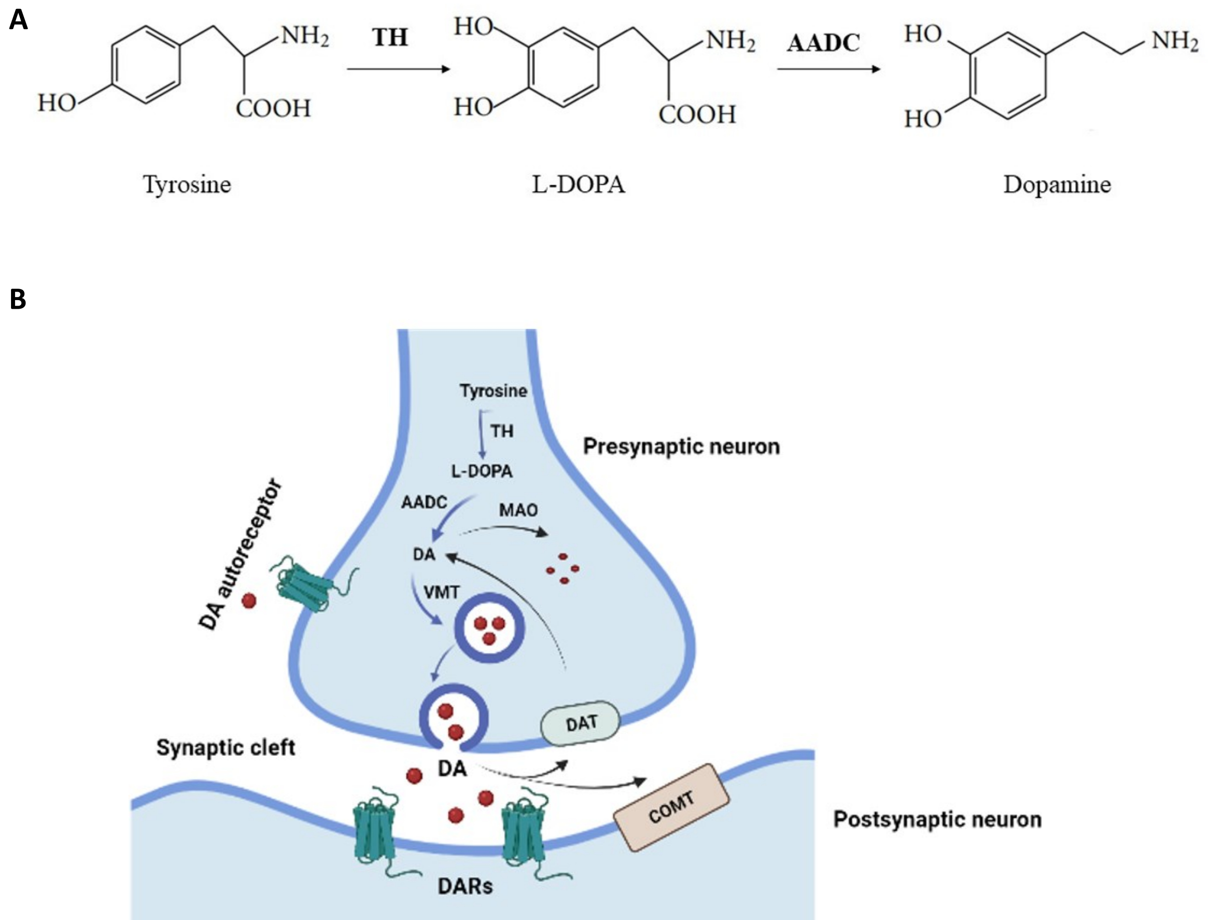

Figure 1. Schematic Representation of DA Synthesis.

A) DA synthesis by TH from tyrosine. Tyrosine is converted to L-DOPA by TH and then AADC converts L-DOPA to DA. B) The key steps in the synthesis and degradation of DA. Once DA is produced, it is stored into vesicles by VMT. Upon an action potential, DA is released into the synaptic cleft where it can bind to postsynaptic DARs or presynaptic autoreceptors. Excess DA can be reuptaken by DAT or metabolised by MAO and COMT. TH, tyrosine hydroxylase; L-DOPA, L-3,4-dihydroxyphenylalanine; AADC, aromatic amino acid decarboxylase; DA, dopamine; VMT, vesicular monoamine transporter; MAO, monoamine oxidase; DAT, dopamine transporter; COMT, catechol-O-methyltransferase; DARs, DA receptors. Created with BioRender.com.

1.2. Dopaminergic Pathways

There are four major dopaminergic neurotransmission pathways in the mammalian brain. In the substantia nigra, the nigrostriatal pathway projects dopaminergic neurons from the substantia nigra pars compacta (SNpc) to the dorsal striatum and plays a primary role in the control of motor function and some forms of motor learning (e.g., dexterous skill). The degeneration of dopaminergic neurons in the nigrostriatal pathway causes a dysregulation of motor control, a hallmark of PD (Darden, 2007).

The mesolimbic pathway links the ventral tegmental area (VTA) of the brain to the nucleus accumbens (NAc) located in the ventral striatum which is involved in motivation, reward, and motor learning. Increased activity in the projections to the nucleus accumbens plays a major role in reinforcement and in more extreme cases with addiction (Nestler & Carlezon, 2006). It is believed that excessive DA activity of the mesolimbic pathway causes the positive symptoms of schizophrenia (Brisch et al., 2014).

The mesocortical pathway connects ventral tegmental area (VTA) to the cerebral cortex. This pathway is important for working memory and cognitive function. Abnormal function in this pathway is implicated in the negative symptoms in schizophrenia patients (Brisch et al., 2014).

In the arcuate nucleus of the hypothalamus, DA neurons make up the tuberoinfundibular pathway which projects to the pituitary gland. DA produced by neurons in the arcuate nucleus inhibits the secretion of the prolactin hormone. Notably, blockade of DA function in tuberoinfundibular pathway by non selective anti dopaminergic anti psychotic drugs causes an increase in prolactin levels (hyperprolatinaemia) (Lyons et al., 2012).

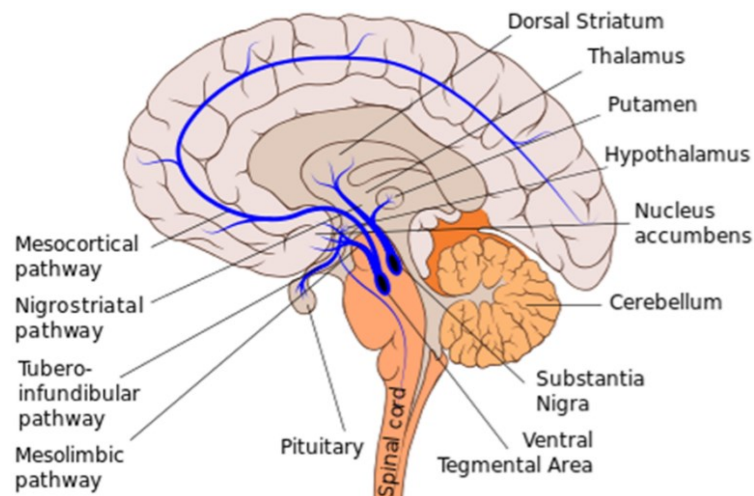

Figure 2. Dopaminergic Neuronal Pathways in the Brain.

There are four major dopaminergic neuronal pathways in the brain: (1) The nigrostriatal pathway connects the substantia nigra with the dorsal striatum; (2) The mesolimbic pathway connects the VTA to the ventral striatum/NAc; (3) The mesocortical pathway connects the VTA to the cerebral cortex; (4) The tuberoinfundibular pathway connects the arcuate nucleus of the hypothalamus to the pituitary gland. VTA, ventral tegmental area; NAc, nucleus accumbens.

1.3. Dopamine Receptors Structure and Classification

DARs belong to the large family of seven-transmembrane receptors (7TMRs) also known as G protein coupled receptors (GPCRs). GPCRs mediate most cellular responses upon activation by light, odorants, neurotransmitters, hormones, peptides, amino acids, and ions. GPCRs signaling pathway starts with interaction and activation of heterotrimeric GTP-binding proteins (Gαβγ subunits) and various effectors like adenylyl cyclases (ACs). Agonist binding to GPCRs promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G protein α-subunit, and this is presumed to allow the dissociation of Gα and Gβγ subunits. Subsequently, Gα and Gβγ subunits can regulate the activity of effector enzymes and ion channels.

DARs can modulate different brain physiological processes including motor activity, learning, memory, and reward. DARs are classically divided into two classes: the D1- class receptors (D1R, D5R) activate ACs through coupling to $G\alpha_s$ and $G\alpha_{olf}$ subunits to raise intracellular levels of adenosine 3′,5′- cyclic monophosphate (cAMP), whereas the D2-class receptors (D2R, D3R, D4R) inhibit ACs through coupling to $G\alpha_i$ and $G\alpha_o$ subunits (Beaulieu & Gainetdinov, 2011; Missale et al., 1998) (Fig. 3). There are some similarities and dissimilarities between D1-class receptors and D2-class receptors. DARs like other GPCRs display an extracellular N-terminal, three extracellular (EL) and three intracellular loops (IL), seven transmembrane regions (TM) and a cytoplasmic tail (CT). Two cysteine residues in EL2 and EL3 of DARs play an important role in stabilizing the receptor structure by forming an intramolecular disulfide bridge (Fig. 4). The D1-class receptors have a shorter IL3 and seven times longer CT in comparison to D2-class receptors. There is 82% amino acid identity between human D1R and D5R in their TM domains. Similarly, D2R shows 75% and 53% identity in the TM domains with D3R and D4R, respectively (Missale et al., 1998). Additionally, D1R-class and D2R-class receptors differ in the post-translational modifications.

The N-terminal of all the DAR subtypes display a similar number of amino acid residues and variable numbers of consensus N-glycosylation sites. Two N-glycosylation sites present in D1-class receptors. However, three, four and one potential glycosylation sites are observed in D2R, D3R and D4R, respectively. DARs can be palmitoylated on cysteine residues located in their CT. The palmitoylated cysteine residues are located near the beginning of the CT in D1-class receptors and near the end of the CT in D2R-class receptors (Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

1.4. Gene Structure of Dopamine Receptors

The D1-class receptors and D2-class receptors mainly differ in the presence or absence of introns in their coding regions. D1R and D5R genes do not contain introns in their coding regions. However, D2R, D3R and D4R genes are interrupted by six, five and three introns, respectively. The presence of introns within the coding region of D2-class receptors provides the basis for the generation of receptor splice variants. Two main variants of D2R, known as D2S (D2-short) and D2L (D2-long) are generated by alternative splicing of an 87-bp exon between introns 4 and 5 of the D2R. These two D2R variants differ in the presence of an additional 29 amino acids in the IL3 (Beaulieu & Gainetdinov, 2011; Missale et al., 1998). At least seven distinct alternatively splicing variants of the D3R have been identified including the full-length D3R, D3S (Fishburn et al., 1993), D3nf (Liu et al., 1994; Schmauss et al., 1993), D3 (TM3-del) (Giros et al., 1991; Snyder et al, 1991), D3 (TM4-del) (Nagai et al., 1993), D3 (O2-del) (Giros et al., 1991), and $rD3^{in}$ (Pagliusi et al., 1993), which will be discussed in more details in D3R section. Researchers also identified several polymorphic variants with a 48-base-pair repeat sequence in the third cytoplasmic loop of D4R containing up to 11 repeats (Beaulieu & Gainetdinov, 2011; Missale et al., 1998).

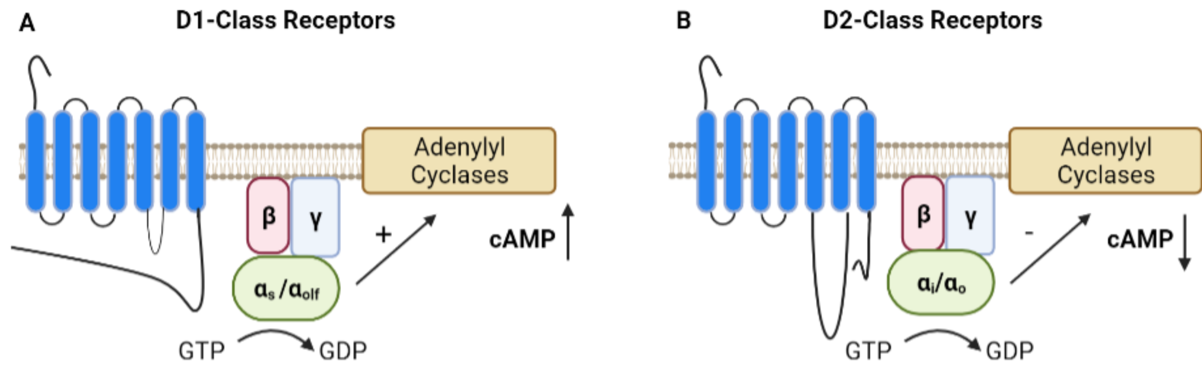

Figure 3. Classification of Dopamine Receptors.
A) Activation of ACs by D1-class receptors (D1R, D5R) through coupling to $G\alpha_s$ and $G\alpha_{olf}$ subunits upon agonist stimulation. B) inhibition of ACs by D2-class receptors (D2R, D3R, D4R) by coupling to $G\alpha_i$ and $G\alpha_o$ subunits after stimulation with agonist. GTP, guanosine triphosphate; GDP, guanosine diphosphate; cAMP, adenosine 3′,5′- cyclic monophosphate. Created with BioRender.com.

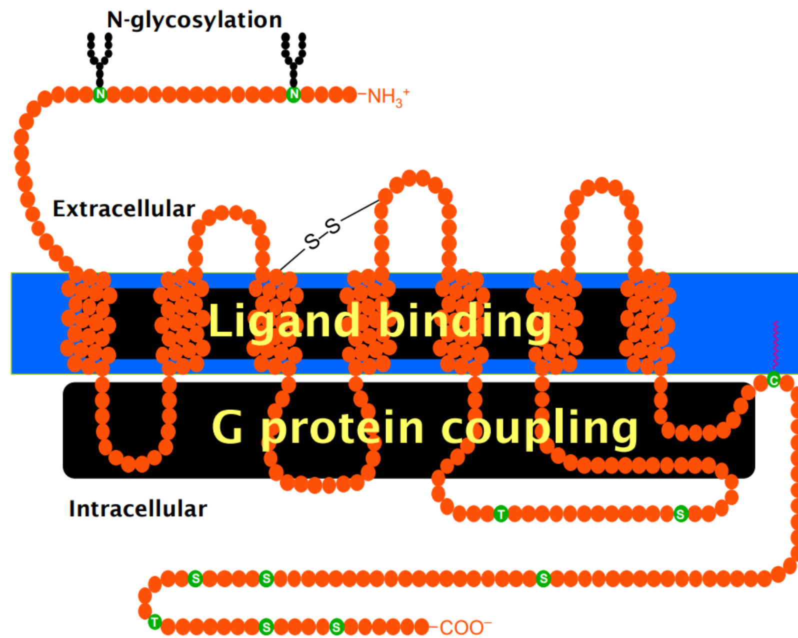

Figure 4. General Structure of GPCRs.
The seven transmembrane domains, intracellular loops (IL), extracellular loops (EL) and cytoplasmic tail (CT) are represented. The extracellular N-terminal of the receptor contains N-glycosylation sites. The CT contains cysteine residues serves as palmitoylation sites. Ligand binding, G protein-coupling domains of GPCRs and disulfide bridge between EL2 and EL3 are also indicated.

1.5. Dopamine D1-Class Receptor Signaling

The D1-class receptors including D1R and D5R are generally coupled to $G\alpha_{s/olf}$ proteins and stimulate the production of cAMP through activation of ACs and activate protein kinase A (PKA). PKA phosphorylates several proteins involved in signal transduction and regulation of gene expression such as DA and cAMP-regulated phosphoprotein, 32kDa (DARPP-32), ion channels and the transcription factor cAMP response element-binding protein (CREB). Notably, phosphorylation of DARPP-32 at Thr34 by PKA leads to inhibition of protein phosphatase 1 (PP1). In contrast, phosphorylation of DARPP-32 at Thr75 by cyclin-dependent kinase 5 (CDK5) prevents the inhibition of PP1 and dampens PKA activity. PKA-DARPP-32 signaling cascade can regulate ion channels and numerous neurotransmitter receptors such as reducing the activity of K^+ channels, Na^+ channels, N and P/Q type Ca^{2+} channels. In contrast, it increases L-type Ca^{2+} channel, N-methyl-D-aspartate receptor (NMDAR), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activities (Beaulieu et al., 2015; Neve et al., 2004). In addition to DARPP-32, CREB is another important PKA substrates which is involved in mediating gene expression and synaptic plasticity. PKA phosphorylates CREB at Ser133, which allows binding of CREB to CREB-binding protein and transcriptional activation of genes with cAMP response elements. Activation of extracellular signal-regulated kinase (ERK) can also increase Ser133-phosphorylated CREB in a PKA independent manner (Neve et al., 2004). Several studies demonstrated the activation of mitogen-activated protein kinases (MAPKs) including ERK, P38 MAP kinase, and c-jun N-terminal kinase (JNK) through D1-class receptors signaling (Neve et al., 2004).

Previous studies suggested that DARs can also regulate phospholipase C (PLC) through coupling to $G\alpha_q$ (Felder et al., 1989; Friedman et al., 1997; Lee et al., 2004; Sahu et al., 2009). Activation of PLC produces inositol trisphosphate (IP3) and diacylglycerol (DAG) through phosphatidylinositol-4,5-biphosphate (PIP2) hydrolysis, which in turn increases the release of

Ca^{2+} through IP3-gated Ca^{2+} channels on the endoplasmic reticulum (ER) and activates protein kinase C (PKC). The high levels of intracellular calcium in the cytoplasm can activate calcium-dependent PKC variants as well as calcium-regulated enzymes, such as the calcium/calmodulin-dependent PK II (CaMKII) and the protein phosphatase 2B (PP2B; also called calcineurin) (Beaulieu et al., 2015). Although several studies have shown the regulation of PKC and intracellular calcium signaling by DA, the molecular mechanism underlying this process is not fully understood. One possible mechanism suggests D5R as the main regulator of PKC activation. However, D1R heterodimerization with other DARs (D2R) also leads to $G\alpha_q$ coupling and PKC activation (Beaulieu et al., 2015; Beaulieu & Gainetdinov, 2011; Neve et al., 2004). Most of the D1-class receptors signaling pathways are summarized in Figure 5.

1.6. Dopamine D2-Class Receptor Signaling

The D2-class receptors (D2R, D3R and D4R) signaling is mediated primarily by coupling to $G\alpha_{i/o}$ proteins to inhibit the cAMP production and decreasing the phosphorylation of PKA substrates. Namely, activation of D2-class receptors decreases PKA-stimulated phosphorylation of DARPP-32 at Thr34, while in contrast it increases phosphorylation at Thr75. In addition to inhibition of ACs, D2-class receptors modulate many other signaling pathways including phospholipases, ion channels, MAPKs, and the Na^+/H^+ exchanger. Some of these regulations are mediated by $G\beta\gamma$ subunits, released from heterotrimeric $G\alpha_{i/o}$ proteins. Namely, D2-class receptors increase K^+ currents, stimulate a G protein-regulated inwardly rectifying K^+ channel (GIRK or Kir3), reduce the activity of L-, N-, P/Q-type Ca^{2+} channels, γ-aminobutyric acid type A receptor ($GABA_AR$) and NMDAR, and stimulate MAPKs. Interestingly, D2-class receptors stimulation could either increase or decrease Na^+ currents depending on the subtypes of D2-class receptors that are expressed by given cells. $G\beta\gamma$ subunits stimulate PLC activity and production of IP3 and DAG through PIP2 hydrolysis. DAG leads to activation of PKC, while IP3 increases the concentration of intracellular calcium. The

enhancement of intracellular calcium activates CaMKII and PP2B resulting in DARPP-32 inhibition and subsequently disinhibition of PP1. Although D2-class receptors inhibit PKA activity, activation of CREB can occur via PP2B, PKC and CaMKII (Beaulieu et al., 2015; Beaulieu & Gainetdinov, 2011; Ginovart & Kapur, 2012; Kingsbury et al., 2007; Neve et al., 2004; Yan et al., 1997). It is worth mentioning that Gβγ subunits display a permissive effect on the AC isoforms AC2, AC4 and AC7, so that the stimulatory effect of other activators, including Gαs and PKC, is enhanced in the presence of free Gβγ (Cooper & Crossthwaite, 2006; Neve et al., 2004). In addition, D2R and D4R can potentiate arachidonic acid release by cytosolic phospholipase A2 (PLA2) activation through Gβγ subunits. Arachidonic acid and its bioactive metabolites are associated with numerous cellular functions including feedback regulation of D2-class receptors signaling and DAT activity (Ginovart & Kapur, 2012; Neve et al., 2004). Some of the D2-class receptors signaling pathways are summarized in Figure 6. In the following section, I will discuss the mechanisms regulating GPCR signaling.

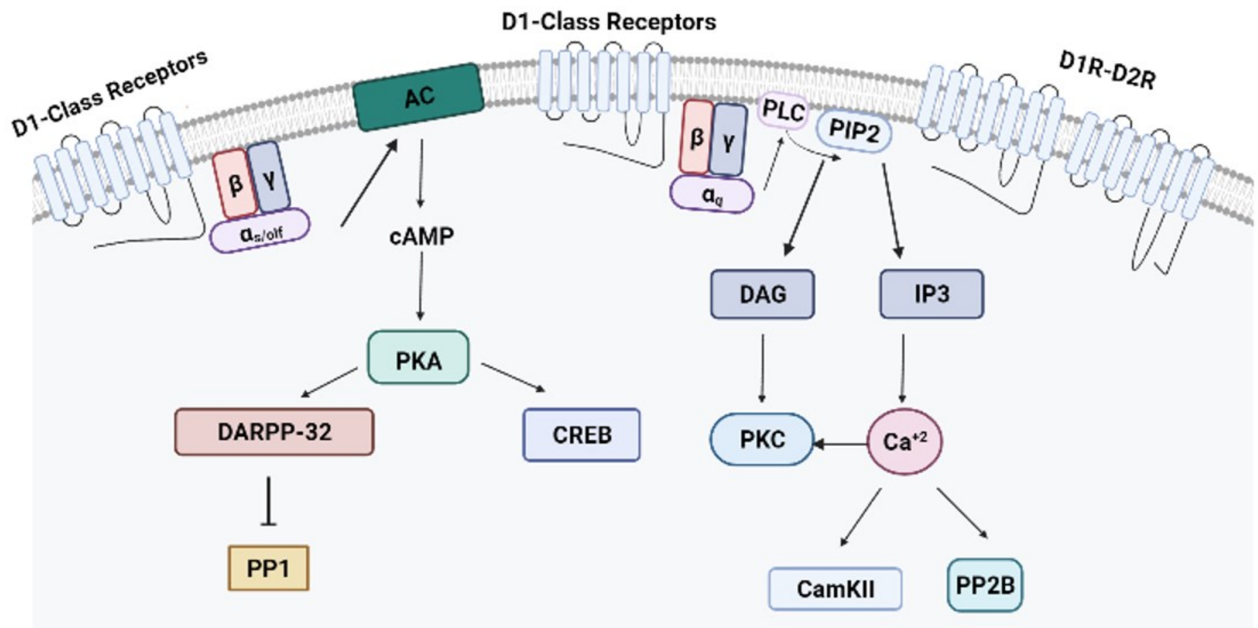

Figure 5. Schematic Representation of the Signalling Cascades Activated by D1-Class Dopamine Receptors.

Upon ligand binding, $G\alpha_{s/olf}$-coupled receptors activate ACs, which increases cAMP production and activates PKA. PKA can activate PKA substrates including CREB and DARPP-32. On the other hand, D1R/D2R heterodimerization regulates the coupling of D1R to $G\alpha_q$ which stimulates PLC. PLC catalyzes the hydrolysis of PIP2 into DAG and IP3. IP3 increases the release of Ca^{2+} through IP3-gated Ca^{2+} channels on the ER. Ca^{2+} and DAG then activate the PKC. The released Ca^{2+} in the cytoplasm can activate CaMKII and PP2B. D1R, D1 dopamine receptor; D1R-D2R, D1-D2 receptor heterodimer; cAMP, adenosine 3′,5′- cyclic monophosphate; AC; adenylyl cyclase; CREB, cAMP response element-binding protein; DARPP-32, dopamine and cAMP-regulated phosphoprotein, 32kDa; PIP2, phosphatidylinositol-4,5-biphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP1, protein phosphatase 1; CaMKII, calcium/calmodulin-dependent PK II; PP2B, protein phosphatase 2B. Created with BioRender.com.

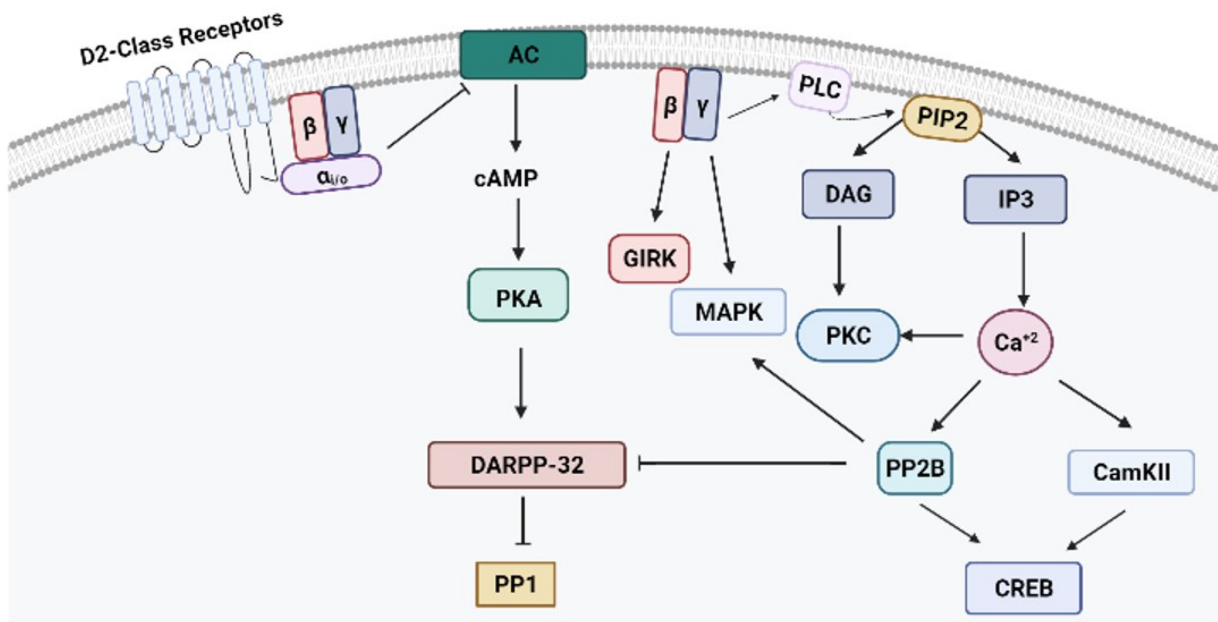

Figure 6. Schematic Representation of the Signalling Cascades Activated by D2-Class Dopamine Receptors.

D2-class receptors signaling pathways are mediated by $G\alpha_{i/o}$ and Gβγ subunits. $G\alpha_{i/o}$ exhibits an inhibitory effect on ACs activity. Reduction of ACs activity leads to inhibition of DARPP-32 and disinhibition of PP1. On the other hand, Gβγ subunits stimulate PLC activity leading to mobilization of intracellular calcium and stimulation of PKC. The enhancement in calcium concentration also activates CAMKII and PP2B (calcineurin) that both increase CREB activity. PP2B activation leads to inhibition of DARPP-32. MAP kinase activation also occurs via Gβγ subunits. cAMP, adenosine 3′,5′- cyclic monophosphate; AC; adenylyl cyclase; CREB, cAMP response element-binding protein; DARPP-32, dopamine, and cAMP-regulated phosphoprotein, 32kDa; PIP2, phosphatidylinositol-4,5-biphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol; GIRK, G protein-regulated inwardly rectifying K^+ channel; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP1, protein phosphatase 1; CaMKII, calcium/calmodulin-dependent PK II; PP2B, protein phosphatase 2B. Created with BioRender.com.

2. Regulation of GPCR Signaling

GPCRs activity can be attenuated at the level of the heterotrimeric G proteins. The role of regulators of G protein signaling (RGS) has been established in modulation of GPCRs signaling pathways. Specifically, RGS binds to activated Gα subunits in G proteins, accelerates the GTP hydrolysis, and thereby rapidly dampens GPCR signaling. On the other hand, most GPCRs undergo three temporally distinct regulatory processes: desensitization, internalization, and downregulation/recycling. Desensitization is defined as attenuation of responsiveness to agonists following prolonged or repeated exposure to an agonist. Internalization or endocytosis is a rapid movement of the phosphorylated GPCR from plasma membrane to intracellular compartments following by receptor stimulation with an agonist. Thus, receptors are sequestered to a compartment where they are unable to interact with hydrophilic ligands in the extracellular environment. Subsequently, the internalized receptors can either be recycled back to the plasma membranes (resensitization) or be degraded (downregulation) through lysosomal or proteasomal pathways. Moreover, endocytosis/recycling might not be mandatory for the resensitization of receptor responsiveness, because receptors can be rapidly dephosphorylated on the cell surface without requiring endocytosis and recycling (Ferguson, 2001; Gainetdinov et al., 2004).

2.1. GPCR Desensitization

The desensitization of GPCRs is generally accomplished through two distinct ways: the heterologous and homologous pathways. In heterologous desensitization, the second messenger dependent kinases (PKA and PKC) phosphorylate not only agonist activated GPCRs, but also those that have not been exposed to the agonist. PKA and PKC phosphorylate the receptor on its IL3 and CT, which directly impairs the coupling of G protein to the receptor (Ferguson, 2001; Kohout & Lefkowitz, 2003; Zhang & Kim, 2017). In contrast, in the

homologous pathway, GPCR kinases (GRKs) (GRK1-7) phosphorylate agonist-bound GPCRs on serine and threonine residues in IL3 and C-terminal domain. The selective phosphorylation of agonist-activated receptors by GRK leads to uncoupling of GPCR from its G protein, and increases the binding of β-arrestins, which connect to adaptors such as adaptor protein (AP)-2 and clathrin. These cellular processes are hallmarks of homologous regulation of receptor responsiveness. Following β-arrestin recruitment, GPCR is internalized through clathrin-coated pits (CCPs) in a process requiring dynamin and β2-adaptin. Some of these receptors can be rapidly resensitized through dephosphorylation, while some of them are downregulated (Kohout & Lefkowitz, 2003; Moore et al., 2007; Zhang et al., 1996; Zhang & Kim, 2017). In addition to clathrin-mediated endocytosis, two different clathrin-independent endocytosis pathways have been established for GPCRs. The first one is caveolae-dependent pathway which contains caveolin-1 as a main component along with cavin, and pacsin (also named syndapin) proteins. Caveolae endocytosis is sensitive to cholesterol depletion. The second clathrin-independent pathway is called **f**ast **e**ndophilin-**m**ediated **e**ndocytosis (FEME). Endophilin has been identified to associate with clathrin-mediated endocytosis (Milosevic et al., 2011; Sundborger et al., 2011), but Boucrot and colleagues suggested endophilin as a critical component of a novel rapid, clathrin-independent internalization pathway. FEME endocytosis pathway is independent of AP-2 and clathrin, but dependent on dynamin, cholesterol, actin, Rac, Rho GTPase, phosphatidylinositol 3-kinase (PI3K) and the serine/threonine protein kinase (PAK1) (Boucrot et al., 2015; Moo et al., 2021). The heterologous and homologous desensitization are summarized in Figure 7.

In contrast to these biochemical pathways, some GPCRs can be desensitized in a phosphorylation-independent manner, such as Leukotriene B4 (LTB4) (Jala et al., 2005) and the corticotropin releasing factor receptor 1 (CRFR1) (Rasmussen et al., 2004). Indeed, some GPCRs employ a specific sequestration pathway called pharmacological sequestration.

Pharmacological sequestration defined as conformation changes in GPCRs without movement to intracellular regions which renders receptors inaccessible to hydrophilic ligands (Fig. 8) (Min et al., 2013; Mostafapour et al., 1996). Overall, studies suggest that one kind of GPCR can employ more than one intracellular trafficking pathway. Some GPCRs can rapidly attenuated on or near the plasma membrane without a long distance migration to the cytosol (Roettger et al., 1995) or as a result of conformational changes (Mostafapour et al., 1996), however some GPCRs undergoes slow but carefully regulated internalization and resensitization (Min et al., 2013).

2.2. Functional Crosstalk Between Homologous and Heterologous Desensitization

Previous studies have suggested the functional interactions between homologous and heterologous pathways. The interaction between two pathways can occur between the major players of each endocytic pathway (PKA/PKC, GRK2/β-arrestins) or through manipulation of them. Some studies reported that GRK2 and β-arrestins are involved in PKA/PKC-mediated regulatory pathways. For instance, in $G\alpha_s$-coupled receptor signaling, β-arrestins promote the recruitment of cAMP phosphodiesterases to ligand-activated receptors which leads to cAMP degradation and subsequently PKA inhibition. In $G\alpha_q$-coupled receptors, β-arrestins can activate DAG kinase and convert DAG to phosphatidic acid (PA) resulting in inhibition of PKC-mediated regulatory pathways. It has also been shown that activated GRK2 can bind to PKCβ and inhibit its activity. On the other hand, some studies have demonstrated the contribution of PKA and PKC in homologous desensitization (Zhang & Kim, 2017). For instance, the role of PKA and PKC in GRK2 activation was demonstrated by facilitating its translocation to the plasma membrane (Cong et al., 2001; Winstel et al., 1996). Furthermore, it has been reported that inhibition of β-arrestin 2 ubiquitination by PKCβII subsequently inhibits homologous desensitization (Zheng et al., 2015).

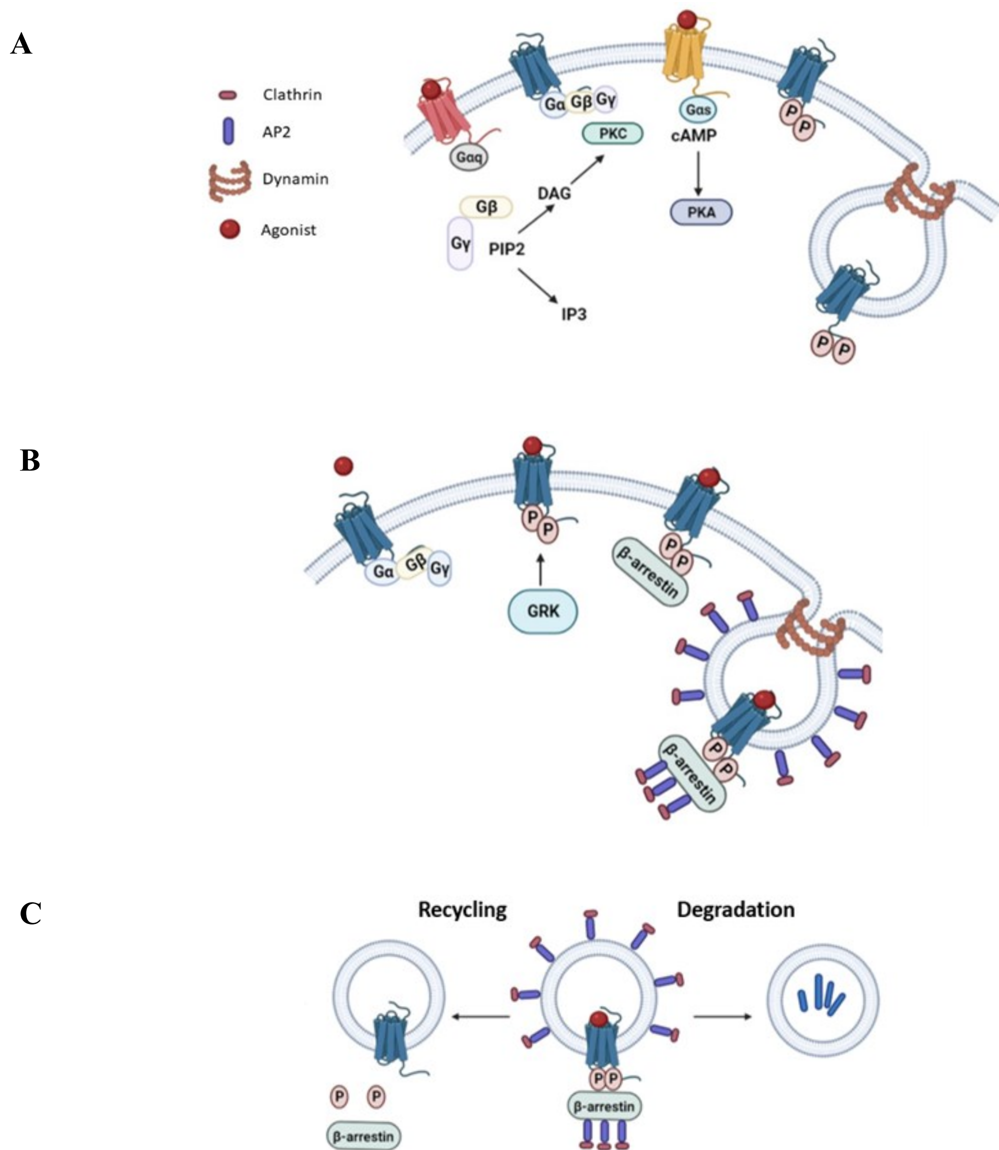

Figure 7. Schematic Representation of GPCR Desensitization.

A) Heterologous desensitization. GPCR uncoupling from G-proteins occurs upon phosphorylation of the receptor by second messenger kinases PKA and PKC**.** Activation of other GPCRs could be important to produce PKA or PKC in this pathway. B) Homologous desensitization. GRKs phosphorylate the agonist activated GPCR on intracellular domains, resulting in arrestin recruitment. Arrestin binding to the receptor inhibits G proteins coupling and leads to desensitization and internalization of the receptor. Receptor/arrestin complex is targeted to clathrin-coated pits, resulting in receptor internalization. C) Internalized GPCR is sorted to either degradation or recycling compartments. PKA, protein kinase A; PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol trisphosphate; PIP2, phosphatidylinositol-4,5-biphosphate; GRK, GPCR kinase. Created with BioRender.com.

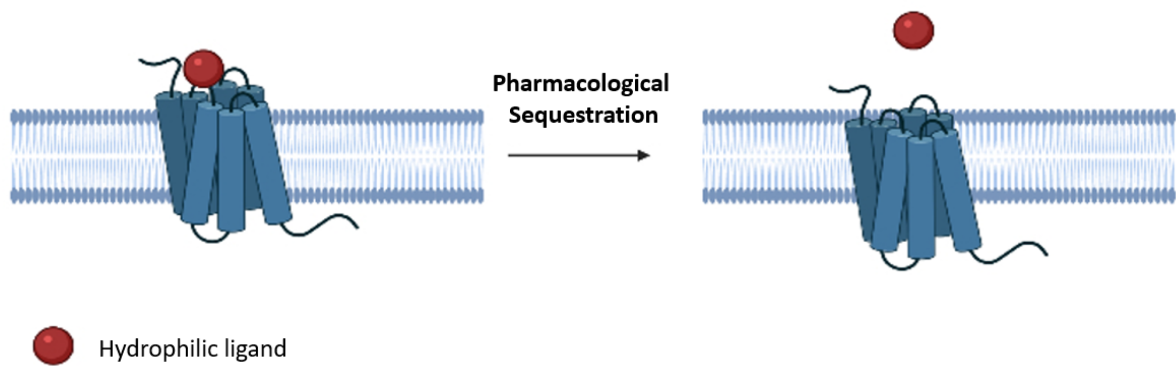

Figure 8. Diagram Showing the Pharmacological Sequestration of GPCRs.
GPCR undergoes conformational changes and translocates toward more hydrophobic regions within the plasma membrane. Although pharmacologically sequestered receptor does not internalize into cytosol, but GPCR is not accessible to hydrophilic ligands, because it is located within more hydrophobic regions of the plasma membrane. Created with BioRender.com.

2.3. Roles of Post-Translational Modifications in GPCR Internalization

Phosphorylation, ubiquitination, glycosylation, and palmitoylation are post-translational modifications occurring in GPCRs. Among all post-translational modifications, the role of receptor phosphorylation in endocytosis has been well established. Several studies investigated the role of ubiquitination, which adds a small regulatory protein called ubiquitin to a substrate protein, in the regulation of mammalian GPCR trafficking. It has been suggested that ubiquitination plays an important role in post-endocytic lysosomal sorting rather than in endocytosis of some GPCRs (Zhang & Kim, 2017).

N-linked glycosylation is the other post-translational modifications that occurs on the Asn-X-Ser/Thr motif on GPCR extracellular domains. It has been shown that N-linked glycosylation on the N-terminus of D2R, D3R, protease-activated receptor type 1 (PAR1) and prostacyclin receptor plays a role in receptor internalization. In addition, N-linked glycosylation is involved in proper cell surface expression, desensitization, and internalization of D2R and D3R. Interestingly, disruption of N-terminus glycosylation either represses or facilitates endocytosis of the D2R and D3R, respectively (Min et al., 2015; Zhang & Kim, 2017).

Palmitoylation requires a characteristic Cys-rich Asp-His-His-Cys domain to add a palmitoyl group on Cys residues of membrane proteins or less frequently on Ser and Thr residues. Palmitoylation exerts different effects on GPCRs endocytosis. Palmitoylation is essential for internalization of some GPCRs such as thyrotropin-releasing hormone receptor, D3R, somatostatin receptor 5 (SSTR5), CB1 cannabinoid receptor, and protease-activated receptor (PAR2). On the other hand, palmitoylation has minimal or no effect on endocytosis of some GPCRs including β_2-adrenergic receptor (β_2AR), α_1-adrenergic receptor (α_1AR), and C-C chemokine receptor type 5. Interestingly, palmitoylation shows inhibitory effect on the endocytosis of luteinizing hormone/human choriogonadotropin receptor and V1A vasopressin

receptor (Zhang & Kim, 2017). It has been shown that agonist affinity, cell surface expression, phorbol-12-myristate-13-acetate (PMA)-induced internalization, and agonist-induced desensitization of D3R is significantly inhibited after treatment with 2-bromopalmitate (2BP), a palmitoylation blocker, or when the potential palmitoylation site was mutated (C400G). In contrast, neither the point mutation of the potential palmitoylation site nor 2BP treatment affect cell surface expression and DA induced internalization of D2R (Zhang et al., 2016).

Overall, these data suggested that changes in the cellular environment and protein-protein interactions that influence posttranslational modifications can play important roles in intracellular GPCR trafficking. For example Gα interacting protein (GAIP) C terminus 1 (GIPC1) decreases D3R palmitoylation levels through interacting with D3R and subsequently diminishes the localization of D3R at the plasma membrane (Arango-Lievano et al., 2016).

2.4. Arrestin Classification

Arrestins family contains two major domains (N and C) which consists large β-sheets and connecting loops. The four identified arrestins can be divided into two groups based on sequence homology, function, and tissue distribution. The first group includes visual arrestin (known as arrestin-1, S-antigen) and cone arrestin (known as arrestin-4, X-arrestin or C-arrestin). The second group includes β-arrestins (β-arrestin 1 and β-arrestin 2, also named arrestin 2 and arrestin 3, respectively). The visual arrestin is localized primarily in the retina and at lower levels in the pineal gland. The cone arrestin is localized primarily in cone photoreceptors in retina as well as in pineal gland. β-arrestins are ubiquitously expressed in all tissues, but are highly expressed in brain and spleen (Ferguson, 2001). The main features of β-arrestins especially β-arrestin 2 are discussed further in the following sections.

2.4.1. β-arrestin Functions

The role of β-arrestins in terminating GPCR signaling is well established. In addition to GPCR desensitization and internalization, β-arrestins have been identified as multifunctional adapter proteins that regulate a variety of cellular functions either independent or in concert with G-proteins. Besides clathrin and AP-2, β-arrestins scaffold different proteins involved in gene expression, receptor transactivation, receptor trafficking and transcriptional regulation. Namely, β-arrestins scaffold components of MAPKs such as the ERK1/2 and JNK3, Src family tyrosine kinases, the Ser/Thr protein phosphatase 2A (PP2A), E3 ubiquitin ligases and deubiquitinases, cyclic nucleotide phosphodiesterases (PDEs), diacylglycerol kinases (DGKs), elements of the nuclear factor kB (NFkB) signaling pathway and regulators of small GTPase activity (Gurevich & Gurevich, 2019; Moo et al., 2021; Moore et al., 2007; Peterson & Luttrell, 2017; Smith & Rajagopal, 2016; Jean-Charles et al., 2017). β-arrestins regulate the transactivation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), insulin-like growth factor type 1 receptor (IGF-1R) and vascular endothelial growth factor receptor (VEGFR) via GPCRs dependant mechanisms (Jean-Charles et al., 2017). Namely, β-arrestins can regulate the transactivation of EGFR in a GPCR dependant manner though activation of a transmembrane matrix metalloprotease that cleaves membrane-bound EGF ligand (Smith & Rajagopal, 2016).

Despite 78% amino acid sequence homology between β-arrestin 1 and β-arrestin 2, important differences between two isoforms are observed (Smith & Rajagopal, 2016). One important difference between β-arrestin 1 and β-arrestin 2 is in the nucleocytoplasmic shuttling. Although both isoforms have nuclear localization sequences at the N- terminus, only β-arrestin 2 additionally has a C-terminal nuclear export sequence. Consequently, in inactive state, β-arrestin 1 can be found both in the nucleus and cytoplasm, whereas β-arrestin 2 is in the

cytoplasm. However, upon activation, both β-arrestin isoforms are transported either to the nucleus or plasma membrane (Markova et al., 2021).

In addition, the role of β-arrestins as a scaffold protein in signaling pathways depends on cell type and receptor (Gurevich & Gurevich, 2019; Markova et al., 2021; Smith & Rajagopal, 2016). Interestingly, β-arrestin 1 and β-arrestin 2 may show opposing effects on activation or inhibition of a specific signaling pathways in the process known as reciprocal regulation. Reciprocal regulation is observed in angiotensin II type 1A (AT_{1A}), where knockdown of β-arrestin 2 and β-arrestin 1 attenuates and potentiates ERK signaling, respectively (Smith & Rajagopal, 2016). Another example is with the metabotropic glutamate 7 (mGlu7) receptor, for which β-arrestin 2 enables JNK activation and inhibits ERK, whereas β-arrestin 1 activates ERK while inhibiting JNK (Jean-Charles et al., 2017).

2.4.2. β-arrestin Interactions

Two different interactions within β-arrestin and phosphorylated GPCRs are observed: one involves phosphorylated residues on C-terminal tail of the GPCRs (RP-tail), called 'tail' interaction, and one involves the transmembrane core of GPCRs, called 'core' interaction. These interactions are mediated by two distinct sites on arrestin called phosphorylation sensor and activation sensor, respectively (Kahsai et al., 2018). Different GPCRs show different phosphorylation patterns leading to various arrestin-mediated signaling effects (Latorraca et al., 2020; Smith & Rajagopal, 2016). GPCRs which are internalized through the clathrin-dependent endocytic pathway are divided into two groups, Class A and Class B receptors. Class A receptors including the β2AR, μ-opioid receptor (MOR), endothelin type A (ETA) receptor, D1R, and α_{1B} adrenergic receptor show higher binding affinity for β-arrestin 2 than β-arrestin 1. On the other hand, class B receptors such as AT_{1A}, vasopressin V2, neurotensin 1 (NT1), thyrotropin-releasing hormone (TRH), and substance-P/neurokinin-1 (SP/NK1) receptors

show equal affinity for β-arrestin 2 and β-arrestin 1. It has been shown that β-arrestin dissociates from the class A receptors at or near the plasma membrane. In contrast, it maintains a stable association with class B receptors and colocalizes in endosomes (Moore et al., 2007). The transient or stable interaction between β-arrestins and GPCRs is correlated with rates of resensitization meaning that class A receptors resensitize more rapidly than class B receptors. The mechanism underlying the different interactions between β-arrestins, and class A or B receptors is still unclear. Although both β1AR and β2AR form transient complexes with β-arrestin 2 and trigger equal β-arrestin 2 recruitments to CCPs, only β2AR internalizes in CCPs in a complex with β-arrestin. Interestingly, β1AR does not internalize with β-arrestin but it can still traffic to CCPs and mediate ERK1/2 phosphorylation, which persists in endosomes (Jean-Charles et al., 2017).

A recent study showed that GPCRs promote a direct interaction between $G\alpha_i$ and β-arrestins, regardless of their canonical $G\alpha_i$ subtype coupling. The tripartite complex between GPCR, $G\alpha_i$ and β-arrestin also bind to ERK and their disruption impaired ERK phosphorylation and cell migration. Different GPCR ligands could induce complex formation between $G\alpha_i$ and β-arrestin. The $G\alpha_i$•β-arrestin complex scaffolds a GPCR, or a signaling effector (ERK), or possibly both, and may form functional signaling complexes (Smith et al., 2021). On the other hand, data obtained from GST pull-down assay showed the involvement of N-terminus of β-arrestin 2 in the interaction with Gβγ. These results showed the important roles of β-arrestin in the modulation of signaling pathways involving Gβγ (Min et al., 2013).

3. D3R and AC5

Previous studies on D2-class receptors, suggest that D2R and D4R inhibit more strongly AC activity in different cell types, whereas D3R shows subtle inhibition or no effect on this second messenger system. However, it has been shown that activation of D3R by quinpirole can

selectively inhibit forskolin (FSK)-stimulated AC5 activity in human embryonic kidney 293 (HEK293) cells co-transfected with D3R and AC5. This effect is limited to AC5, because D3R could not inhibit other AC isoforms, including AC6 which is related to AC5 (Robinson & Caron, 1997). Furthermore, another study has demonstrated that D3R could inhibit the formation of intracellular cAMP in SH-SY5Y cells, which express AC5 endogenously (Zaworski et al., 1999). On the other hand, northern blots and in-situ hybridization studies in rats have shown that AC5 is expressed in the striatum, nucleus accumbens and olfactory tubercle and at lower levels in islands of Calleja, where the D3R is also expressed (Glatt & Snyder, 1993; Mons & Cooper, 1994).

Considering the co-expression of D3R and AC5 in brain regions which modulate normal and pathological motor function, and the selective inhibition of AC5 activity by D3R, therefore, AC5 may be intimately linked to the regulation of D3R activity. During my master's project, I have assessed the D3R and AC5 interaction, and the role this interaction may play in D3R signaling and trafficking. In the following sections, the main functional properties of D3R and AC5 will be described in greater detail.

3.1. Dopamine D3 Receptor

The rat D3R was first cloned and characterized by Sokoloff and colleagues (Sokoloff et al., 1990). The D3R as other GPCRs shows an extracellular N-terminal, three ELs and three ILs, seven TM regions and a C-tail. The D3R gene is located on chromosome 3 in the q13.3 band (Jeanneteau et al., 2006). As mentioned earlier, different alternatively splicing variants of the D3R have been identified including the full-length D3R, D3S (Fishburn et al., 1993), D3nf (K. Liu et al., 1994; Schmauss et al., 1993), D3 (TM3-del) (Giros et al., 1991; Snyder et al, 1991), D3 (TM4-del) (Nagai et al., 1993), D3 (O2-del) (Giros et al., 1991), and rD3^{in} (Pagliusi et al., 1993). Although D3R and D3S are able to bind DA with high affinity, the five additional D3R

variants including D3nf do not bind DA with high affinity, and are believed to regulate receptor dimerization (Fishburn et al., 1993; Prieto, 2017; Pritchard et al., 2006; Richtand, 2006).

D3S lacks 63 bp nucleotides, which encode 21 amino acids in the putative third cytoplasmic loop of the D3R. D3S has been identified in mouse brain and is able to bind dopaminergic ligands (Fishburn et al., 1993). D3 (TM3-del), another D3R splice variant has been identified in rat and human brain, which contains a 113 bp deletion in the first extracellular loop and TM3 (Giros et al., 1991; Snyder et al, 1991). D3 (TM4-del) has been identified in human and has a 143 bp deletion mainly encompassing the TM4. Thus, D3 (TM4-del) codes 138 amino acids protein containing the first three transmembrane domains (Nagai et al., 1993). D3 (O2-del) has been identified in rat and has a 54 bp deletion which corresponds to the ten last amino acids of the second extracellular loop (O2) and the eight first amino acids of TM5 (Giros et al., 1991). In addition to mentioned splice variants, a longer form of D3R ($rD3^{in}$) also has been identified in rat. $rD3^{in}$ contains an insertion of 84 bp resulting in a 28 amino acids insertion in the first extracellular loop (Pagliusi et al., 1993). The D3nf isoform is one of the best characterized D3R splice variants and its expression detected in post-mortem cortical tissues from schizophrenic patients. A deletion of 98 bp nucleotides that code for the carboxyl-terminal region of the third cytoplasmic loop of the receptor encodes D3nf protein lacking TM6 and 7 (Liu et al., 1994; Schmauss et al., 1993). D3nf mRNA has been shown to be as abundant as D3R mRNA and is expressed in same regions of human brain (Liu et al., 1994). Interestingly, D3R mRNA expression is decreased in the cortex of patients with chronic schizophrenia (Schmauss et al., 1993), whereas increased D3nf splicing efficiency was observed in the cortex of post-mortem tissue from schizophrenic patients (Schmauss, 1996). D3nf mRNA and protein are expressed in rat, monkey, and human brain (Liu et al., 1994; Nimchinsky et al., 1997). Previous studies have shown that D3nf may regulate D3R localization and function through dimerization (Elmhurst et al, 2000; Karpa et al., 2000; Nimchinsky et al., 1997). On the other hand, D3nf

interacts with D3R and decreases the capacity of D3R to bind ligand (Elmhurst et al., 2000). This interaction also appears to result in the mislocalization of D3R from the plasma membrane to an intracellular compartment (Karpa et al., 2000).

In addition, Lannfelt et al. first described a single nucleotide polymorphism (SNP) in the first exon of the D3R corresponding to a serine to glycine substitution at position 9 in the extracellular N-terminal domain of D3R (i.e., Ser9Gly; rs6280) (Fig. 9) (Lannfelt et al., 1992). The DNA alteration creates a BalI (MscI) restriction enzyme site, which can be used to detect this genetic polymorphism (Sivagnanasundaram et al., 2000; Steen et al., 1997). The frequency of the Ser9/Ser9, Ser9/Gly9 and Gly9/Gly9 genotypes among healthy Swedish people were 51%, 41.5% and 7.5%, respectively (Lannfelt et al., 1992). Nevertheless, the allele frequencies could vary among ethnicities (Li et al., 2020; Qi et al., 2017).

Few studies have investigated the effect of Ser9Gly polymorphic variant on ligand binding affinity, signal transduction pathways including MAPK activation, prostaglandin E2 (PGE2) and FSK-stimulated cAMP signaling pathways.

D3Ser9 and D3Gly9 showed similar pharmacological properties for 7-hydroxydipropylaminotetralin (7-OH-DPAT), chlorpromazine, GR103691, GR218231, haloperidol, raclopride except for DA and the D3R selective ligand GR99841 using transfected chinese hamster ovary (CHO) cells. Gly9/Gly9 homozygote displayed significantly higher DA binding affinity, although the Ser9/Gly9 heterozygote binding did not differ from the Ser9/Ser9 homozygote. Moreover, the D3-selective ligand GR99841 displayed a significantly increased binding affinity for both the Ser9/Gly9 heterozygote and Gly9/Gly9 homozygote compared to the Ser9/Ser9 (Lundstrom & Turpin, 1996). Hellstrand et al demonstrated that D3R polymorphic variants show same binding affinity for [^{3}H]-spiperone in CHO transfected cells. They reported that Gly9 allele induces a marked shift in intracellular transduction mechanisms.

In their study, D3Ser9 more robustly mediated DA-induced inhibition of FSK-stimulated cAMP production than D3Gly9 in transfected CHO cells in a pertussis toxin (PTX) sensitive manner. In contrast, the D3Gly9, but not D3Ser9, mediated an inhibitory effect of DA on PGE2-induced cAMP production which was not sensitive to PTX (Hellstrand et al., 2004). The molecular mechanisms underlying Ser9Gly switches in the inhibition of FSK and PGE2-induced cAMP pathways remains unclear. Jeanneteau and colleagues investigated the signaling properties of D3R polymorphic variants in transfected HEK293 cells. Interestingly, same levels of glycosylation and anterograde trafficking in both D3R variants were observed. On the other hand, their findings indicated a stronger DA-induced cAMP inhibition by Gly9 than Ser9 variant. Indeed, although both D3R variants showed same levels of DA-induced activation of MAPK, the MAPK signaling mediated by the Gly9 variant was prolonged in comparison to that of the Ser9 variant (Jeanneteau et al., 2006). Different results observed for D3R polymorphic variants mediated cAMP accumulation in these two studies can be explained by using two different cell lines as well as FSK-stimulated endogenous ACs activity (in transfected CHO cells) versus overexpression of AC5 (in transfected HEK293 cells) to assess DA-induced inhibition of cAMP production.

Although D3R and D2R are the main targets of most currently used neuroleptics and share considerable structural homology, they show some differences in brain distribution, pharmacological features, trafficking and signaling pathways. Compared to the D2R, the D3R distribution is more restricted in the brain (Ahlgren-Beckendorf & Levant, 2004). The D3R shows much higher agonists affinity (between 20 to 100-fold) than D2R, particularly for DA, quinpirole, and 7-OH-DPAT. In contrast, lower affinity for some antagonists including spiperone, domperidone, and haloperidol (between 10 to 30-fold) is observed for D3R compared to D2R in HEK293 cells (Robinson et al., 1994).

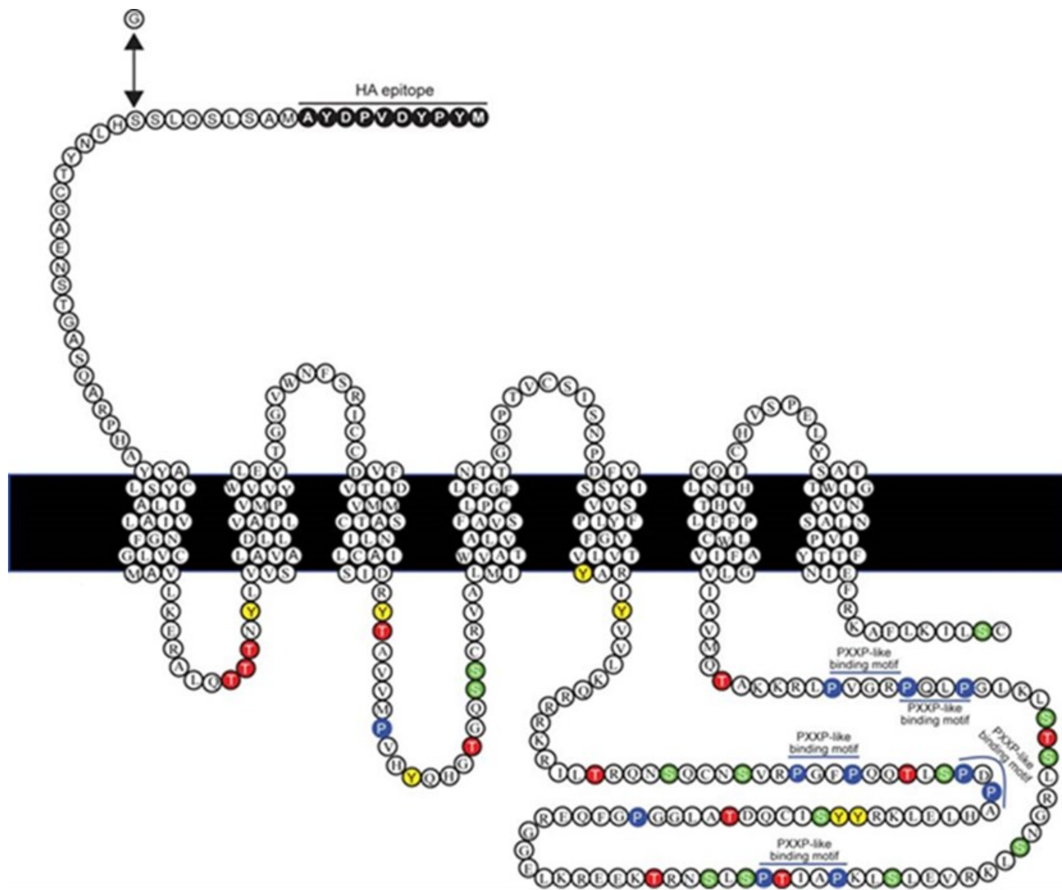

Figure 9. Schematic Representation of the Secondary Structure of the HA-tagged hD3R. Black circles represent amino acid sequence of HA epitope. The double arrowhead indicates a serine to glycine substitution at position 9 of N-terminal domain of D3R. Green and red circles represent potential serine and threonine phosphorylation sites. Potential tyrosine phosphorylation sites are indicated using yellow circles.

3.2. D3R Desensitization and Internalization

In most of the literature reviews discussed here, it is not clear which D3R polymorphic variants has been used except for Cho et al. (2007) and Min et al. (2013), which they used D3Ser9 polymorphic variant. As mentioned above, D2R and D3R show differences in several signaling aspects including desensitization and internalization. Internalization of human D2R occurs through CCPs which involves GRKs, β-arrestin and dynamin molecules. In contrast, only a small fraction of human D3R (~10%) is internalized after DA stimulation via CCPs in the presence of overexpressed GRKs or β-arrestin 2. Data obtained from chimeric D2/D3 receptors demonstrate the role of IL2 and IL3 of D2R in β-arrestin 2 translocation and sequestration of the receptor (Kim et al., 2001). Therefore, only a negligible fraction of D3R is internalized through homologous internalization (agonist-specific) and that a significant fraction of D3R undergoes heterologous desensitization and internalization (second messenger-dependent kinase-specific). Homologous and heterologous desensitization of human D2R and D3R, respectively, have been confirmed in different cell types including two neural-derived cells, Neuro2a and NG108–15 as well as in HEK293 and CHO cells. However, heterologous internalization of D3R was not observed in SH-SY5Y cells (Cho et al., 2007; Xu et al., 2019). Cells treatment with PMA (a PKC activator), induced D3R phosphorylation and desensitization (Cho et al., 2007; Thompson & Whistler, 2011; Zhang et al., 2016). However FSK (a PKA activator) treatment did not affect D3R phosphorylation and desensitization showing the specific involvement of PKC in D3R regulation and desensitization (Cho et al., 2007). Results obtained from treatment with specific inhibitors of different PKC isoforms or co-expression of active forms of PKC demonstrated that PKC-βII and PKC-δ but not PKC-α or PKC-ζ significantly increase PMA-induced internalization of D3R. In addition, co-immunoprecipitation (co-IP) results suggest a stronger interaction between human D3R and PKC-βII compared to D3R and PKC-δ. Site-directed mutagenesis revealed serine residues at

positions 229 and 257 on the IL3 of D3R as putative phosphorylation sites in PMA-induced D3R phosphorylation, desensitization, internalization, and degradation (Cho et al., 2007; Zhang et al., 2016). PKC-mediated heterologous internalization of D3R is dynamin dependent but GRK, β-arrestin and caveolin-1 independent (Cho et al., 2007; Xu et al., 2019). Studies using CHC (clathrin heavy chain) knockdown and treatment with methyl-β-cyclodextrin (MβCD) as a caveolar pathway inhibitor showed that PKC-mediated internalization of D3R selectively occurs through clathrin-mediated pathway (Xu et al., 2019; Zhang et al., 2016). It has been also suggested that interaction between D3R and filamin is critical for PMA-induced D3R internalization (Cho et al., 2007). In addition, internalized D3R is not able to recycle back to the plasma membrane and instead undergoes degradation after PMA-induced internalization. Down-regulation of the D3R appears to be mediated, at least in part, by an interaction between the GPCR-associated sorting protein-1 (GASP-1) and C-terminal domain of D3R, because knockdown of GASP-1 or disruption of GASP-1 binding through receptor mutation inhibited D3R down-regulation (Thompson & Whistler, 2011).

As mentioned earlier, proteasomal and lysosomal pathways are two main degradation pathways for endocytosed proteins. It has been reported that phosphorylation of D3R by PKC is required for D3R ubiquitination and degradation through lysosomal pathway. Overall, PKC-mediated phosphorylation of D3R is involved in clathrin-mediated internalization, which is critical for the lysosomal degradation of D3R (Zhang et al., 2016).

However, none of the commonly known D3R agonists promotes significant D3R internalization. Nonetheless, agonist stimulation of human D3R leads to pharmacological sequestration, a process defined as the sequestration of cell surface receptors into a more hydrophobic fraction within the plasma membrane without undergoing into intracellular regions. Pharmacological sequestration renders D3R inaccessible to hydrophilic ligands (Min et al., 2013). Pharmacological sequestration is closely related to desensitization of D3R in a

Gβγ- and β-arrestin 2 dependent process, but GRK2, GRK-interacting protein (GIT1), β-arrestin 1 and $G\alpha_i$ independent process (Min et al., 2013; Xu et al., 2019; Zheng et al., 2020). It has been shown that residues S145/S146 (IL2), C147 (IL2) and N12/19 (N-terminus) are involved in pharmacological sequestration and desensitization of D3R. Studies using HEK293 cells and different agonists showed different levels of pharmacological sequestration and desensitization. Namely, 7-OH-DPAT induced the highest level of D3R sequestration and desensitization of D3R, followed by quinpirole and DA. Pharmacological sequestration and desensitization can provide a critical cellular mechanism for D3R as an autoreceptor by performing highly flexible regulatory mechanism. Keeping the D3R near the plasma membrane during prolonged agonist activation can provide rapid resensitization according to the cellular needs (Min et al., 2013).

Interestingly, a recent study by Xu and colleagues identified a novel class of D3R agonists called SK608 which induces homologous internalization of human D3R stably expressed in CHO, HEK 293 and SH-SY5Y cells. GRK2, clathrin, dynamin I/II are critical molecules in the SK608-induced homologous internalization of D3R, while $G\alpha_{i/o}$, β-arrestin 1/2, GIT1, caveolin are not involved in this process (Xu et al., 2019).

Overall, D3R undergoes various regulation mechanisms, which depend on activation by different agonists and molecular players involved in D3R internalization and trafficking such as PMA-induced heterologous internalization, agonist-induced pharmacological sequestration, and SK608-induced homologous internalization.

3.3. D3R and β-arrestins

The possibility of β-arrestins interacting with GPCRs to regulate different cellular responses is supported by studies showing a role of β-arrestins in the modulation of signaling beyond desensitization and internalization. Intriguingly, although D3R internalization is not correlated

with β-arrestin translocation, a constitutive interaction between human D3R and β-arrestin 2 has been reported (Kim et al., 2005; Min et al., 2013). The basal interaction between D3R and β-arrestin 2 might be important for pharmacological sequestration and desensitization as discussed above. Interestingly, an interaction between human D3R/β-arrestin 2/filamin A was observed, and the formation of this tripartite complex is under the control of basal GRK activity (Kim et al., 2005).

As mentioned earlier, confocal microscopy results demonstrated subtle translocation of β-arrestin 2 to the plasma membrane and D3R internalization only occurs in the presence of overexpressed GRKs (Kim et al., 2001). However, one recent study using a sensitive split luciferase-based assay with the ability to quantify β-arrestin 2 recruitment to human D3R showed agonist-induced β-arrestin 2 recruitments to D3R that was not affected by GRK2 overexpression. Instead, this process was sensitive to PKC inhibitor, which decreased the maximum response and potency of agonist. Overall, β-arrestin 2 recruitment to D3R is facilitated by a PKC-dependent phosphorylation (Forster et al., 2020).

3.4. D3R Expression in the Brain

The D3R serves both postsynaptically on DA target cells and presynaptically as an autoreceptor on dopaminergic neurons (Kim et al., 2005; Le Foll et al., 2014). D3R controls impulse flow and release of DA (Gobert et al., 1995; Tepper et al., 1997). Interestingly, D3R-null mice exhibit higher extracellular levels of DA compared to wild-type (WT) littermates, which confirms that D3R would modulate DA release (Joseph et al., 2002; Koeltzow et al., 1998).

The distribution of D3R in the brain is limited in comparison to the other DARs. The highest levels of D3R are found in the nucleus accumbens, the olfactory tubercle and the islands of Calleja. At lower levels, the D3R is also observed in the striatum, the substantial nigra pars compacta, the septal area and in various cortical areas. In these brain regions, D3R modulates

mesolimbic and nigrostriatal dopaminergic systems (Ahlgren-Beckendorf & Levant, 2004; Beaulieu & Gainetdinov, 2011; Le Foll et al., 2014; Missale et al., 1998; Nakajima et al., 2013; Sokoloff et al., 1992).

3.5. D3R Involvement in Neuropsychiatric Disorders and Current Treatments

D3R is involved in the regulation of behavioral activity, emotion, memory, movement, anxiety, reward, cognition, and endocrine functions (Cho et al., 2003; Li et al., 2020). D3R dysfunction has been reported in some neuropsychiatric disorders such as addiction, cognitive deficits, depression, schizophrenia, Alzheimer's disease (AD), and PD (Le Foll et al., 2014; Maramai et al., 2016; Sokoloff et al., 1992).

Piggott et al. demonstrated higher D3R binding in the striatum of AD compared to healthy controls in a [^{3}H]-7-OH-DPAT autoradiography study of post-mortem brains (Piggott et al., 1999). On the other hand, studies on D3R knockout (KO) mice showed the reduced levels of neurofibromin (NF1) versus enhanced levels of amyloid precursor protein (APP), which are involved in the pathogenesis of AD (Nakajima et al., 2013).

The elevated D3R expression has been found in the central nervous system and blood lymphocytes in untreated schizophrenia patients (Gurevich et al., 1997). In contrast, low levels of D3R expression were observed in long-term hospitalized patients with chronic schizophrenia. These data suggested that long-term antipsychotic medication may modify brain D3R expression in schizophrenia (Prieto, 2017).

Previous studies suggested that DA depletion in PD may increase D3R expression and activity (Prieto, 2017). Moreover, alteration of D3R is involved in the early stages of PD and it can be considered as an early-stage biomarker for PD occurrence. The D3R is involved in various actions in both dyskinesia and motor recovery following L-DOPA treatment in PD. Agonist activation of D3R is thought to play critical roles in PD treatment, notably by increasing DA

concentration, decreasing α-Syn accumulation, enhancing secretion of brain derived neurotrophic factors (BDNF), ameliorating neuroinflammation and oxidative stress, promoting neurogenesis and interaction with D1R (Yang et al., 2020).

Several studies have identified the role of D3R not only in drug-related reward and drug intake, but also in behavioral sensitization including reinstatement and drug-seeking behavior (Le Foll et al., 2005; Leggio et al., 2016; Zhan et al., 2018). Higher levels of D3R has been observed in the substantia nigra pars compacta, hypothalamus, and amygdala of cocaine addicts, in the substantia nigra pars compacta of methamphetamine users, as well as in the hypothalamus of alcohol-dependent patients (Prieto, 2017).

Moreover, D3R downregulation has been found following chronic stress, and this effect can be reversed by antidepressant treatments or electroconvulsive therapy. This suggests a role of enhanced dopaminergic neurotransmission mediated by D3R in adaptive changes to stress (Leggio et al., 2016).

The role of D3R in depression has been indicated by downregulation of BDNF. It has been shown that antidepressant drugs could re-establish BDNF levels (Leggio et al., 2016). The D3R KO mice displayed depressive-like symptoms via increasing tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 levels, and altering BDNF expression in selected mesolimbic dopaminergic regions (Moraga-Amaro et al., 2014; J. Wang et al., 2020). Wang et al (2018) revealed the antidepressant effects of D3R activation in inflammation-associated depressive-like model. On the other hand, inhibition of D3R by systemic injection of a D3R antagonist resulted in a depression-like phenotype in normal mice (Wang et al., 2018).

The Ser9Gly polymorphism has been reported to be associated with or play a role in the phenotypic expression of neurological and psychiatric disorders such as acute pain in sickle

cell disease, schizophrenia, bipolar disorder, PD, familial essential tremor (ET) and suicidal behaviors (Jeanneteau et al., 2006; Jhun et al., 2014; Li et al., 2020; Zai et al., 2015).

For the association between Ser9Gly and schizophrenia, there are still conflicting results with out a consistent conclusion. Several molecular epidemiological studies have investigated the putative association between Ser9Gly polymorphic variant and risk of schizophrenia (Crocq et al., 1992; Kennedy et al., 1995; Sivagnanasundaram et al., 2000; Spurlock et al., 1998; Mant et al., 1994), whereas others showed no association (Chen, 1997; Jönsson et al., 2004; Li et al., 2020; Qi et al., 2017; Tanaka et al., 1996). The inconclusive findings could be attributed to small sample size, inclusion of various genetic backgrounds, various frequencies of the polymorphic variant among different populations and environmental factors. Moreover, the gene-gene, protein-protein interactions and epigenetics were not assessed in these studies (Lee et al., 2008; Li et al., 2020; Qi et al., 2017). In addition, studies reported that Ser9Gly shows higher affinity (almost five-fold) for endogenous DA (González et al., 2021; Li et al., 2020; Lundstrom & Turpin, 1996). Taken together, there is a possibility that Ser9Gly variant can play a role in the degree of response to neuroleptic drugs (Eichhammer et al., 2000), tardive dyskinesia (Basile et al., 1999; Jönsson et al., 2004; Lovlie et al., 2000), age of onset or other aspects of schizophrenia or psychosis.

Few studies have explored the relationship between cognitive function and D3R polymorphism in individuals with substance use disorders. In particular, Gly9/Gly9 carriers exhibited more cognitive dysfunction than the Ser9/Ser9 genotypes in human immunodeficiency virus (HIV)-positive individuals who were dependent on methamphetamine (Gupta et al., 2011).

A recent study in women with anorexia nervosa who carried the homozygous variant Gly9/Gly9 genotype displayed significantly worse eating disorders-related symptomatology than those with other genotypes (González et al., 2021).

Overall, D3R can be considered as a potential target for the treatment of addiction, cognitive deficits, depression, schizophrenia, AD and PD (Ginovart & Kapur, 2012; Maramai et al., 2016; Sokoloff et al., 1992). Most of the current drug treatments for these diseases target both D2R and D3R and cause severe side effects. For instance, current antipsychotic drugs for schizophrenia patients inhibit D2R, which cause extrapyramidal side effects and increase prolactin release. Pre-clinical studies suggested that administration of D3R selective antagonists could potentially reduce these side effects in patients (Maramai et al., 2016; Millan et al., 2000). This could be explained by contradictory involvement of D2R and D3R in pathogenesis. For instance, D2R appears to be downregulated in the brains of addictive people. However, upregulation of D3R is observed in the brain of cocaine-addicted individuals. Thus, D3R preferential ligands can be used as a strategy for the treatment of these disorders by causing fewer side effects. However, the high sequence homology between D3R and D2R challenges the development of D3R selective compounds. Taking advantages of new technologies such as crystallography, PET imaging, ligand docking, and data obtained from protein-protein interactions may develop our knowledge for discovery of D3R selective compounds. Although some D3R drugs have been synthesized, but there are currently no FDA approved treatments for these disorders (Cortés et al., 2016; Leggio et al., 2016).

3.6. D3R-Protein Interactions

Previous findings demonstrated novel direct interactions between GPCRs and other signaling proteins or receptors, which can augment GPCR functionality and modulate their signaling pathways, binding affinities, and receptor trafficking. Here, I described briefly the proteins interacting with D3R.

Several studies identified the interaction between D3R and other proteins or GPCRs mainly through intracellular domains, specially IL3 and C-terminal domain. These interactions may

have important consequences for receptor functions, since significant changes in receptor activation, maturation, ligand binding, signaling and trafficking have been observed for instance with several D3R heterodimers (Cortés et al., 2016; Guo et al., 2010). Lin et al identified the interaction between Filamin A (a protein widely expressed in neurons) with the IL3 of D3R that controls the cell surface expression of D3R using the yeast two-hybrid approach followed by pull-down and co-IP experiments (Lin et al., 2001). Studies using yeast two-hybrid screens, pull-down and co-IP assays demonstrated an interaction between C-terminal domain of protein 4.1N (one of the 4.1 family of cytoskeletal proteins which is specifically enriched in neurons of mammalian brain) and N-terminal segment of the IL3 of D3R. Thus, protein 4.1N•D3R interaction is required for localization or stability of D3R at the neuronal plasma membrane (Binda et al., 2002). In another study, a direct interaction between elongation factor-1Bβγ (eEF1Bβγ) and D3R was identified. D3R activation leads to phosphorylation of eEF1Bγ subunit on serine residue(s). PKC is believed to be critical for D3R-mediated regulation of eEF1Bγ (Cho et al., 2003). An interaction between C-terminal domain of D3R and the PDZ domain-containing protein, GIPC (GAIP interacting protein, C terminus) has been demonstrated using yeast two-hybrid system, pull-down, and affinity chromatography assays. GIPC has been suggested as a selective scaffold protein to assist receptor trafficking and signaling (Jeanneteau et al., 2004). Using the same methods, Basile et al revealed an interaction between IL3 of D3R and paralemmin which is a lipid anchored synaptic protein. This interaction reduces D3R plasma membrane expression and decreases basal, isoproterenol (ISO) and FSK-stimulated AC activity (Basile et al., 2006). GST fusion pull-down assays demonstrated that CaMKII binds to the IL3 of D3R. This interaction is Ca^{2+} sensitive and is sustained by autophosphorylation of CaMKII, which can modulate D3R regulation and signaling. Interaction between CaMKII and D3R promotes phosphorylation of D3R at specific serine site (S229) and reduces D3R efficacy in inhibiting cAMP formation (Liu et al., 2010).

In addition, the D3R may form heterodimers with other DARs such as the D1R and the D2R. Functional D2R•D3R heterodimer has been demonstrated using domain swapping approach and co-IP (Scarselli et al., 2001). The direct interaction between D3R and D1R in both striatal membranes and transfected HEK293 cells has been reported using co-IP and bioluminescence resonance energy transfer (BRET) assays. As a result of dimerization, the affinity of DA for D1R and the potency of DA in activating AC is increased. Moreover, D3R is switched to the D1R trafficking mechanisms (Fiorentini et al., 2008). Beside its interaction with DAR subtypes, the D3R may also form complexes with other GPCRs. Fluorescence resonance energy transfer (FRET) analysis demonstrated that D3R and adenosine A_{2A} receptor ($A_{2A}R$) form heteromeric complex in which $A_{2A}R$ antagonistically modulates both the affinity and the signaling of the D3R (Fuxe et al., 2005). Using BRET assays, an interaction between D3R and β2 subunit of nicotinic acetylcholine receptors (nAChR) was demonstrated. D3R•nAChR heterodimer in cultured DA neurons and mouse mesencephalic brain sections as well as in hiPSCs-derived DA neurons was shown using the proximity ligation assay (PLA). The D3R•nAChR heterodimer plays an important role in supporting DA neuron growth and survival (Mutti et al., 2020). A recent study using confocal microscopy and FRET assays demonstrated that orphan receptor G protein-coupled receptor 143 (GPR143) and D3R colocalize and interact at intracellular membranes. The GPR143•D3R complex may negatively modulate D3R activity in response to DA by changing affinity for DA or postponing delivery of the D3R to the plasma membrane (Bueschbell et al., 2021). Budzinski and colleagues demonstrated that bivalent ligands shift the D3R trafficking to a β-arrestin-dependent endocytosis through D3R and neurotensin receptor 1 (NTSR1) heterodimerization (Budzinski et al., 2021). Some of the D3R-protein interactions are summarized in Figure 10.

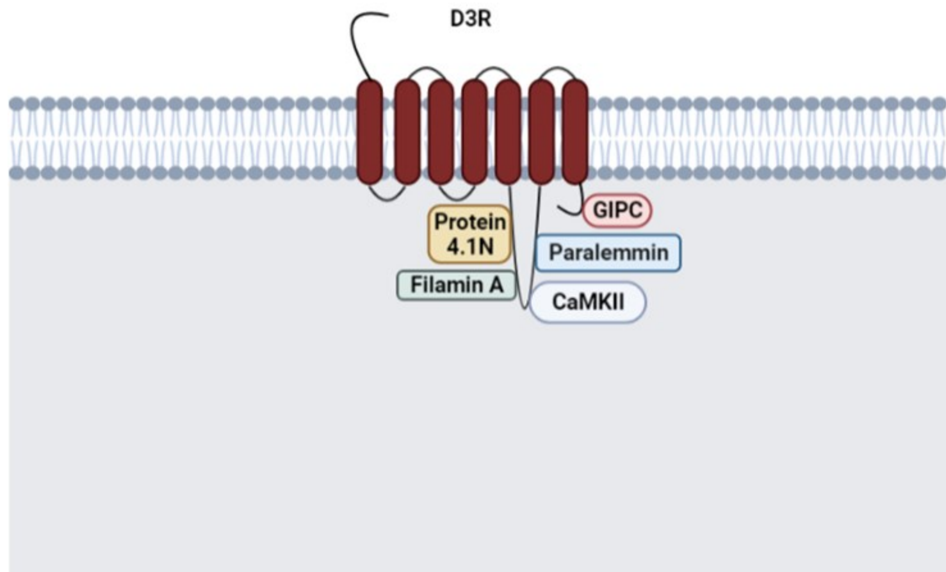

Figure 10. Proteins Interacting with D3R.

Protein 4.1N, Filamin A, CaMKII and Paralemmin interact with IL3 of D3R while GIPC interacts with C-terminal region of the receptor. These protein-protein interactions modulate D3R signaling and trafficking. D3R, dopamine D3 receptor; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; GIPC, GAIP interacting protein, C terminus. Created with BioRender.com.

4. Adenylyl Cyclases (ACs)

ACs are membrane-bound enzymes and comprise a family of molecules involved in the conversion of adenosine triphosphate (ATP) to cAMP, a second messenger that exerts a wide variety of effects via several intracellular signaling pathways. Nine membrane-bound AC isoforms and one soluble AC form (sAC) exist in mammals. ACs are divided into five groups based on primary amino acid sequences and distinct functional features (Table. 1). The nine membrane-bound ACs are large proteins that share a similar structure with an intracellular N-terminus followed by two repeats of six transmembrane helices domains (TM1 and TM2) alternating with two intracellular catalytic domains (C1 and C2) and an intracellular C-terminus (Fig. 11). The C1 and C2 domains act as catalytic sites and participate in specific intracellular regulations of AC isoforms. The catalytic activity, as well as the sites of interaction with FSK and $G\alpha_s$, require both cytoplasmic moieties (Cooper & Crossthwaite, 2006; Hanoune & Defer, 2001). Each isoform is regulated in a unique manner by Gα and Gβγ subunits, divalent cations, posttranslational modifications, protein kinases (PKA, PKC, and Ca^{2+}/CaM), phosphatases (calcineurin), calcium and can support and integrate differential regulatory pathways through cross-talk with other signal transduction systems (Beazely & Watts, 2006; Hanoune & Defer, 2001; Zimmermann et al., 1998). $G\alpha_s$ subunit activates all membrane bound ACs, while $G\alpha_{i/o}$ subunits only inhibit the Group 1 (AC1, AC3, AC8) and Group 3 (AC5, AC6) isoforms. The Gβγ subunits activate Group 2 (AC2, AC4, AC7) and potentially Group 3, whereas promote an inhibition of the Group 1. On the other hand, calcium stimulates the Group 1 of ACs in a calmodulin-dependent fashion. The AC9 is the only isoform which is inhibited by Ca^{2+}-dependent phosphatase calcineurin. Moreover, AC9 is insensitive to FSK while other membrane-bound ACs can be directly stimulated by FSK to increase intracellular cAMP (Beazely & Watts, 2006). AC2, AC7, but not AC4, are activated by phosphorylation by PKC. The mRNA expression results suggest that all AC isoforms can be found in brain cells. Tissue

expression profiles and/or selective subcellular localizations of ACs, enable AC isoforms to mediate their specific pathophysiological functions. For instance, AC3 which is highly expressed in olfactory neurons mediates the odorant-induced transduction cascade and behavior. In contrast, sAC, which is highly expressed in testes, is important for sperm fertilization. In addition, the role of AC1 and AC8 have been shown in learning and memory (Wang et al., 2009). On the other hand, AC1 and AC2 which are highly expressed in hippocampus, cerebral cortex and cerebellum may modulate synaptic plasticity (Hanoune & Defer, 2001).

Two mechanisms have been proposed for the recruitment of different proteins by ACs in signaling pathways. The first mechanism is the pre-existing physical interaction between proteins that helps signal transmission. The second mechanism is based on the proximity of signaling proteins to ACs without pre-existing interactions. Both mechanisms apply to the regulation of ACs by GPCRs and Ca^{2+}, which relies not only on the makeup of the immediate protein environment, but also on the discrete membrane localization (Cooper & Crossthwaite, 2006). Previous studies revealed the specific localization of Ca^{2+}-sensitive isoforms AC3, AC5, AC6 and AC8 in lipid rafts. Lipid rafts are characterized as plasma membrane domains that are rich in cholesterol and sphingolipid. A subset of rafts is caveolae, which is associated with the scaffolding protein caveolin. In contrast, the Ca^{2+}-insensitive isoforms AC2, AC4 and AC7 are present in the plasma membrane. This different localization may be directly related to specific properties of AC regulation. Alterations in membrane lipid and cholesterol content can modify AC activity. It has been shown that cytoplasmic loops (C1 and C2) are required for the localization of AC5 or AC8 to the lipid rafts. Interestingly, the colocalization between a given GPCR and AC on plasma membrane could be important for regulation of ACs. For instance, colocalization of β-adrenoreceptors and AC6 in caveolae in cardiac myocytes causes the AC6 preferential stimulation. In contrast, prostanoid EP2 receptors, which are localized outside of

caveolae, stimulate AC6 less efficiently (Beazely & Watts, 2006; Cooper, 2003; Cooper & Crossthwaite, 2006; Crossthwaite et al., 2005).

4.1. Group 3 of Adenylyl Cyclases

AC5 and AC6 belong to the calcium-inhibited family of AC isoforms. AC5 is highly expressed in cardiac muscle and striatal neurons, however AC6 is found in many other tissues. Although AC5 and AC6 share a very high amino acid sequence identity and several regulatory properties such as robust stimulation by $G\alpha_s$ and FSK, and inhibition by Ca^{+2} and PKA, they also show some differences in their regulation. Although other AC isoforms are inhibited by high concentrations of Ca^{+2}, but only sub-micromolar concentrations of Ca^{+2} is sufficient to inhibit AC5 and AC6 activity. The capacitative calcium entry (CCE) of calcium influx into intact cells inhibits cAMP accumulation in cells that express endogenous or recombinant AC5 or AC6. The *in vitro* studies using solubilized proteins showed that $G\alpha_i$ inhibits AC5 and AC6, but $G\alpha_o$ cannot efficiently inhibit them. On the other hand, other studies on recombinant cell lines and tissues showed that $G\alpha_o$ can inhibit cAMP production in cells that express AC6. These observations suggest that inhibition of AC5 or AC6 by $G\alpha_o$ requires an intact cell membrane or perhaps additional protein interactions that are absent in a reconstituted *in vitro* system. In addition, based on *in vitro* studies, $G\alpha_q$ enhances AC6 activation but not AC5. Specific localization of AC5 and AC6 in lipid rafts may serve to functionally localize these enzymes with other GPCR signaling components such as calcium and CCE channels, nitric oxide signaling proteins, phosphodiesterases and the Na^+/H^+ exchanger that serves to protect AC6 from changes in intracellular pH (Beazely & Watts, 2006; Hanoune & Defer, 2001). Since my master's project is focused on AC5, the main characteristics of AC5 are described in the following sections.

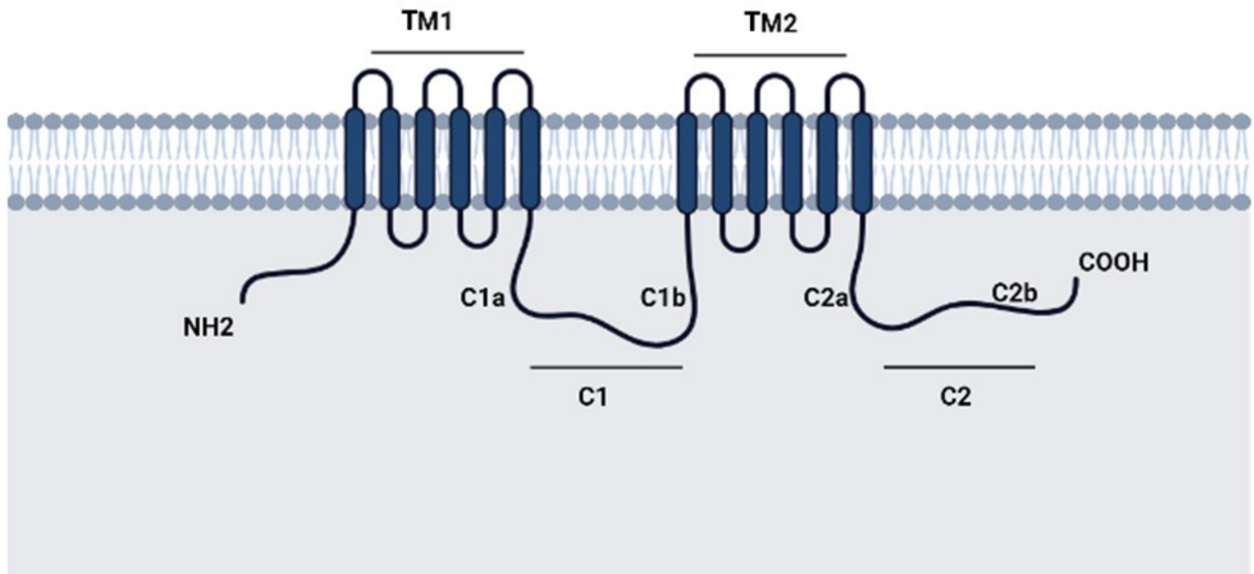

Figure 11. Schematic Representation of Membrane Bound ACs.

The membrane bound ACs display an intracellular N-terminus, followed by a set of six transmembrane domain (TM1), a large intracellular catalytic domain (C1), which is divided in to a conserved C1a region important for the formation of the catalytic unit, and the more divergent C1b region, a second set of six transmembrane domains (TM2) and an additional large intracellular catalytic domain (C2). Created with BioRender.com.

Group	Isoform
1	AC1, AC3, AC8
2	AC2, AC4, AC7
3	AC5, AC6
4	AC9
5	sAC

Table 1. The ACs Family.
The AC isoforms can be divided into five groups based on their primary amino acid sequences and distinct functional features.

4.2. Adenylyl Cyclase 5 (AC5)

AC5 gene located on chromosome 3p21.1 includes 21 exons coding for a 1261-amino acid protein (Chen, 2012). AC5 has the longest N-terminal domain (239 amino acid) among AC isoforms (Wang et al., 2009). AC5 is the dominant AC isoform in the brain and myocardium. AC5 is highly expressed in the striatum, nucleus accumbens, and olfactory tubercle and at lower levels in all other brain regions (Chen, 2012; Y. Chen et al., 2014; Dy et al., 2016). Assessing AC5 mRNA expression by Iwamoto et al showed that AC5 is expressed at least 10-20 fold more abundantly in the striatum than in other brain regions such as the cortex and the cerebellum in WT mice (Iwamoto et al., 2003). AC5 in striatal medium spiny neurons (MSNs) mediates striatal dopaminergic signaling and controls not only initiation of voluntary movements, but also prevention of involuntary movements (Bruce et al., 2019).

4.3. AC5-Related Disorders and Current Specific Treatment

Several studies have shown the involvement of AC5 mutations in complex movement disorders. Most of the inheritance of AC5 mutations are autosomal dominant. However autosomal recessive inheritance has been reported in some studies. Moreover, there are some reports of somatic mosaicism where mosaic carriers could be either symptomatic or asymptomatic (Ferrini et al., 2021). Most of the identified AC5 mutations are found in C1 and C2 domains, which likely affect the formation of catalytic pocket. It is possible that gain of function mutation facilitates the interaction between C1 and C2, resulting in increasing cAMP production. Moreover, mutations in other region of AC5 such as M1029L in the TM2 domain could modify the protein structure and bring C1 and C2 close together. The majority of identified AC5 mutations are missense but some frameshift mutations such as the deletion p.K694_M696 in the intracellular catalytic portion also have been reported (Ferrini et al., 2021). Interestingly, both gain and loss of function mutations in AC5 are observed in AC5-

related dyskinesia. Chen and colleagues using exome sequencing combined with linkage information demonstrated that AC5 is a strong candidate gene for a rare autosomal dominant mendelian disease familial dyskinesia with facial myokymia (FDFM) (Chen, 2012).

A missense mutation as well as two gain of function mutations (p.A726T and p. R418W) cause FDFM. These mutations in different AC5 domains increase intracellular cAMP in transfected cells upon stimulation of β-adrenergic receptors with ISO (Chen et al., 2014).

In a recent study, Crispr-Cas9 was used to characterize five AC5 gain of function mutations, which have been observed in AC5 related dyskinesia. In the AC5 mutants, enhanced cAMP production in response to $G\alpha_s$-mediated stimulation and reduced inhibition following D2R activation were observed, respectively. Their results suggested that patients with AC5 gain-of-function mutations may exhibit enhanced direct pathway activity, in combination with reduced disinhibition from the D2R-mediated indirect pathway (Doyle et al., 2019).

AC5 mutations have been reported as a cause of early onset hyperkinetic movements, and deep brain stimulation may improve its symptoms (Dy et al., 2016). The distribution of AC5 in the specific brain regions as well as progressively enhancement of AC5 gene transcription throughout development in human brain, may suggest the impact of AC5 dysfunction in movement disorders that worsen with age (Dy et al., 2016).

In contrast to the gain of function mutations, Iwamoto et al showed AC5 KO mice develop a parkinsonian-like disorder with abnormal coordination, bradykinesia and locomotor impairment that worsened with stress. Interestingly, selective stimulation of D1R or D2R improved some but not all the motor deficits such as the movement coordination. This suggests a specific role of AC5 because other AC isoforms present in striatum cannot fully compensate the functions of AC5 (Iwamoto et al., 2003). Furthermore, AC5 KO mice exhibit extended lifespan through oxidative stress protection. Inhibition of AC5 activates Raf/MEK/ERK

signaling pathway that upregulates the antioxidant mitochondrial enzyme manganese superoxide dismutase, resulting in resistance to oxidative stress, apoptosis and osteoporosis during ageing (Yan et al., 2007).

It has been shown that AC5 KO mice exhibit attenuated L-DOPA-induced dyskinesia (LID) in PD animal models (Park et al., 2014). The role of AC5 as an upstream mediator of LID is supported by studies in mice lacking AC5, which exhibit suppressed activation of key molecules in LID such as PKA, ERK, MSK1 (mitogen- and stress-activated protein kinase 1), and histone H3 (Park et al., 2014). Moreover, using the lentivirus shRNA-AC5 to inhibit AC5 expression in the dorsal striatum confirmed the role of AC5 in LID (Park et al., 2014).

There are currently no disease-modifying therapies for AC5-related diseases that show proven long-term efficacy (Ferrini et al., 2021). A study showed that administration of neuroleptic drugs such as sulpiride, haloperidol, and clozapine produced enhanced locomotion in AC5 KO mice (Park et al., 2014). This study suggests that AC5 is an essential component for neuroleptic effects of antipsychotic drugs (Lee et al., 2002).

It has been reported that deep brain stimulation may improve some AC5-related movement disorders (Dy et al., 2016). This study considered AC5 as an important component in the diagnosis of early onset hyperkinetic movement disorders that may respond to deep brain stimulation (Dy et al., 2016).

A case report study showed drinking coffee could reduce the hyperkinetic movement disorder in patients with AC5-related dyskinesia. This effect could be explained by antagonism of A_{2A} receptors by caffeine that results in inhibition of AC5 in patients with AC5 gain of function mutations (Méneret et al., 2019).

Another recent insight into a targeted therapeutic approach has been provided by Doyle and colleagues (2019). They suggested an AC P-site inhibitor, called SQ 22,536, as an effective

future therapeutic approach for treatment of AC5-related disorders. P-site inhibitors are adenosine nucleotide analogues that bind to the catalytic pocket of ACs. In this study, SQ 22,536 inhibited all five AC5 gain of function mutations to a significantly greater degree compared to WT (Doyle et al., 2019).

Based on all these studies and effort to find novel therapeutic approaches for treatment of AC5-related disorders, these findings are still not entirely specific in targeting the core underlying pathogenesis of these disorders. The increasing awareness of AC5-related disorders and the mechanisms involved in pathogenesis will likely yield novel therapeutic approaches, specifically targeting AC5 to reduce side effects.

4.4. AC5-Interacting Proteins

As mentioned earlier, physical interaction between proteins is one of the mechanisms that helps signal transduction. AC5 can interact with other proteins and modulate cAMP signaling pathway or other signaling molecules involved in regulation of cAMP signaling. Yeast two-hybrid assays revealed the interaction between the protein associated with Myc (PAM) and C2 domain of AC5, which inhibits the AC5 activity (Scholich et al., 2001). A study by Salim et al. (2003) identified the direct interaction of regulators of the G protein signaling 2 (RGS2) with C1 domain of AC5. This interaction inhibits the cAMP production independent of inhibition of $G\alpha_i$ (Salim et al., 2003). Pull-down assays, co-IP analyses, and co-localization in the brain showed that Ric8a (RIC8 guanine nucleotide exchange factor A), directly interacts with N-terminal of AC5 and consequently suppresses the activity of AC5 in a $G\alpha_i$-dependent manner. The inhibition of AC5 by Ric8a is highly selective and isoform specific (Wang et al., 2007). An interaction between A-kinase anchoring protein (AKAP79/150) and AC5 also has been identified. AKAP79/150 facilitates phosphorylation of AC5 by recruiting PKA to the AC5 and creates a negative feedback loop for the tight control of cAMP production (Bauman et al.,

2006). A recent study using a novel bimolecular fluorescence complementation (BiFC) method and co-IP assay identified the interaction between AC5 and PPP2CB (PP2A catalytic subunit) or NAPA [the intracellular trafficking associated protein-NSF (N-ethylmaleimide-sensitive factor) attachment protein alpha] under normal (vehicle) and sensitizing (quinpirole) conditions. The main finding of this work revealed the constitutive interaction between PPP2CB and presumably PP2A with AC5, whereas NAPA associated with AC5 following quinpirole pre-treatment (Doyle et al., 2019). Improving our knowledge on protein interacting with AC5 may help to develop new treatments for AC5-related disorders using high-throughput drug screening. Some of the AC5-protein interactions are summarized in Figure 12.

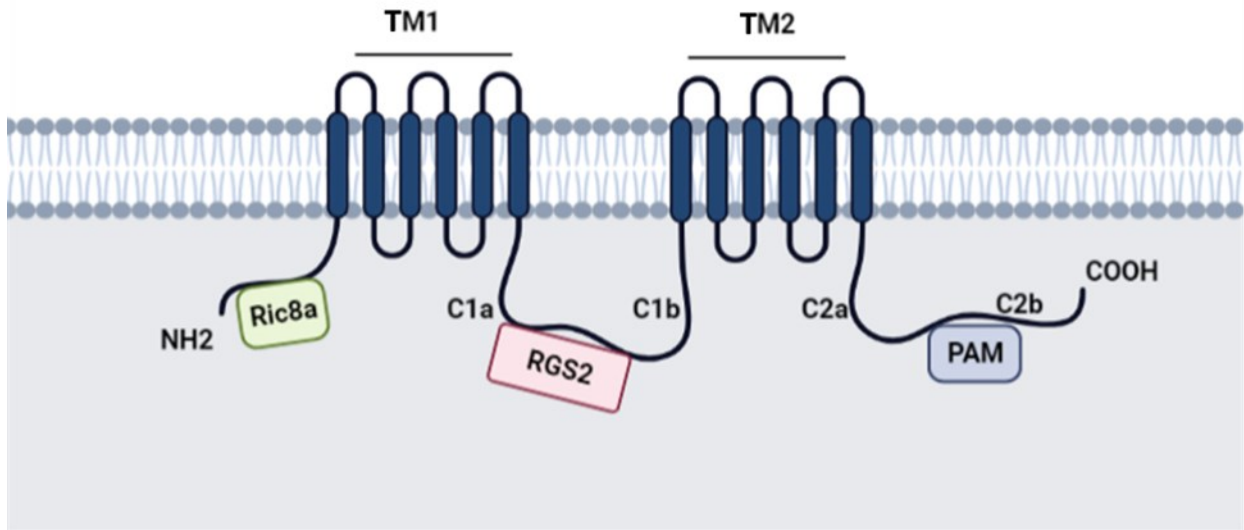

Figure 12. Proteins Interacting with AC5.
Ric8a, RGS2 and PAM interact with N-terminal, C1 and C2 domains of AC5, respectively. These protein-protein interactions inhibit the AC5 activity. Ric8a, RIC8 guanine nucleotide exchange factor A; RGS2, regulators of the G protein signaling 2; PAM, protein associated with Myc. Created with BioRender.com.

5. Rationale, Hypothesis and Objectives

Co-expression of D3R and AC5 in striatum, nucleus accumbens, olfactory tubercle and islands of Calleja which are major coordinators of normal and pathological movement, and the selective inhibition of AC5 activity by D3R (Robinson & Caron, 1997; Zaworski et al., 1999) raise the possibility of a functional link between AC5 and D3R in the modulation of signal transduction and trafficking. Indeed, the ability of D3R and AC5 to interact with other proteins suggests that the functional link between D3R and AC5 could be through a direct interaction. In addition, pharmacological, genetic, and human post-mortem studies have demonstrated a central role of D3R in the pathophysiology and treatment of different neuropsychiatric disorders as discussed above. Most of the current treatments for these disorders target D2R and D3R, which may cause severe side effects. This emphasizes the importance of designing specific drugs and improving drug target selectivity to improve treatment and reduce presumptive side effects. However, the high sequence homology between D3R and D2R challenges the development of D3R selective compounds. As discussed earlier, heterodimers and protein-protein interactions play important roles in modulating D3R signaling or trafficking. Further investigation in mediators of D3R signaling and trafficking, and protein interacting with this receptor is required to understand the pathogenesis of psychiatric and neurological disorders. This could be meaningful for novel therapeutic discoveries targeting specifically D3R signaling complexes for the treatment of diseases displaying D3R dysfunction.

Considering these points, my M.Sc. thesis project investigated the potential roles of AC5 in modulating D3R signaling and trafficking. However, it is also important to consider the role of AC5 in the modulation of D3Ser9 and D3Gly9 polymorphic variants to assess if AC5 plays a biased effect on D3Ser9 versus D3Gly9. Specifically, I hypothesize that AC5 plays an

important role in modulating D3R trafficking and signaling pathways through interacting with D3R.

To address my hypotheses, I propose the following objectives:

- Characterize the interaction between D3R and AC5 and test whether DA stimulation regulates this interaction.

- Assess whether having a physical complex between D3R and AC5 will modify ligand binding properties of the receptor.

- Assess the role of AC5 in the modulation of D3R signaling cascade; specifically, the inhibition of AC5 by D3R polymorphic variants following activation of AC5.

- Assess the role of AC5 in modulating D3R trafficking; specifically, cell surface expression, total expression and internalization of the receptor following agonist exposure.

- Assess the interaction between D3R, AC5 and β-arrestin 2.

Materials and Methods

1. Materials

Eagle's minimal essential medium (EMEM) and phosphate-buffered saline (PBS) were obtained from Wisent Bioproducts (Saint-Jean-Baptiste, QC, Canada). Hanks' Balanced Salt Solution (HBSS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), bovine calf serum (BCS), gentamicin, tween-20, Cytiva HyClone bovine serum albumin (BSA) and puromycin were purchased from Gibco (Burlington, ON, Canada). Dulbecco's modified eagle medium (DMEM) was obtained from Corning (Canada). Bio-Rad protein assay dye reagent concentrate, polyvinylidene difluoride (PVDF**)** membrane, precision plus protein dual colors standards, ammonium persulfate (APS), and triton X-100 detergent were purchased from Bio-Rad laboratories (Mississauga, ON, Canada). [N-metyl-^{3}H]-spiperone and [^{3}H]-spiperone were obtained from PerkinElmer (Boston, MA, USA). Bio-Safe II biodegradable scintillation cocktail from Research Products International (Mount Prospect, IL, USA). (+)-Butaclamol hydrochloride, (S)- (-)-sulpiride, S (-)-raclopride (+)-tartrate, dimethyl sulfoxide (DMSO), FSK, (+)-sodium L-ascorbate (AA), DA , aprotinin, benzamidine, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and soybean trypsin inhibitor were purchased from Sigma-Aldrich (Oakville, ON, Canada). (-)-Quinpirole hydrochloride, D-luciferin sodium salt and PMA, Calbiochem® were from Bio-Techne Canada (Toronto, ON), GoldBio (St Louis, MO, USA) and VWR International (Mississauga, ON, Canada), respectively. The paraformaldehyde aqueous solution was from Electron microscopy sciences (Hatfield, PA, USA).

2. DNA Constructs

Different plasmids DNA have been used for transfection based on experiments, including WT human D3Ser9/Gly9 (untagged, HA-tagged, Flag-tagged), untagged human AC5 (kind gift of Dr. Carmen Dessauer, The University of Texas Health Science Center at Houston, TX, USA),

human Myc-Flag-tagged AC5 and AC6 (Origene Ð ¢ Technologies, Rockville, MD, USA), untagged β-arrestin 2, β-arrestin 2-V54D, and HA-tagged dynamin-1-K44A.

3. Cell Culture

3.1. Human Embryonic Kidney 293 Cells (HEK293)

HEK293 cells were cultured between passages 40-52 in a humidified atmosphere containing 5% CO_2 incubator at 37°C in EMEM supplemented with 10% (v/v) heat-inactivated FBS and 40 μg/ml gentamicin (complete EMEM) for binding studies (saturation and competition), ELISA (enzyme-linked immunosorbent assays) experiments, co-immunoprecipitation/ immunoblotting (co-IP/ IB).

3.2. HEK293T Cells

HEK293T cells which are stably expressing GloSensor were grown in DMEM supplemented with 5% (v/v) FBS, 5% (v/v) BCS and 10 μg/ml puromycin at 37°C in a humidified atmosphere containing 5% CO_2 to be used in cAMP GloSensor assays. Cells were grown between passages 10-30.

4. Transfection

Cells were seeded in 100-mm culture dishes (2.5 million cells/dish) and transiently transfected with different plasmids DNA as described below using a modified calcium phosphate precipitation method (Plouffe et al., 2010). The amount of D3R DNA was adjusted to obtain the desired receptor B_{max}. Empty vector (pCMV5) was used to adjust the total amount of DNA per dish based on different experiments. Cells were incubated overnight following the transfection, then washed with PBS, detached with trypsin, resuspended in desired culture media, and reseeded in assay dishes/plates.

5. *In vivo* Co-immunoprecipitation

Striatal tissues from Sprague Dawley rats were kindly provided by the laboratory of Dr. Jean-Claude Béïque. The brain striatal structures were collected from postnatal day 21 (P21) and quickly snap frozen in liquid nitrogen. Striatal tissues were homogenized using a Dounce tissue homogenizer (10 strokes) in a buffer containing: 20 mM Tris-HCl (pH 7.4), 320 mM sucrose, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 2 mM Na_3VO_4, and protease inhibitors (10 µg/ml benzamidine, 10 µg/ml soybean trypsin inhibitor, 20 µg/ml PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A). The total homogenate was centrifuged at 800 *g* for 10 min. The resulting supernatant was subjected to further centrifugation for 15 min at 10,000 *g*. The protein concentration was measured from supernatant using Bio-Rad protein assays. 1 mg of lysates were immunoprecipitated with 5 µg/ml of: mouse monoclonal anti-AC5 antibodies (MilliporeSigma; 19D5.C1), normal mouse IgG (Santa Cruz Biotechnology; SC-2025), rabbit polyclonal anti D3R antibodies (Abcam; ab42114), or normal rabbit IgG (MilliporeSigma; 12-370). Following overnight incubation in end-over-end rotation at 4°C, 50 µl of protein A/G plus agarose (Santa Cruz Biotechnology; SC-2003) were added to the immunoprecipitated samples for overnight. Beads were pelleted, washed four times with 1 ml of ice-cold $RIPA^+$ buffer (50 mM Tris; pH 8.0; 150 mM NaCl; 0.1% (W/V) SDS; 5 mM of EDTA; 1% (w/v) NP-40; 0.5% (w/v) deoxycholate sodium salt; 10 mM sodium fluoride; 10 mM disodium pyrophosphate) and protease inhibitors. The protein complexes were eluted by adding 40 µl SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) loading buffer (25 mM Tris-HCl, pH 6.5; 10% [v/v] glycerol; 8% [w/v] sodium dodecyl sulfate [SDS]; 5% [v/v] β-mercaptoethanol; and 0.004% [w/v] bromophenol blue) and the eluted protein complexes were saved at -20°C until further analysis. In addition, inputs of 50 µg of proteins from striatal tissues were prepared in SDS-PAGE loading buffer.

6. *In vitro* Co-immunoprecipitation

HEK293 cells were transfected with 1 µg HA-D3Ser9 or HA-D3Gly9 with and without 2.5 µg Flag-AC5 and/or 1 µg β-arrestin 2. Empty vector (pCMV5) was added to normalize the total amount of DNA to 5 µg per 100-mm dish. The following day, cells were washed with PBS, trypsinized, and reseeded in 100-mm dishes for co-IP and binding studies. 48 hours after reseeding, media was aspirated and cells were incubated in incubation buffer (EMEM without phenol red; 20 mM HEPES; and 10 µg/ml gentamicin) with either DA (10 µM final, dissolved in AA) or AA as a vehicle (0.1 mM final) for 15 min at 37°C in a humidified atmosphere containing 5% CO_2. At the end of incubation, cells were put on ice, media was aspirated, and cells were washed twice with cold PBS. Then cells were collected in 0.8 ml of cold $RIPA^+$ buffer with protease inhibitors, and lysates solubilized using end-over-end rotation for 1 hour at 4°C. The solubilized extracts were centrifuged at 12,500 rpm for 15 min at 4°C. Protein concentration of lysates was measured using Bio-Rad protein assays and BSA as a protein standard. 500 µg lysates were incubated with 50 µl of either pierce anti-HA agarose (Thermo Fisher Scientific; 26181) over night or anti-Flag M2 affinity gel (MilliporeSigma; A2220) for 2 hours at 4°C using the rotating wheel. At the end of incubation time, the antibody matrix was washed five times with cold $RIPA^+$ buffer, followed by eluting samples with 60 µl of SDS-PAGE loading buffer overnight at room temperature. Based on the protein concentration, 20 µg of protein were aliquoted as input samples combined with SDS-PAGE loading buffer. D3R expression was assessed using radioligand binding assays as described in following parts.

7. Western Blotting

Western blots were performed for all the experiments to confirm the expression of AC5, Flag-AC6, β-arrestin 2, β-arrestin 2-V54D, HA-dyn-1-K44A. Transfected cells were reseeded in 6-well plates. On the day of experiment, plates were placed on ice, media was aspirated, cells

were washed with cold PBS, scraped with lysis buffer (10 mM Tris-HCl, pH 7.4; and 5 mM EDTA, pH 8.0) including protease inhibitors and sonicated for 15 seconds. Protein concentration of lysates were measured as described above and 20 μg of protein was used for SDS-PAGE.

Co-IP samples, inputs and western blot samples were loaded into 10% (v/v) acrylamide gels for SDS-PAGE. Bio-Rad's Trans-Blot Turbo System and transfer buffer (Bio-Rad laboratories) were used to transfer proteins onto PVDF membranes at 25 V for 10 min, then incubated in blotto solution (50 mM Tris-HCl, pH 8.0; 80 mM NaCl; 2 mM CaCl2; 5% [w/v] nonfat dry milk; 0.2% [v/v] NP-40; and 0.02% [w/v] NaN3) overnight on a rocking platform shaker at 4°C. The following day, membranes were rinsed with Tris-buffered saline containing Tween-20 (TBS-T) (20 mM Tris-HCl, pH 7.4; 137 mM NaCl; and 0.2% [v/v] Tween-20), then incubated with desired antibodies on a rocking platform shaker at 4°C overnight. To probe D3R and AC5 immunocomplex following D3R IP, membranes were incubated with mouse a monoclonal anti-Flag M2-peroxidase (HRP) (MilliporeSigma; A8592) or a mouse monoclonal anti-AC5 antibodies (MilliporeSigma; 19D5.C1) diluted 1:2000 in TBS-T. To probe D3R and AC5 immunocomplex following AC5 IP, membranes were incubated with biotin anti-HA 11 epitope tag (Biolegend; 16B12) or rabbit polyclonal anti D3R antibodies (Abcam; ab42114) diluted 1:2000 in TBS-T. The next day, membranes were washed three times with TBS-T for 10 min and incubated for 1 hour at room temperature with appropriate secondary antibodies; such as ECL anti mouse IgG, horseradish peroxidase linked species specific whole antibody from sheep (GE Healthcare; NA931V) diluted in 1:5000; ECL anti-rabbit IgG, horseradish peroxidase-linked species specific whole Ab from donkey (GE Healthcare; NA934V) diluted 1:10000 in TBS-T; and streptavidin-horseradish peroxidase conjugate (GE Healthcare; RPN1231V) diluted 1:2000 in TBS-T. It is worth mentioning that blots for co-IP also were probed to confirm the immunoprecipitation of AC5 and D3R. Membranes were washed three

times after incubation time with TBS-T for 40 min and protein bands were visualized using Amersham ECL reagents (GE Healthcare; RPN2236). Images were acquired using MicroChemi DNR Bio-Imaging System and GelCapture Chemi acquisition software.

To probe the membranes for α-tubulin, membranes were incubated with stripping buffer (500 mM glacial acetic acid; 100 mM β-mercaptoethanol; and 0.5 % [w/v] SDS) for 30 min on shaker at room temperature to remove antibody bound to HA-D3R, then rinsed with TBS-T and incubated with blotto solution followed by other steps as described above. Rabbit monoclonal anti-α-tubulin antibodies (Cell Signaling Technology; 11H10) diluted 1:2000 in TBS-T was used as a primary antibody to detect α-tubulin. To detect dyn-1-K44A, β-arrestin 2/ β-arrestin 2-V54D, membranes were probed with mouse monoclonal anti-dynamin-1 (Cell Signaling Technology; 3G4B6), or rabbit monoclonal anti-β-arrestin 2 antibodies (Cell Signaling Technology; C16D9) diluted 1:2000 in TBS-T, respectively. To assess an interaction between HA-D3R, Flag-AC5 and β-arrestin 2, Flag-AC5 and β-arrestin 2 were probed in HA immunocomplexes with monoclonal anti-Flag M2-peroxidase (HRP) (Millipore Sigma; A8592), or rabbit monoclonal anti-β-arrestin 2 antibodies (Cell Signaling Technology; C16D9), respectively. Blots were also reprobed to confirm IP of HA-D3R.

8. Radioligand Binding Assays

8.1. Binding Studies

Binding assay was performed to assess the D3R expression level in transfected cells by determining the maximal number of binding sites (B_{max}) for all experiments. On the day of experiment, cells seeded in 100-mm dishes were put on ice, washed with cold PBS, scraped with cold lysis buffer, and centrifuged for 20 min at 18,000 rpm and 4°C. The pellets were detached with cold lysis buffer and homogenized with a Kinematica Brinkmann Polytron 3,000 at 15,000 rpm for 20 sec, then centrifuged again for 20 min at 18,000 rpm and 4°C. The final

pellets were resuspended with resuspension buffer (62.5 mM Tris-HCl, pH 7.4; and 1.25 mM EDTA, pH 8.0) and homogenized again. Binding assay was carried out with 100 µl of cell membrane preparations in a total volume of 500 µl containing 50 µl of [^{3}H]-spiperone or [N-metyl-^{3}H]-spiperone (~3 nM) as non-selective D3R antagonist in the presence or absence of 50 µl of 10 µM (+)-butaclamol in milli-Q-water containing 0.01% [v/v] ethanol (to determine non-specific binding). Membranes were incubated for 75 min at room temperature and harvested onto glass fiber filters using a Brandel M-48 Semiautomated Harvesting System. Filters were washed three times with cold washing buffer (50 mM Tris-HCl, pH 7.4; and 100 mM NaCl) and were added to scintillation vials containing Bio-Safe II biodegradable scintillation cocktail. Finally, receptor-bound radioactivity trapped on the filters was quantified using a Beckman LS 6,500 liquid scintillation counter. Protein concentration of the membranes were assessed using Bio-Rad protein assays as described above. Total membrane protein concentration and [^{3}H]-spiperone or [N-metyl-^{3}H]-spiperone radioactivity in dpm were used to calculate B_{max} in pmol/mg membrane proteins.

8.2. Saturation and Competition Studies

HEK293 cells were transfected with 4.5 µg of untagged-D3Ser9 or D3Gly9 with and without 2.5 µg of AC5 and reseeded in 150-mm culture dishes for these assays. On the day of experiment, cells were put on ice, washed with cold PBS, scraped with cold lysis buffer, and centrifuged for 20 min at 18,000 rpm and 4°C. The pellets were detached with cold lysis buffer and homogenized, then centrifuged again for 20 min at 18,000 rpm and 4°C. The final pellets were resuspended with 3 ml lysis buffer and homogenized again. 2.4 ml of the membranes were snap frozen in liquid nitrogen and stored at -80°C to be rethawed for competition studies and the rest of fresh membrane preparations were used in saturation studies. Membrane samples for saturation or competition were diluted in 3- or 5-ml resuspension buffer, respectively.

For saturation studies, 100 µl of membrane in a total volume of 500 µl, including binding buffer, increasing gradient concentrations of [^{3}H]-spiperone (~0.01–3 nM), either with or without 10 µM (+)- butaclamol were used to determine equilibrium dissociation constant (K_d) and B_{max} of D3R.

For competition studies, 100 µl of membrane in a total volume of 500 µl, including binding buffer, constant concentration of [^{3}H]-spiperone and increasing concentrations of agonists (DA and quinpirole), and antagonists [(S)- (-)-sulpiride, S (-)-raclopride (+)-tartrate salt] were used to assess inhibitory constants (K_i) of the ligands. DA was prepared in AA and quinpirole, raclopride and sulpiride were prepared in milli-Q-water. Membranes were incubated for 75 min at room temperature and harvested onto glass fiber filters, washed three times with cold washing buffer, then added to scintillation vials containing Bio-Safe II biodegradable scintillation cocktail, and receptor-bound radioactivity trapped on the filters was quantified using a Beckman LS 6,500 liquid scintillation counter. Protein concentration of membranes were evaluated using Bio-Rad protein assays as described above. Total membrane protein concentration and [^{3}H]-spiperone radioactivity in dpm were used to calculate B_{max} in pmol/mg membrane proteins.

9. cAMP GloSensor Assays

HEK293T cells were co-transfected with 0.5 µg D3Ser9 or D3Gly9 and 2.5 µg AC5 or Flag-AC6 versus D3Ser9 or D3Gly9 alone. The total amount of transfected DNA was consistently kept as 5 µg per dish and empty vector (pCMV5) was used to reach to this amount. In the following day, HEK293T cells were reseeded (15,000 cells/well) into poly-L-lysine (PLL; 25 µg/ml)-coated 384-well plates. After 24 hours, the media was removed from 384-well plates and cells were incubated with 0.3 mg/ml luciferin (dissolved in 1X HBSS + 20 mM HEPES; pH 7.4) for 15 min at room temperature. Cells then were stimulated with either DA at a final

concentration of 10 μM, quinpirole at a final concentration of 1 μM, and vehicle for 15 min at room temperature. After this incubation, FSK and isoproterenol (ISO) were added at a final concentration of 5 μM and 1 μM, and cells incubated for another 15 min. The luminescence activity was measured using Synergy H1Multi-Mode Plate Reader. To confirm D3R and AC5/Flag-AC6 expression, radioligand binding assays and western blots were performed, respectively.

10. Enzyme-Linked Immunosorbent Assays (ELISA)

HEK293 cells were transfected with (either 1 μg for internalization or 4.5 μg for cell surface and total expression) Flag-D3Ser9 and Flag-D3Gly9 with and without 2.5 μg AC5 and other DNA plasmids (1 μg β-arrestin 2, 1 μg β-arrestin 2-V54D, 1 μg HA-dyn-1-K44A). Cells were reseeded in 12-well plates after transfection. For internalization assay, on the day of experiment, culture media was replaced with incubation buffer and cells were treated with either DA (10 μM final) or AA as a vehicle (0.1 mM final) to assess DA-induced internalization at 37°C in a humidified atmosphere containing 5% CO_2. Also, to assess PMA-induced internalization, cells were incubated with either PMA (1 μM final) or DMSO as a vehicle (0.02% v/v) for 15 min at 37°C in a humidified atmosphere containing 5% CO_2. Afterwards, the media was aspirated, and cells were fixed with 3.7% (v/v) paraformaldehyde in PBS for 10 min at room temperature. For cell surface and total expression, culture media was aspirated and replaced with 3.7% (v/v) paraformaldehyde in PBS for 10 min at room temperature.

Following fixation with paraformaldehyde, cells were washed twice with 0.2% (w/v) BSA in PBS for 10 min followed by blocking with 1% (w/v) BSA in PBS for 30 min. Then, cells were incubated with monoclonal anti-Flag M2-peroxidase (HRP) antibody (MilliporeSigma; A8592) diluted 1:20000 in 1% (w/v) BSA for 1 hour. Cells then were washed two times with 0.2% (w/v) BSA in PBS for 10 min. For total expression, 1% (v/v) Triton-X-100 detergent

added in 0.2% or 1% (w/v) BSA for washing and blocking steps to detect total expression of the receptors (present either in cytosol or located on the cell surface). Subsequently, cells were treated with 400 µl o- phenylenediamine dihydrochloride (OPD) (0.5 mg/ml dissolved in stable peroxide substrate buffer; Thermo Fisher Scientific) and covered with aluminium foil for 15 min. The reaction was stopped by adding 100 µl of 3N HCL. The solution in each well was transferred to 96 well-plates. The optical density (OD) at 490 nm was read using a SpectraMaxM5. The background reading obtained from mock-transfected cells is subtracted to calculate the corrected values. To determine receptor internalization by ELISA, detection of an extracellular N-terminal epitope tag on the receptor is assessed. This epitope is no longer recognized by the cognate antibody once the receptor is internalized. The percentage of receptor endocytosis is calculated from the OD values of vehicle- and agonist-treated cells [(vehicle-treated−agonist-treated)/(vehicle-treated)]. Radioligand binding assays were used to determine D3R expression. In addition, western blots were used to determine AC5, β-arrestin 2, β-arrestin 2-V54D, HA-dyn-1-K44A expressions.

11. Statistical Analysis

GraphPad Prism (version 9) was used for statistical analyses and curve fitting. All data are expressed as means ± SEM. For co-IP/IB assays, immunoreactive bands were quantified by Fiji software and then quantifications were normalized to vehicle. For radioligand binding assays, non-linear regression curve fitting was used to analyse equilibrium dissociation constant (K_d), inhibitory constants (K_i) and maximal number of binding sites (B_{max}). For cAMP assays, cAMP accumulation in the presence of FSK or ISO was normalized to 100%, and cAMP levels in the presence of FSK/ISO plus quinpirole or DA were normalized to the cAMP accumulation stimulated by FSK or ISO. Unpaired-*t* test, one-sample *t*-test and two-way ANOVA were performed where indicated in the results section, and differences with $p \leq 0.05$ deemed statistically significant.

Results

1. Co-IP of a Protein Complex between D3R and AC5 *In vivo*

As mentioned in the Introduction, D3R and AC5 can interact with different proteins (Basile et al., 2006; Bauman et al., 2006; Binda et al., 2002; Budzinski et al., 2021; Bueschbell et al., 2021; Cho et al., 2003; Fiorentini et al., 2008; Fuxe et al., 2005; Guo et al., 2010; Lin et al., 2001; Mutti et al., 2020; Salim et al., 2003; Scarselli et al., 2001; Scholich et al., 2001; Wang et al., 2007). Therefore, I investigated whether D3R has the potential to form a hetero oligomer with AC5 using co-IP/IB assays. To test the protein complex formation *in vivo*, rat brain striatal lysates were used for co-IP of D3R or AC5, followed by immunoblotting with AC5 and D3R antibodies. First, D3R was immunoprecipitated using anti-D3R antibodies and A/G agarose beads, followed by immunoblotting with anti-AC5 antibodies. AC5 immunoreactivity was detected in D3R immunocomplex, which suggests a potential interaction between D3R and AC5 (Fig. 13A). In addition, AC5 and D3R inputs shown in figure 13A confirm the expression of AC5 and D3R in this brain region. The theoretical molecular weight of non-glycosylated rat D3R (446 aa) and AC5 (1262 aa) are predicted to be 50 and 139 kDa, respectively. Reciprocally, AC5 was immunoprecipitated using anti-AC5 antibodies and A/G agarose beads, followed by immunoblotting with anti-D3R antibodies (Fig. 13B). D3R and AC5 inputs are shown in figure 13B. Detection of D3R immunoreactivity in AC5 immunocomplex supports the protein complex formation between D3R and AC5. Overall, IP of D3R, followed by immunoblotting of AC5, and reciprocally IP of AC5, followed by immunoblotting of D3R, provide evidence for the existence of a D3R•AC5 protein complex in the striatum. Co-IP/IB assays also were performed using rat brain cortical lysates for further validation of the existence of a D3R•AC5 protein complex in the brain (data are not shown).

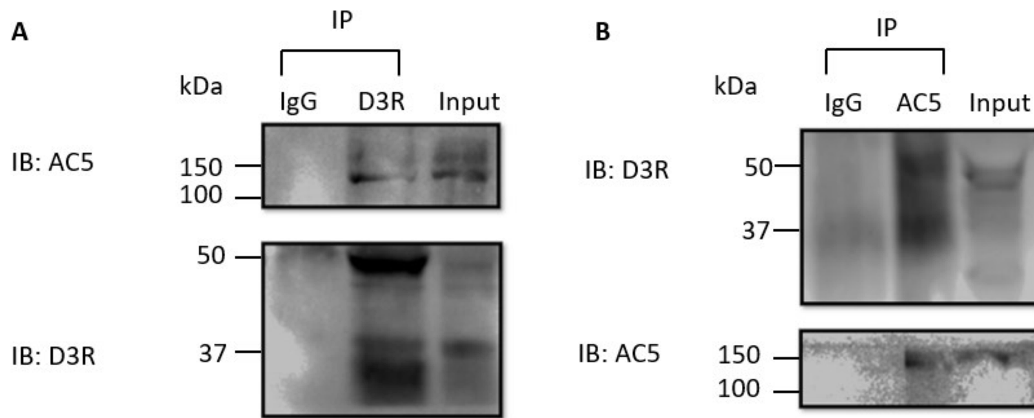

Figure 13. Formation of a Protein Complex between D3R and AC5 in Striatal Lysates of Rats.
A) A representative blot of three independent experiments. Top, D3R was immunoprecipitated using anti-D3R antibodies and A/G agarose beads, followed by immunoblotting with anti-AC5 antibodies using striatal brain lysates from P21 rats. Bottom, immunoblotting with anti-D3R antibodies. B) A representative blot of three independent experiments. Top, AC5 was immunoprecipitated using anti-AC5 antibodies and A/G agarose beads, followed by immunoblotting with anti-D3R antibodies. Bottom, immunoblotting with anti-AC5 antibodies. Blots represent two independent experiments. IP, immunoprecipitation; IB, immunoblot.

2. Validation of the Formation of Protein Complex between D3R and AC5 in Co-Transfected HEK293 Cells: Evidence for a Facilitatory Role of DA

To confirm the complex formation *in vitro* and to examine the effect of DA-induced D3R activation on the D3R•AC5 complex formation, co-IP/IB experiments were performed in HEK293 cells transfected with human HA-D3Ser9 or HA-D3Gly9 polymorphic variants with and without human Flag-AC5 stimulated for 15 min with 10 µM DA or AA (vehicle). First, HA-D3R was immunoprecipitated and Flag-AC5 was detected in immunocomplexes in the absence and presence of DA (Fig. 14A). Importantly, Flag-AC5 was not detected in HA-tagged immunocomplexes from cells transfected with Flag-AC5 alone, HA-D3R alone and empty pCMV5 vector (mock). The HA-D3R was probed in immunocomplexes using anti-HA antibodies to confirm IP of HA-D3R (Fig. 14B). The theoretical molecular weight of human HA-D3R (410 aa) and Myc-Flag-tagged AC5 (1295 aa, Origene) are predicted to be 45 and 145 kDa, respectively. The results showed two broad electrophoretic mobility bands for HA-D3R (~ 40- 45 kDa) and (~ 65- 70 kDa). To validate the expression of Flag-AC5 and to confirm the amount of protein loaded, inputs of lysates were probed with anti-FLAG-HRP and anti-α-tubulin antibodies (Fig. 14C and D). Radioligand binding assays were used to determine HA-D3R expression. Densitometric analysis of the co-IP of HA-D3Ser9•Flag-AC5 and HA-D3Gly9•Flag-AC5 were corrected from Flag-AC5 inputs and normalized relative to basal. Activation of HA-D3R by DA significantly increased the abundance of HA-D3Ser9•Flag-AC5 (49% ± 0.2) and HA-D3Gyl9•Flag-AC5 (41% ± 0.1) protein complexes (Fig. 14E and F).

In a separate set of experiments, Flag-AC5 was immunoprecipitated and HA-D3R probed in immunocomplexes with anti-HA antibodies. HA-D3R was detected in immunocomplexes obtained from HEK293 cells co-transfected with HA-D3R and Flag-AC5 in the absence and presence of DA. However, no HA-D3R was detected in Flag immunocomplexes from cells

transfected with HA-D3R alone, Flag-AC5 alone or mock (Fig. 15A). To validate HA-D3R and Flag-AC5 expressions and amounts of protein loaded, inputs of lysates were probed with anti-HA, anti-FLAG-HRP and anti-α-tubulin antibodies (Fig. 15B, C, D). HA-D3R expression was also confirmed with radioligand binding assays. The results support the formation of protein complex between Flag-AC5 and both HA-D3R polymorphic variants under basal and DA-stimulated conditions in co-transfected HEK293 cells. Quantification of the immunoreactive bands of HA-D3Ser9•Flag-AC5 and HA-D3Gly9•Flag-AC5 in HEK293 lysates were corrected for Flag-AC5 inputs and normalized relative to basal. Densitometric analysis showed that activation of HA-D3R with DA significantly increases the abundance of HA-D3Ser9•Flag-AC5 (22% ± 0.05) and HA-D3Gyl9•Flag-AC5 (15% ± 0.04) protein complexes (Fig. 15E and F).

Overall, the results demonstrate the constitutive interaction between both HA-D3R polymorphic variants and Flag-AC5 in co-transfected HEK293 cells. These results also show that activation of HA-D3R by DA may facilitate the formation of protein complex between D3R and AC5.

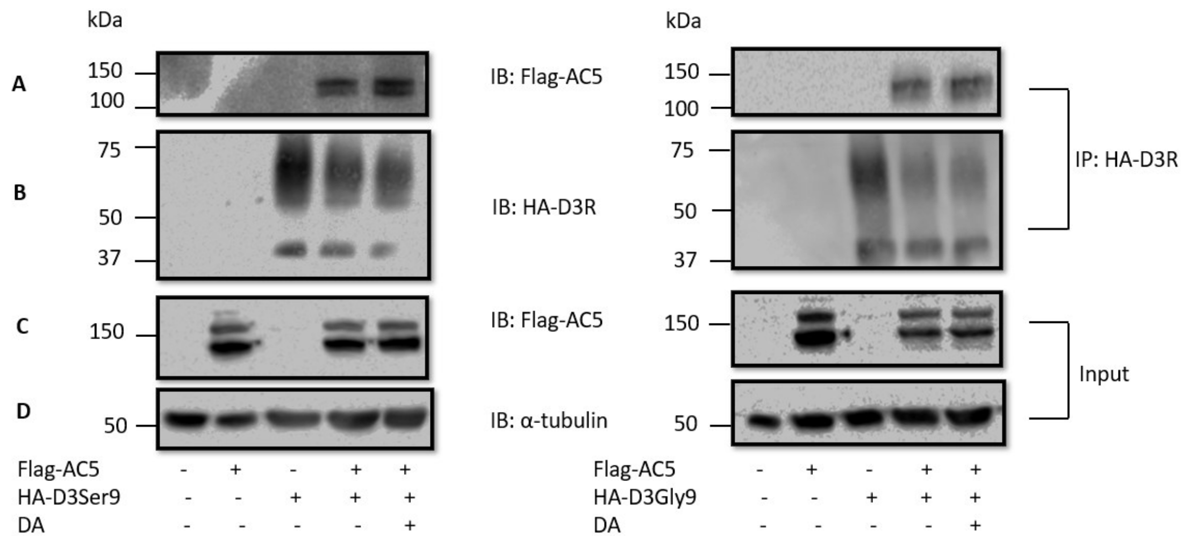

Figure 14. Complex Formation between HA-D3Ser9 or HA-D3Gly9 and Flag-AC5 under Basal and DA Stimulation.

A representative example of a co-IP/IB experiment using HEK293 cells transfected with HA-D3R and Flag-AC5. A) HA-D3R was immunoprecipitated using HA-antibody beads and Flag-AC5 was probed with anti-FLAG-HRP antibodies. B) HA-D3R was probed in immunocomplexes using biotinylated anti-HA antibodies. C) Flag-AC5 inputs were probed using anti-FLAG-HRP antibodies. D) α-tubulin inputs were probed with anti α-tubulin antibodies. E, F) Densitometric analyses of the co-IP of HA-D3Ser9+Flag-AC5 (E) and HA-D3Gly9+Flag-AC5 (F) from HEK293 lysates stimulated with DA (10 µM for 15 min) and compared to basal. Bars represent means ± SEM from n=6 independent experiments. Statistical analysis was done using one sample *t*-test. * $p < 0.05$ as compared to basal. The B_{max} values in pmol/mg for [^{3}H]-spiperone in cells transfected with HA-D3Ser9, HA-D3Ser9+Flag-AC5, HA-D3Gly9 and HA-D3Gly9+Flag-AC5 expressed as mean ± SEM were 2.70 ± 0.47, 2.22 ± 0.63, 2.28 ± 0.40, 1.88 ± 0.42, respectively. DA: dopamine; IP, immunoprecipitation; IB, immunoblot

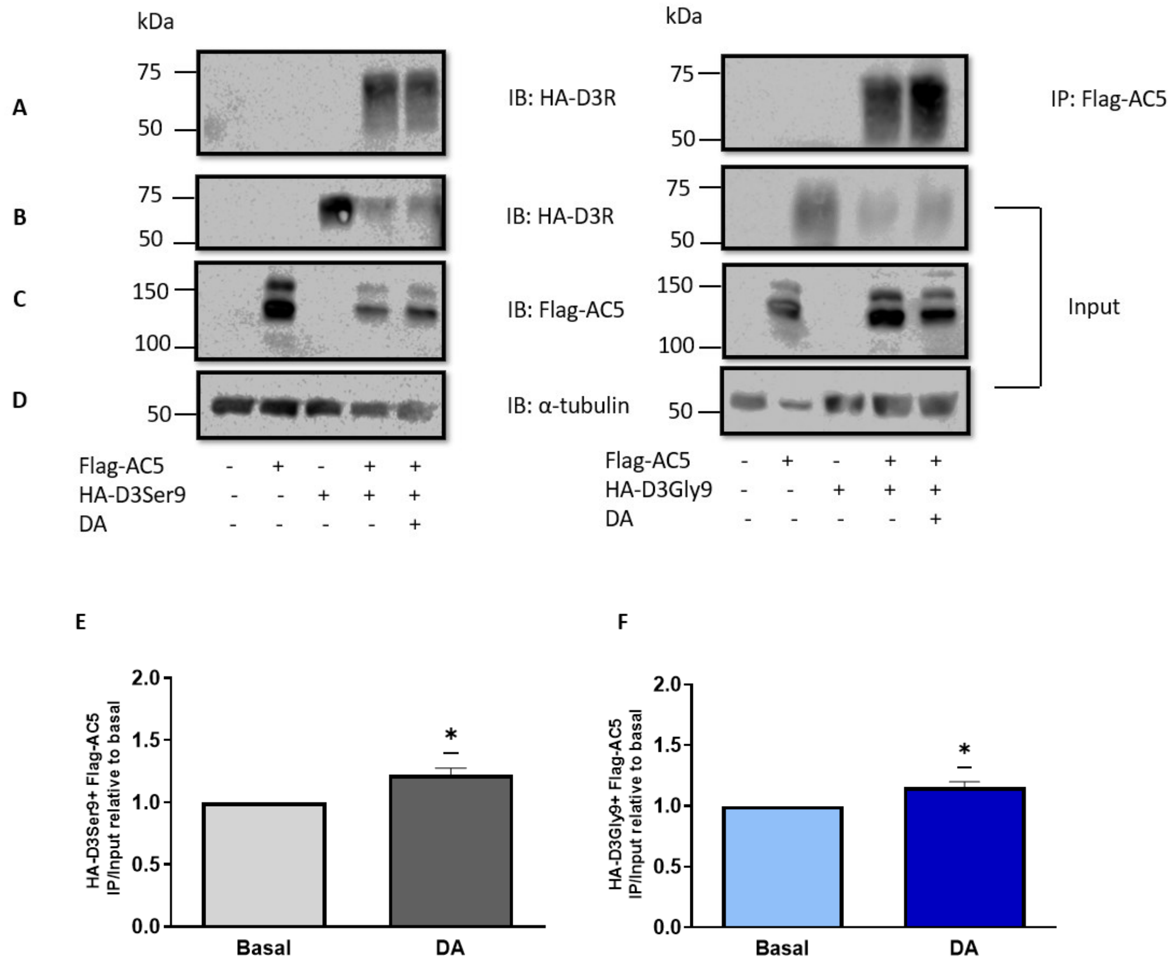

Figure 15. Complex Formation between HA-D3Ser9 or HA-D3Gly9 and Flag-AC5 under Basal and DA Stimulation.

A representative example of a co-IP/IB experiment using HEK293 cells transfected with both HA-D3R polymorphic variants and Flag-AC5. A) Flag-AC5 was immunoprecipitated using Flag-antibody beads and HA-D3R was probed using biotinylated anti-HA antibodies. B) HA-D3R inputs were probed using biotinylated anti-HA antibodies. C) AC5 inputs were probed using anti-FLAG-HRP antibodies. D) α-tubulin inputs were probed with anti α-tubulin antibodies. E, F) Densitometric analysis of co-IP of HA-D3Ser9+Flag-AC5 (E) and HA-D3Gly9+Flag-AC5 (F) from HEK293 lysates stimulated with DA (10 µM for 15 min) and compared to basal. Bars represent means ± SEM from n=4 independent experiments. Statistical analysis was done using one sample *t*-test. * $p < 0.05$ as compared to basal. The B_{max} values in pmol/mg for [^{3}H]-spiperone in cells transfected with HA-D3Ser9, HA-D3Ser9+Flag-AC5, HA-D3Gly9 and HA-D3Gly9+Flag-AC5 expressed as means ± SEM were 2.63 ± 0.73, 1.908 ± 0.51, 2.69 ± 0.95, 2.82 ± 0.71, respectively. DA: dopamine; IP, immunoprecipitation; IB, immunoblot

3. D3R Displays Similar Ligand Binding Properties in the Presence and Absence of AC5

It is important to assess if the complex formation between D3R and AC5 modifies ligand binding and expression properties of the D3R. To test whether complex formation between D3Ser9 or D3Gly9 and AC5 modifies ligand binding properties of the receptor, saturation and competition assays were performed using HEK293 cells transfected with D3Ser9 or D3Gly9 with and without AC5. Specifically, saturation studies were used to determine the equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) of D3R. Competition studies were done to determine the inhibitory constant (K_i) for agonists (DA and quinpirole) and antagonists (raclopride and sulpiride) to assess whether AC5 alters the receptor affinity for agonists and antagonists. Representative saturation curves for cells transfected with D3Ser9 or D3Gly9 with and without AC5 are shown in figure 16. The K_d, B_{max} and K_i values of D3R polymorphic variants with and without AC5 are summarized in Table 2 and 3, respectively. The K_d values of D3Ser9 and D3Gly9 did not change significantly in the presence of AC5, which suggests that AC5 does not change the ability of D3Ser9 and D3Gly9 to bind [^{3}H]-spiperone (Fig. 17A). In addition, B_{max} obtained from saturation curves showed that AC5 displays no significant effect on the expression of D3Ser9 and D3Gly9 (Fig. 17B). Furthermore, competition studies showed no significant change in the affinity of D3Ser9 and D3Gly9 for neither agonists (DA, quinpirole) nor antagonists (sulpiride, raclopride) in the presence of AC5 compared to receptors transfected alone (Fig. 18). Overall, the results indicate that both D3R polymorphic variants display similar ligand binding properties and expression in the presence and absence of AC5, suggesting that AC5 has no major impact on the ligand binding affinity and expression of the D3R.

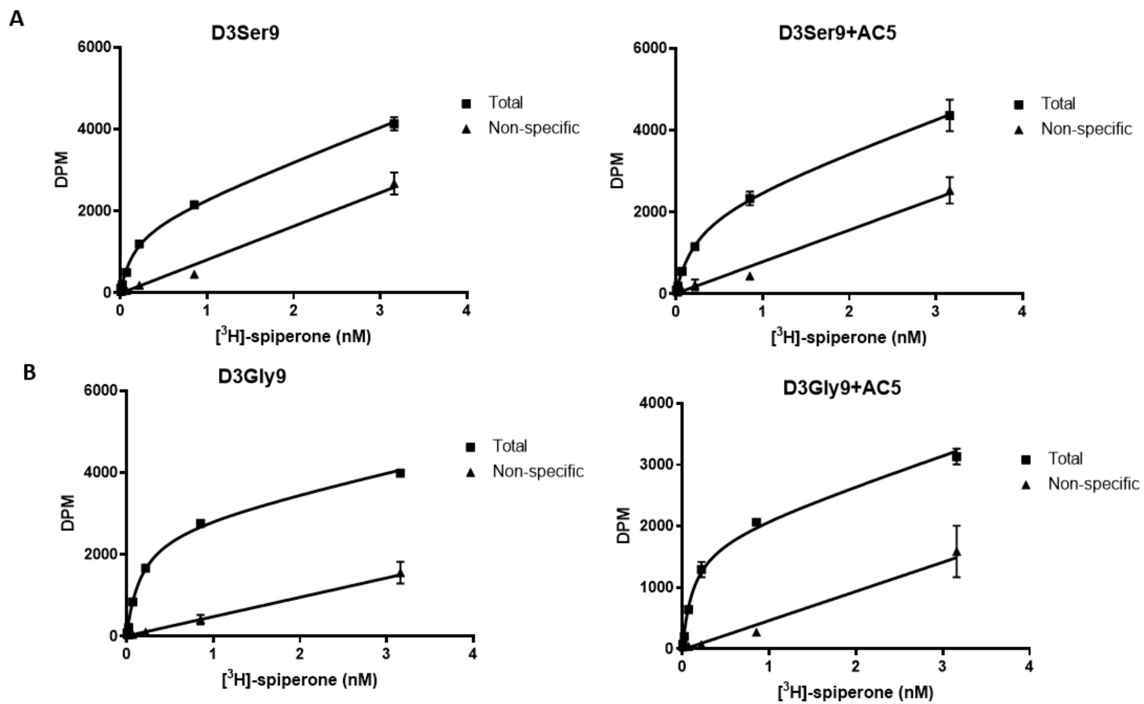

Figure 16. Saturation Binding Curves of [^{3}H]-Spiperone on Membrane Preparations from HEK293 Cells Transfected with D3Ser9 or D3Gly9 in the Presence or Absence of AC5.

A representation of saturation binding curves of [^{3}H]-spiperone performed in duplicate for A) HEK293 cells transfected with D3Ser9 ± AC5. B) D3Gly9 ± AC5. Saturation curves were analyzed using GraphPad Prism 9.0. DPM, disintegrations per minute.

Receptor	[^{3}H]-spiperone K_d (nM)	B_{max} pmol/mg protein
D3Ser9	0.29 ± 0.09	11.05 ± 1.42
D3Ser9+AC5	0.28 ± 0.13	8.66 ± 0.99
D3Gly9	0.33 ± 0.11	11.12 ± 1.15
D3Gly9+AC5	0.35 ± 0.17	11.20 ± 1.23

Table 2. Equilibrium Dissociation Constants (K_d) and B_{max} Values for [^{3}H]-Spiperone and of D3Ser9 and D3Gly9 in the Presence and Absence of AC5.

K_d values and B_{max} values obtained from saturation curves done in duplicate are shown as means ± SEM of n=5 independent experiments. Statistical analysis showed no significant difference in K_d values and B_{max} values of D3Ser9 and D3Ser9+AC5 or D3Gly9 and D3Gly9+AC5.

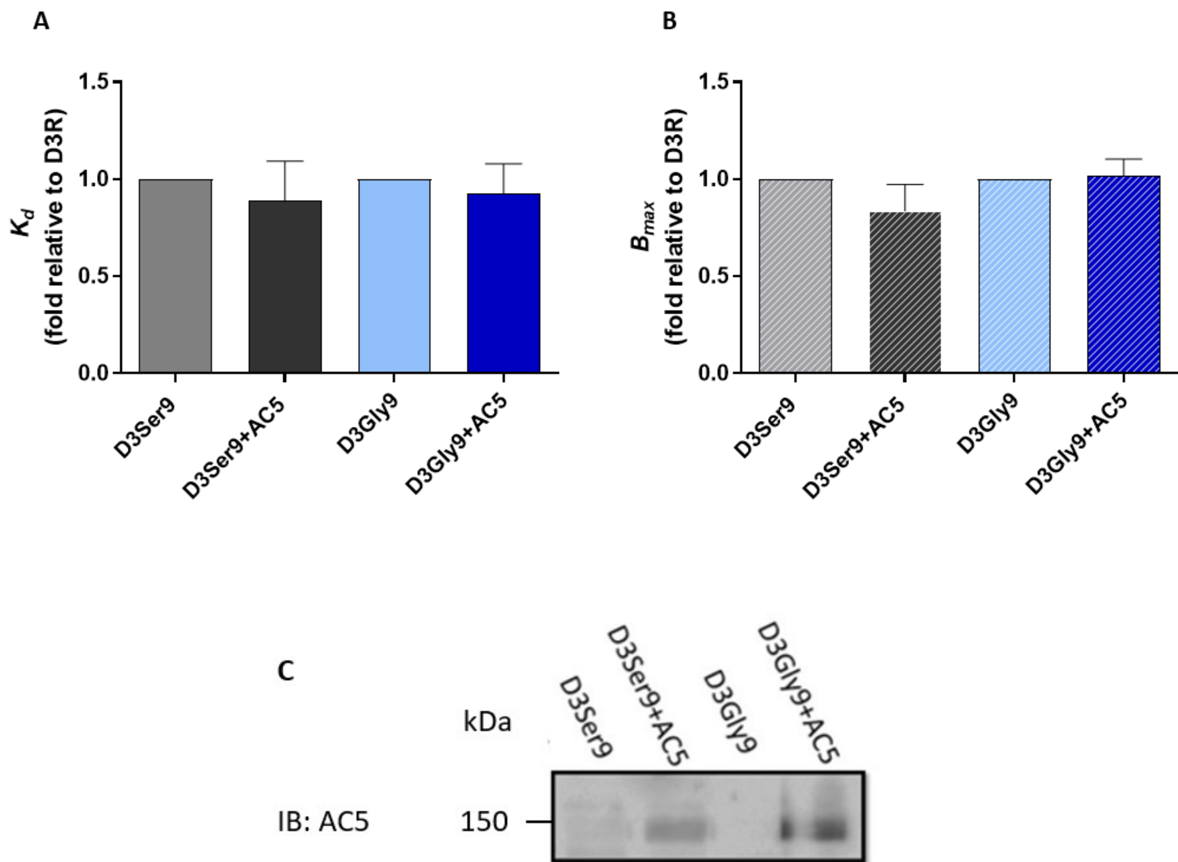

Figure 17. Fold Change Values of Equilibrium Dissociation Constants (K_d) and B_{max} of D3Ser9 and D3Gly9 for [^{3}H]-Spiperone in the Presence and Absence of AC5.

Bars represent the K_d values (A) and B_{max} values for [^{3}H]-spiperone (B) of D3Ser9 and D3Gly9 in the presence of AC5 obtained from saturation curves and expressed as fold change relative to D3Ser9 and D3Gly9 alone. Data were analysed using a one-sample *t*-test. C) Representative blots for membrane preparation from HEK293 cells transfected with D3Ser9 and D3Gly9 in the presence or absence of AC5 using anti-AC5 antibodies as described in Materials and Methods.

Receptor	Dopamine K_i (nM)	Quinpirole K_i (nM)	Raclopride K_i (nM)	Sulpiride K_i (nM)
D3Ser9	13.23 ± 3.73	15.82 ± 4.48	6.36 ± 1.60	17.18 ± 3.72
D3Ser9+AC5	10.64 ± 2.25	12.53 ± 3.67	3.87 ± 1.13	14.51 ± 8.42
D3Gly9	14.44 ± 3.03	19.86 ± 3.72	4.99 ± 1.66	12.70 ± 3.33
D3Gly9+AC5	16.14 ± 5.33	18.49 ± 7.00	3.23 ± 0.69	9.39 ± 4.35

Table 3. Inhibitory Constants (K_i) Values for Agonists (Dopamine and Quinpirole) and Antagonists (Raclopride and Sulpiride) at D3Ser9 and D3Gly9 in the Presence and Absence of AC5.

K_i values obtained from competition curves done in duplicate are shown as means ± SEM of n=5 independent experiments. Statistical analysis showed no significant difference in K_i values of D3Ser9 and D3Ser9+AC5 or D3Gly9 and D3Gly9+AC5.

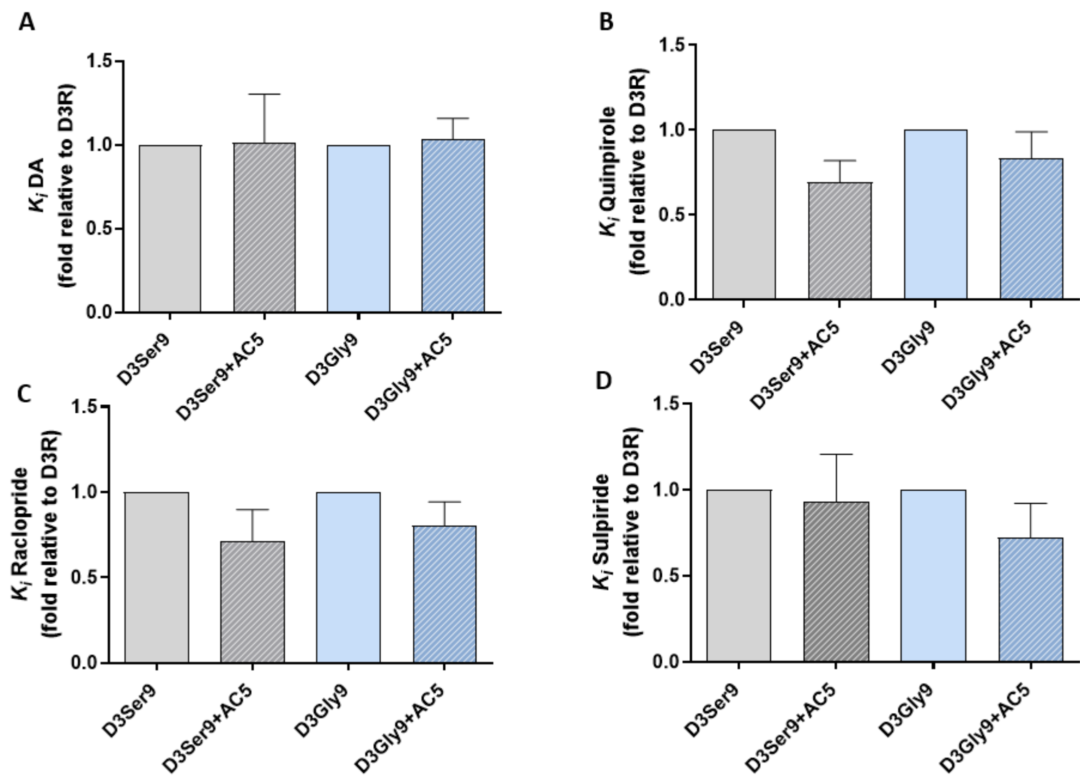

Figure 18. Fold Change Values of Inhibitory Constants (K_i) of Agonists and Antagonists for D3Ser9 and D3Gly9 in the Presence and Absence of AC5.

Bars represent the K_i values of D3Ser9 and D3Gly9 in the presence of AC5 obtained from competition curves and expressed as fold change relative to D3Ser9 and D3Gly9 alone. Data were analysed using a one-sample *t*-test. DA, dopamine

4. Selective Inhibition of AC5 Activity by D3R

As discussed in the Introduction, inhibition of ACs by D3R is subtle in comparison to other D2-class receptors. Moreover, quinpirole-stimulated D3R can specifically inhibit FSK-stimulated cAMP levels in HEK293 cells overexpressing AC5 (Robinson & Caron, 1997). GloSensor-based cAMP assays were used to assess the specific inhibition of AC5 by both D3R polymorphic variants in GloSensor-HEK293T cells co-transfected with D3Ser9 or D3Gly9 and AC5 or Flag-AC6 versus D3Ser9 or D3Gly9 alone. In GloSensor assays, changes in intracellular cAMP levels can be measured as luminescence output resulting from D-luciferin substrate conversion to oxyluciferin. $G\alpha_s$-coupled GPCRs can increase luminescence response following stimulation with agonists, whereas stimulation of $G\alpha_i$-coupled GPCRs decreases cAMP levels in the cells. This change in cellular cAMP levels is hard to detect at a basal cellular condition in which cAMP levels are already low (Kumar et al., 2017). In my studies, FSK and ISO were used to increase basal cellular cAMP levels. FSK is a direct activator of ACs, and ISO increases cAMP levels through endogenously expressed β-adrenergic receptors. I tested the inhibition of FSK and ISO-stimulated AC5 and AC6 activity by both D3R polymorphic variants following activation by quinpirole and DA. DA and quinpirole respectively induced 28 ± 10.9% and 26.4% ± 5.3% inhibition of FSK-stimulated cAMP in cells co-expressing D3Ser9 and AC5. However, in these cells, DA and quinpirole respectively induced 36.4 ± 5.6 % and 20.7 ± 7.9% inhibition under ISO-stimulated cAMP production. In contrast, in cells transfected with D3R alone or with Flag-AC6, DA and quinpirole did not significantly alter the intracellular cAMP levels following treatment with FSK or ISO. On the other hand, the inhibition induced by DA and quinpirole in FSK-treated conditions in cells transfected with D3Gly9 and AC5 were 34.7 ± 6.2% and 36.2 ± 7%, respectively. In addition, inhibition of AC5 activity by DA and quinpirole in cells treated with ISO was 41.4 ± 2.3% and 24.1 ± 6.2%, respectively. Interestingly, no inhibition of endogenous AC activity was detected in cells

expressing D3Gly9 alone or with Flag-AC6 under FSK or ISO-stimulated AC5 conditions (Fig. 19). Overall, the results indicate that D3R mediates inhibition of FSK and ISO-stimulated AC5 activity following activation by DA or quinpirole in cells co-transfected with D3R and AC5. Interestingly, D3Gly9 elicits slightly stronger inhibition of cAMP production in cells treated with FSK and ISO in the presence of AC5 versus D3Ser9, although it was not significant as determined by statistical analysis. In addition, DA induced slightly stronger inhibition of ISO-stimulated AC5 activity in comparison to FSK in cells transfected with D3Ser9 or D3Gly9 and AC5, albeit not statistically significant. Importantly, no inhibition was observed, when D3R was transfected alone or with Flag-AC6. These findings further support a selective inhibition of AC5 isoform by D3R polymorphic variants. In all experiments, the levels of receptor expression between conditions were measured using radioligand binding assays. The expression of AC5 and Flag-AC6 were confirmed using western blot assay (Fig. 19D).

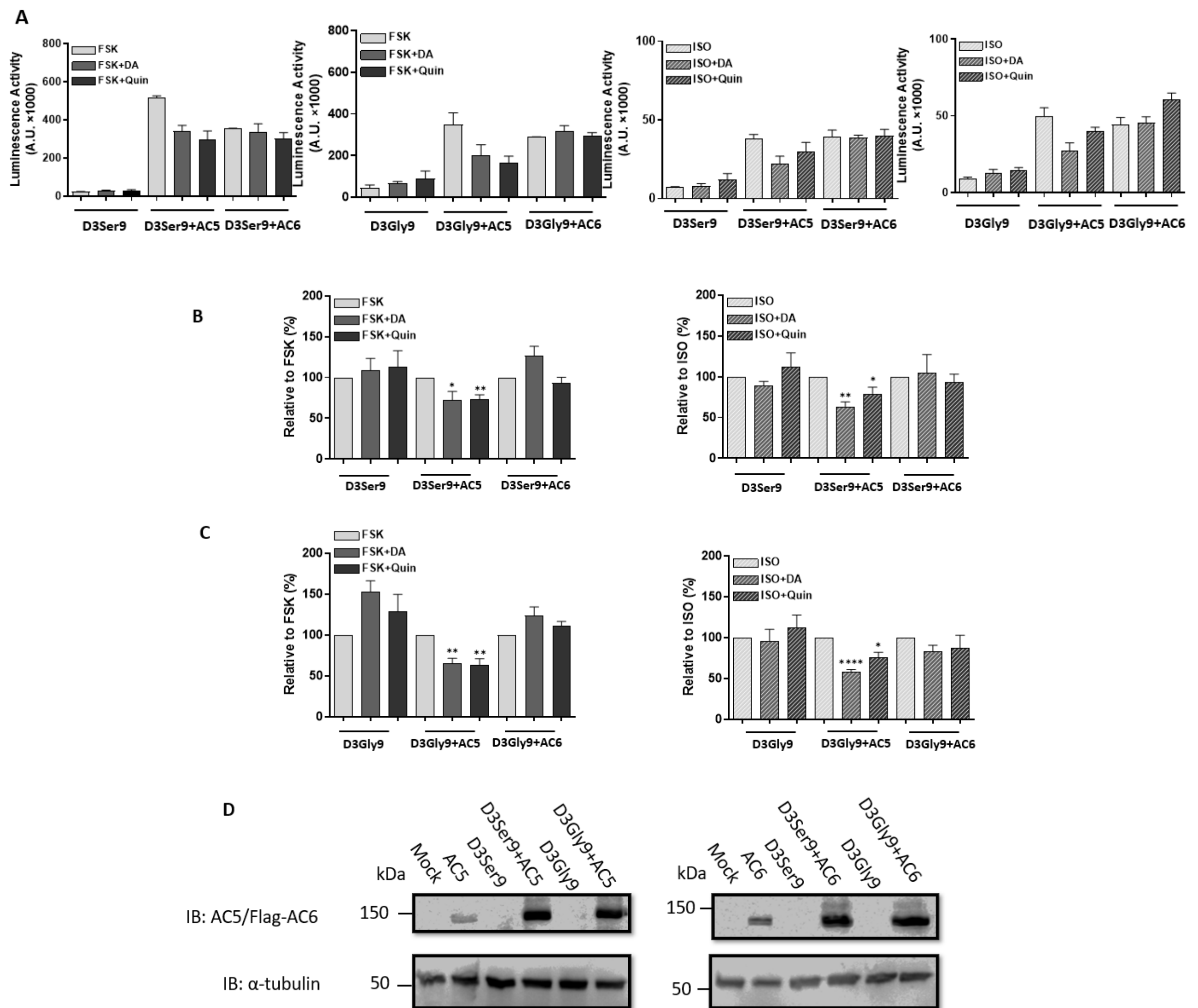

Figure 19. Inhibition of FSK or ISO-stimulated cAMP Production in HEK293T Cells Transfected with D3R in the Presence or Absence of AC5 or Flag-AC6.

HEK293T cells co-transfected with D3Ser9 or D3Gly9 and AC5 or Flag-AC6 versus D3Ser9 or D3Gly9 alone were stimulated with either 10 µM DA or 1 µM quinpirole for 15 min, then followed by 15 min treatment with 5 µM FSK or 1 µM ISO. A) An example of raw data done in triplicates B, C) relative values of inhibition of FSK or ISO stimulated AC5 or 6 activity by DA or quinpirole in cells transfected with D3Ser9 versus D3Ser9+AC5 or Flag-AC6 (B) and D3Gly9 versus D3Gly9+AC5 or Flag-AC6 (C). Bars represent means ± SEM of n=6 independent experiments done in triplicate. Data were normalized as described in experimental procedures. Statistical significance was assessed by one-sample *t*-test (100%). *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ when compared to D3R. D) Representative blots of transfected HEK293T cells using anti-AC5 or anti-FLAG-HRP antibodies as described in Materials and Methods. The B_{max} value in pmol/mg for [^{3}H]-spiperone expressed as means ± SEM were as follows: D3Ser9, 4.303 ± 0.79; D3Ser9+AC5, 4.052 ± 0.77; D3Ser9+Flag-AC6, 4.382 ± 0.66; D3Gly9, 5.108 ± 1.240; D3Gly9+AC5, 4.488 ± 0.68; D3Gly9+Flag-AC6, 5.56 ± 1.11. DA, dopamine (10µM); Quin, quinpirole (1µM); FSK, forskolin (5µM); ISO, isoproterenol (1µM).

5. AC5 Modulates D3R Trafficking

5.1. AC5 Promotes Total Expression and Surface Expression of D3Ser9 and D3Gly9

It has been shown that interactions between Filamin A, protein 4.1N and Paralemmin with the third loop or C-terminal of the D3R can modify surface expression of the D3R (Guo et al., 2010). Herein, I tested the hypothesis that AC5 may control intracellular sorting and trafficking of D3R through its interaction with D3R. To test this hypothesis, HEK293 cells were transfected with Flag-D3Ser9 or Flag-D3Gly9 with and without AC5 and ELISA assessed whether AC5 plays a role in cell surface expression or total expression of the D3R. Cell surface expression of Flag-D3Ser9 and Flag-D3Gly9 increased by about 54% and 46%, respectively in cells expressing AC5 in comparison to cells transfected with Flag-D3Ser9 or Flag-D3Gly9 alone. On the other hand, in Triton-X permeabilized cells, an enhancement in total expression of Flag-D3Ser9 and Flag-D3Gly9 by about 31% and 34% also was observed in the presence of AC5. However, AC5 expression did not cause a significant change in the intracellular levels of Flag-D3Ser9 or Flag-D3Gly9 (Fig. 20).

These results reveal that AC5 promotes an increase in total expression and surface expression of both D3R polymorphic variants. Data obtained from radioligand binding assays show the same levels of receptor expression between conditions. Western blots confirmed expression of AC5.

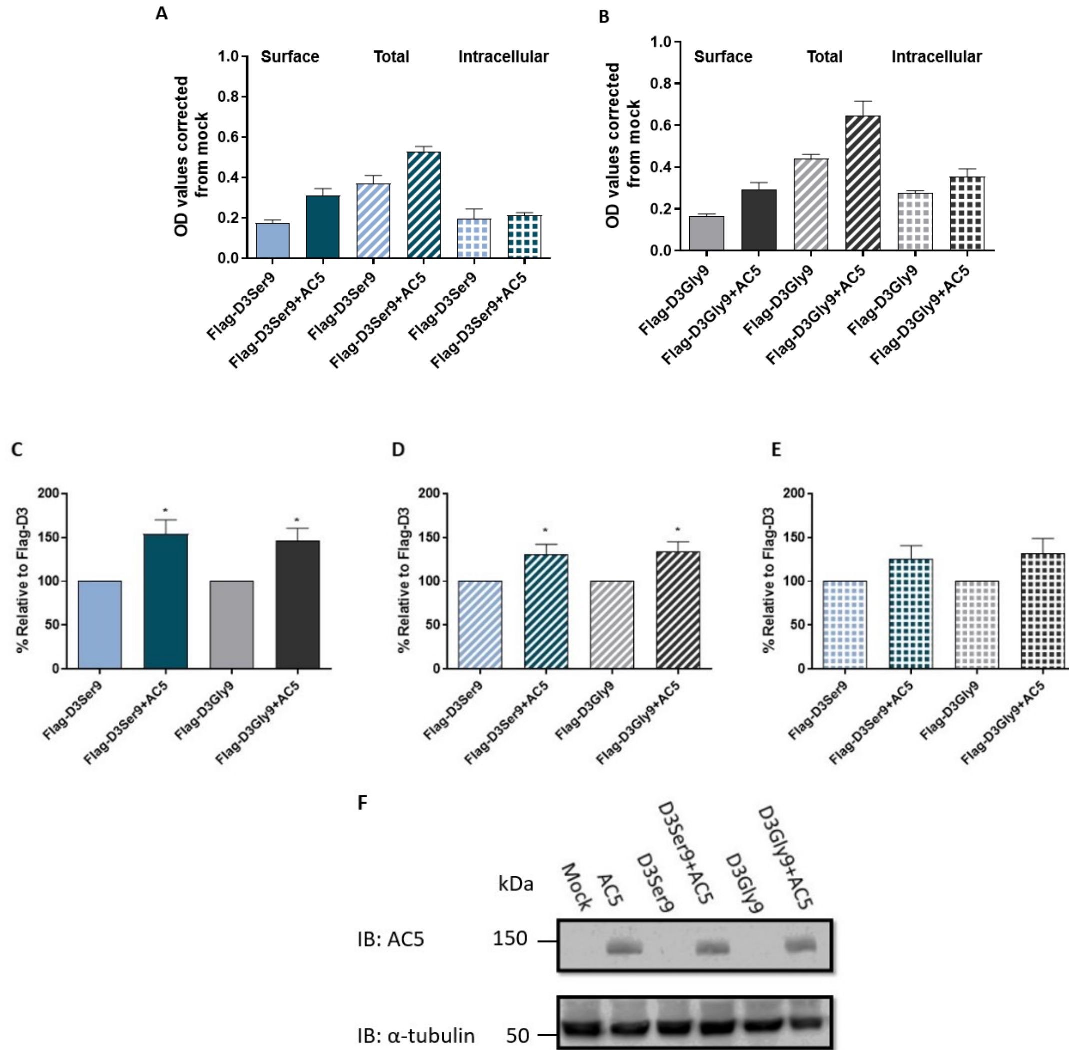

Figure 20. Role of AC5 in the Modulation of Cell Surface Expression and Total Expression of Flag-D3R Polymorphic Variants in HEK293 Cells.

A and B) Representative OD values of one experiment done in triplicate for cell surface and total expression of Flag-D3Ser9 (A) and Flag-D3Gly9 (B). OD values subtracted from mock OD values. OD values for intracellular receptors were obtained from subtracting the corrected cell surface values from the corrected total values. C) Normalized cell surface expression values obtained from ELISA were subtracted from mock and expressed as percentage relative to the D3R. D) Normalized total expression values of the Flag-D3Ser9 and D3Gly9±AC5 after Triton-X permeabilization were subtracted from mock values and expressed as percentage relative to D3R alone. E) Normalized intracellular receptors values were obtained from subtracting the corrected cell surface values from the corrected total values and expressed as percentage relative to D3R alone. Bars represent the means ± SEM of n=7 experiments done in triplicate. Data were analysed using a one-sample *t*-test (100%). *, $p < 0.05$ compared to D3R. F) Representative blots for HEK293 cells transfected with Flag-D3Ser9 and Flag-D3Gly9 with and without AC5 using anti-AC5 antibodies as described in Materials and Methods. B_{max} in pmol/mg membrane proteins for [^{3}H]-spiperone expressed as means ± SEM were as follows: Flag-D3Ser9, 6.19 ± 0.46; Flag-D3Ser9+AC5, 5.59 ± 0.81; Flag-D3Gly9, 5.77 ± 1.05; Flag-D3Gly9+AC5, 5.28 ± 0.76.

5.2.1. AC5 Promotes D3Ser9 but not D3Gly9 Endocytosis Following DA Treatment

As discussed in the Introduction, desensitization of D3R is weakly associated with homologous internalization, while D3R mainly undergoes heterologous desensitization and internalization (Cho et al., 2007). To test whether AC5 can modulate D3R internalization, HEK293 cells were transfected with Flag-D3Ser9 or Flag-D3Gly9 with and without AC5 and were stimulated with either vehicle or DA for 15 min and then subjected to ELISA. Following DA stimulation, Flag-D3Ser9 internalization was found to be 2-fold greater in the presence of AC5 (26.8 ± 3.1 %) compared to Flag-D3Ser9 alone (12.1± 2.4 %), while no change in internalization of Flag-D3Gly9 was observed with (9.1 ± 2.8 %) and without (9 ± 2.5 %) AC5. Overall, data obtained from ELISA show that AC5 promotes D3Ser9 internalization following DA treatment. In contrast, no effect was observed in D3Gly9 internalization by AC5 following DA exposure (Fig. 21).

5.2.2. AC5 Mediates DA-Induced D3Ser9 Endocytosis in Dynamin and β-arrestin 2 Dependent Process

To assess the molecular players involved in AC5-induced internalization of D3Ser9, the effect of dynamin and β-arrestin 2 were evaluated. HEK293 cells were transfected with dominant-negative mutants of dynamin I (K44A) or β-arrestin 2 (V54D) as along with Flag-D3Ser9 with or without AC5. Co-expression of dyn-1-K44A with AC5 significantly decreased (~2.5-fold) AC5-facilitated DA-induced Flag-D3Ser9 internalization, while no significant difference was observed between this condition and cell transfected with Flag-D3Ser9 and dyn-1-K44A. Taken together, the results show that co-expression of dyn-1-K44A markedly inhibits the AC5-facilitated DA-induced internalization of D3Ser9, suggesting that functional dynamin is required for AC5-facilitated internalization of D3Ser9 following DA activation (Fig. 22).

Moreover, over expression of β-arrestin 2-V54D inhibited AC5-facilitated Flag-D3Ser9 internalization following DA treatment (Fig. 23). This suggests that AC5 failed to facilitate DA-induced Flag-D3Ser9 internalization in the presence of dominant-negative mutant of β-arrestin 2. This suggests that DA-mediated endocytosis of Flag-D3Ser9 is dependent on β-arrestin 2. The effect of overexpressed WT β-arrestin 2 was also assessed to test whether more internalization can occur in the presence of exogenous β-arrestin 2 in Flag-D3Ser9 conditions. Interestingly, no significant effect was observed in cells transfected with Flag-D3Ser9, AC5 and β-arrestin 2 in comparison to cells transfected with Flag-D3Ser9 and AC5. This suggests that endogenous β-arrestin 2 expressing in HEK293 cells is sufficient to induce internalization of D3Ser9 but required the presence of AC5 to enable DA-induced D3R internalization (Fig. 23).

Furthermore, to test whether overexpression of WT β-arrestin 2 can promote internalization of Flag-D3Gly9 by AC5, cells were transfected with WT β-arrestin 2 as well as Flag-D3Gly9 with or without AC5. The same levels of Flag-D3Gly9 internalization (~10%) in all conditions were observed. These results show that AC5 failed to promote D3Gly9 internalization even in the presence of overexpression of β-arrestin 2. Thus, overexpression of β-arrestin 2 had no effect on D3Gly9 internalization (Fig. 23).

Herein, results obtain from ELISA studies imply that AC5 facilitates DA-induced internalization of D3Ser9 through a dynamin and β-arrestin 2-dependant process without playing a role in D3Gly9 internalization.

5.2.3. AC5 Attenuates PMA-Induced D3Ser9 Internalization without any Effect on D3Gly9 Internalization

I also further investigated the role of AC5 in heterologous (PKC-induced) internalization of D3R. To determine whether AC5 can modulate PKC-mediated internalization of D3R,

transfected cells were treated with 1 μM PMA versus vehicle for 15 min. Same levels of internalization for Flag-D3Ser9 (38.4 ± 6.7 %), Flag-D3Gly9 (38.5 ± 3.1 %), and Flag-D3Gly9+AC5 (39.3 ± 4.8 %) were observed, while Flag-D3Ser9 with AC5 displayed 17.9 ± 2.7 % internalization, suggesting that AC5 significantly reduces PMA-induced internalization (20.6 ± 6.3%, ~ 2-fold). These results thus reveal that AC5 does not play a role in PMA-induced internalization of D3Gly9, however it significantly reduces the internalization of D3Ser9 under PKC activation (Fig. 24). Thus, data obtained from ELISA studies suggest that AC5 modulates DA-induced internalization of D3Ser9, and PMA-induced heterologous internalization of D3Ser9, while having no effect on D3Gly9 polymorphic variant.

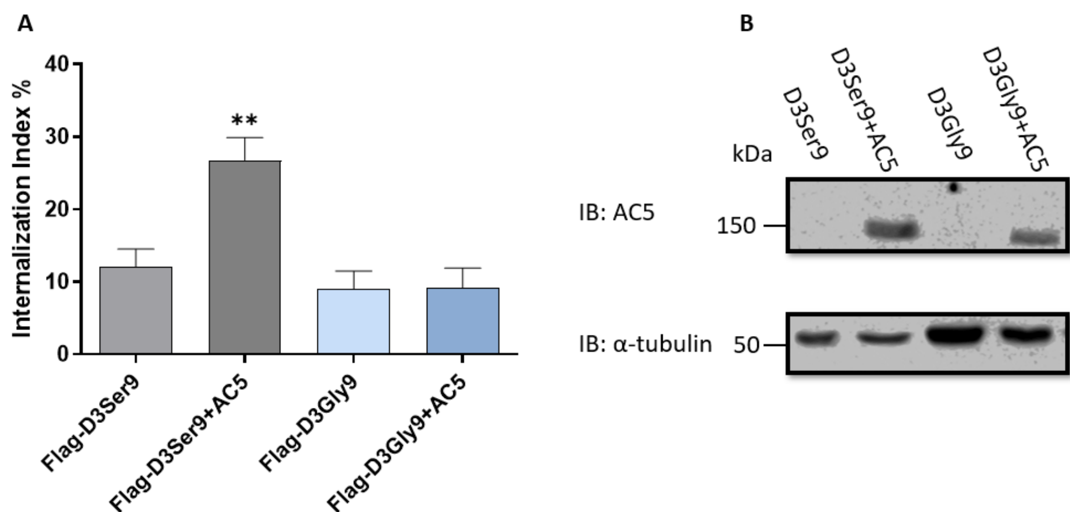

Figure 21. Internalization of Flag-D3R Alone or with AC5 Expressed in HEK293 Cells. HEK293 cells transfected with Flag-D3Ser9 or Flag-D3Gly9 with and without AC5 were stimulated with either 10 μM DA or 0.1 mM AA for 15 min then subjected to ELISA. A) Internalization is represented as the % decrease in cell surface expression following 15 min treatment with DA relative to vehicle. Bars represent the means ± SEM from nine independent experiments done in triplicate. Data were analysed using two-way ANOVA, followed by Tukey multiple comparison test. **, $p < 0.01$ compared to Flag-D3Ser9. B) Representative blots for HEK293 cells transfected with Flag-D3Ser9 or Flag-D3Gly9 ± AC5 using anti-AC5 antibodies as described in Material and Methods. B_{max} values (pmol/mg of membrane proteins) for [^{3}H]-spiperone in means ± SEM expressed were as follows: Flag-D3Ser9, 4.46 ± 0.38; Flag-D3Ser+AC5, 3.80 ± 0.5; Flag-D3Gly9, 3.07 ± 0.54; Flag-D3Gly9+AC5, 3.05 ± 0.47.

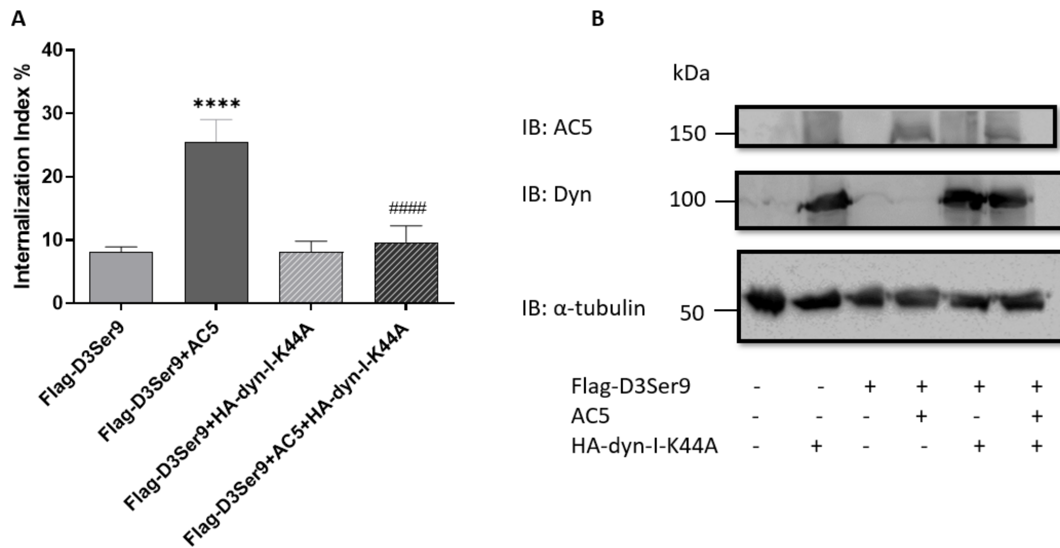

Figure 22. Role of Dynamin in AC5-Facilitated Internalization of Flag-D3Ser9 in HEK293 Cells.

HEK293 cells transfected with Flag-D3Ser9 with and without AC5 and HA-dyn-I-K44A were stimulated with either 10 μM DA or 0.1 mM AA for 15 min then subjected to ELISA. A) Internalization represented as the % decrease in cell surface expression following 15 min treatment with DA relative to vehicle in HEK293 cells transfected with Flag-D3Ser9 with and without AC5 and dominant-negative mutant of dynamin (K44A). Bars represent the means ± SEM from five independent experiments done in triplicate. Data were analysed using two-way ANOVA, followed by Tukey multiple comparison test. ****, $p < 0.0001$ when compared to Flag-D3R and ####, $p < 0.0001$ when compared to Flag-D3R+AC5. B) Representative blots for HEK293 cells transfected with Flag-D3Ser9 ± AC5 and HA-dyn-1-K44A using anti-AC5, and anti-dynamin-1 antibodies as described in Material and Methods. B_{max} values (pmol/mg of membrane proteins) for [^{3}H]-spiperone in means ± SEM were as follows: Flag-D3Ser9, 4.24 ± 1.52; Flag-D3Ser9+AC5, 3.95 ± 0.75; Flag-D3Ser9+HA-dyn-I-K44A, 3.46 ± 1.15; Flag-D3Ser9+AC5+ HA-dyn-I-K44A, 3.12 ± 0.60.

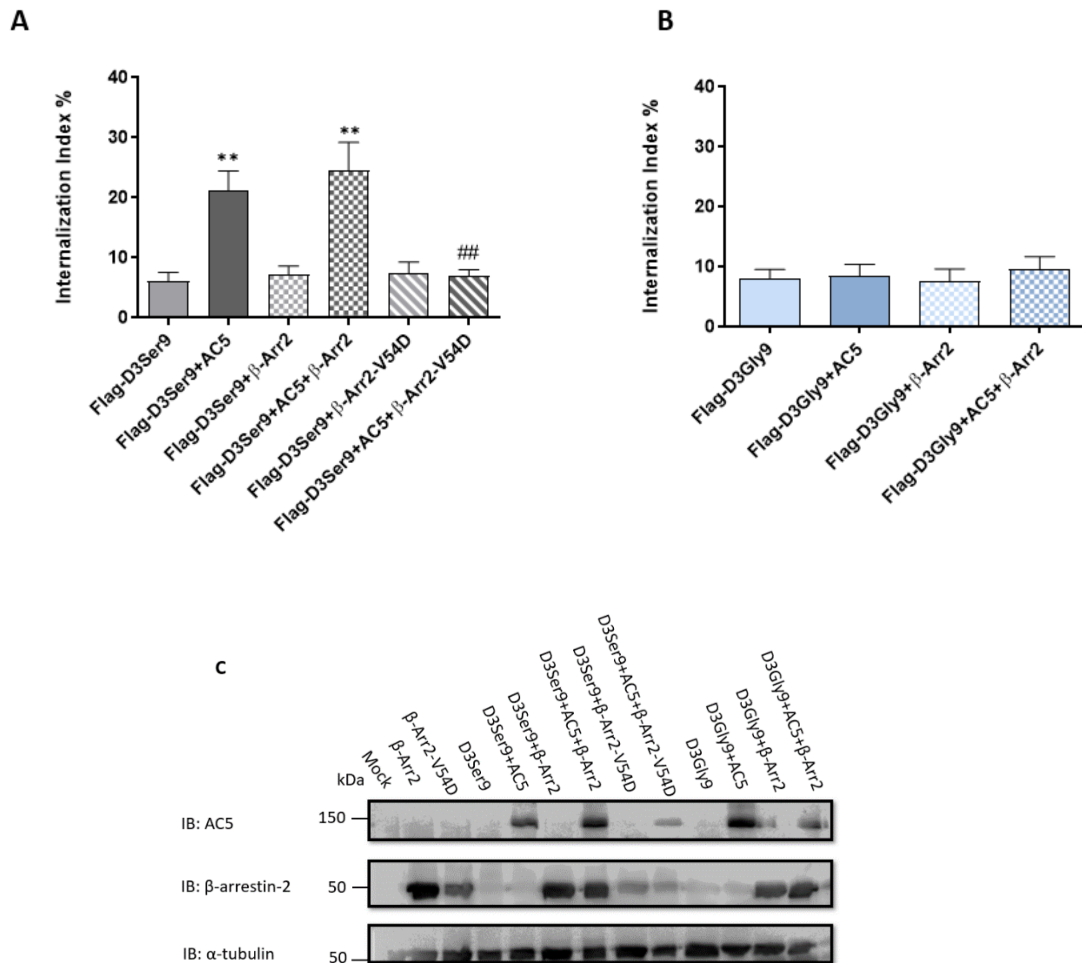

Figure 23. Role of β-arrestin 2 in AC5-Facilitated Internalization of Flag-D3Ser9 or Flag-D3Gly9 in HEK293 Cells.

A, B) Internalization is represented as the % decrease in cell surface expression following 15 min treatment with 10 µM DA relative to vehicle. HEK293 cells transfected with Flag-D3Ser9 and dominant-negative mutant of β-arrestin 2-V54D or WT β-arrestin 2 with and without AC5 (A) and Flag-D3Gly9 and WT β-arrestin 2 with and without AC5 (B). Bars represent the means ± SEM from six independent experiments done in triplicate. Data were analysed using two-way ANOVA, followed by Tukey multiple comparison test. **, $p < 0.01$ as compared to Flag-D3R and Flag-D3R+ β-arrestin 2 and ##, $p < 0.01$ as compared to Flag-D3R+AC5. C) Representative blots for HEK293 cells transfected with Flag-D3Ser9 or Flag-D3Gly9 ± AC5 and β-arrestin 2 or β-arrestin 2-V54D using anti-AC5, and anti-β-arrestin 2 antibodies as described in Material and Methods. B_{max} values (pmol/mg of membrane proteins) for [^{3}H]-spiperone or [N-metyl-^{3}H]-spiperone expressed as means ± SEM were: Flag-D3Ser9, 3.02 ± 0.52; Flag-D3Ser9+AC5, 3.04 ± 0.36; Flag-D3Ser9+β-arrestin 2, 2.23 ± 0.47; Flag-D3Ser9+AC5+β-arrestin 2, 3.02 ± 0.59; Flag-D3Ser9+β-arrestin 2-V54D, 3.07 ± 0.96; Flag-D3Ser9+AC5+β-arrestin 2-V54D, 2.91 ± 0.76; Flag-D3Gly9, 2.24 ± 0.62; Flag-D3Gly9+AC5, 2.48 ± 0.34; Flag-D3Gly9+β-arrestin 2, 2.78 ± 0.55; Flag-D3Gly9+AC5+β-arrestin 2, 2.42 ± 0.51.

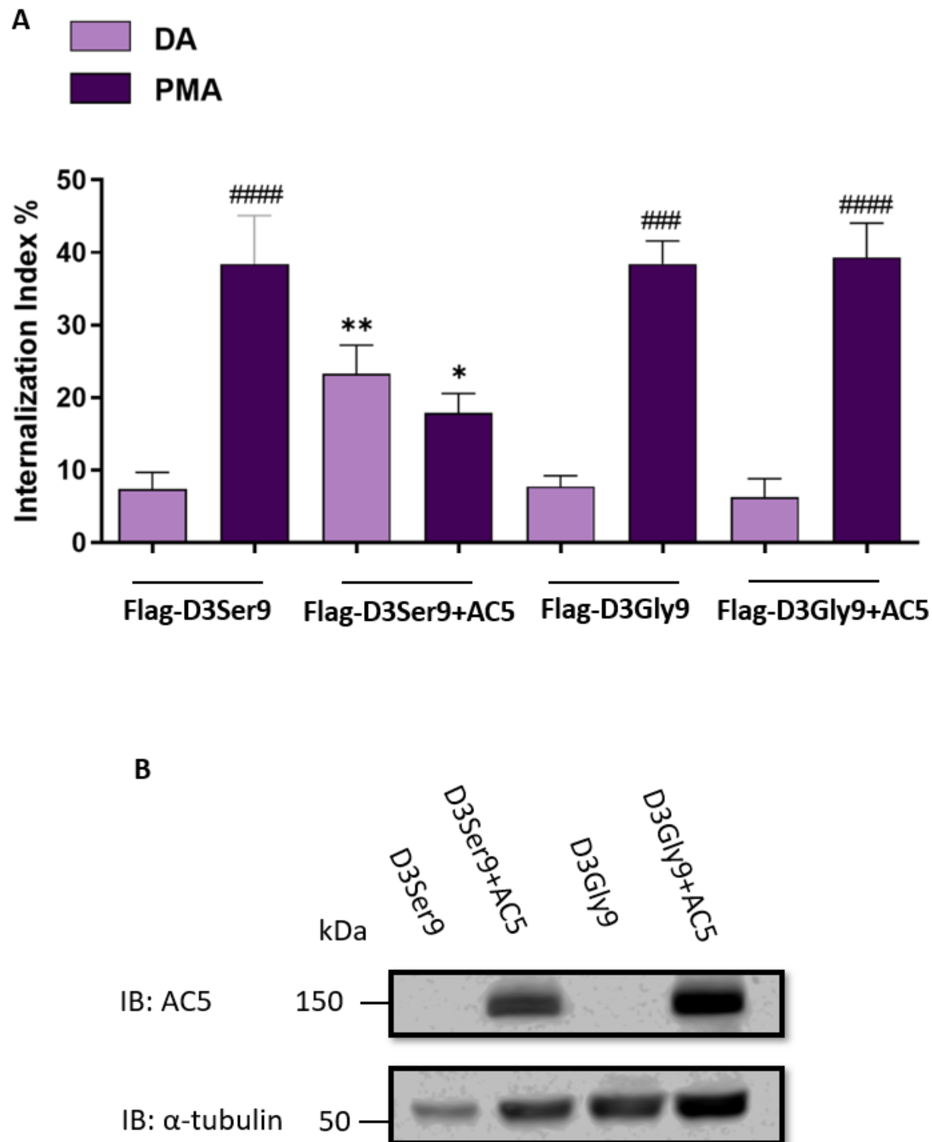

Figure 24. Comparison of the Effect of AC5 on D3R Internalization upon DA Stimulation Versus PKC Activation in HEK293 Cells.

A) Internalization is represented as the % decrease in cell surface expression following 15 min treatment with either 10 μM DA or 1 μM PMA relative to vehicle (AA or DMSO) in HEK293 cells transfected with Flag-D3Ser9 or Flag-D3Gly9 with and without AC5. Bars represent the means ± SEM from five independent experiments done in triplicate. Data were analysed using two-way ANOVA, followed by Tukey multiple comparison test. *, $p < 0.05$; **, $p < 0.01$ when compared to Flag-D3Ser9 upon PMA and DA treatment, respectively. ###, $p < 0.001$; ####, $p < 0.0001$ when compared to DA. B) Representative blots for HEK293 cells transfected with Flag-D3Ser9 or Flag-D3D3Gly9 ± AC5 using anti-AC5 antibodies as described in Material and Methods. B_{max} values (pmol/mg of membrane proteins) for [^{3}H]-spiperone or [N-metyl-^{3}H]-spiperone in means ± SEM were Flag-D3Ser9, 3.29 ± 0.46; Flag-D3Ser9+AC5, 2.75 ± 0.15; Flag-D3Gly9, 2.46 ± 0.49; Flag-D3Gly9+AC5, 2.61 ± 0.39.

6. Interaction Between D3R, AC5 and β-arrestin 2 under Basal and DA Stimulation

Data obtained from ELISA showed the involvement of β-arrestin 2 in AC5-facilitated D3Ser9 internalization upon DA stimulation. Moreover, previous studies have shown that β-arrestin 2 is required for pharmacological sequestration of the D3R (Min et al., 2013; Xu et al., 2019; Zheng et al., 2020). Considering the results obtained above and the role of β-arrestin in receptor signaling regulation as discussed earlier, the interaction between β-arrestin 2, AC5 and D3R was examined in more details.

Co-IP/ IB was performed in HEK293 cells transfected with HA-D3Ser9 or HA-D3Gly9 with and without Flag-AC5 and β-arrestin 2 to test the interaction between them. HA-D3R was immunoprecipitated and β-arrestin 2 in HA immunocomplexes was detected with anti-β-arrestin 2 antibodies in HEK293 cells co-transfected with D3Ser9 or D3Gly9 in the absence and presence of AC5. However, no β-arrestin 2 band was observed in empty pCMV5 vector (mock) (Fig. 25A). To validate the expression of β-arrestin 2 and to confirm the amount of protein loaded, inputs of lysates were probed with anti-β-arrestin 2 and anti-α-tubulin antibodies (Fig. 25B and C). Radioligand binding assays were used to determine D3R expression. The results reveal a constitutive interaction between both D3R polymorphic variants and β-arrestin 2 in cells co-transfected with HA-D3R and β-arrestin 2 with and without Flag-AC5.

To assess whether receptor activation can modify the interaction, cells were treated with 10 μM DA for 15 min and compared to vehicle treatment. Flag-AC5 was detected in HA immunocomplexes in HEK293 cells co-transfected with HA-D3R and Flag-AC5 in the presence and absence of β-arrestin 2 under basal and DA exposure (Fig. 26A). In addition, Flag-AC5 band was not detected in cells transfected with Flag-AC5, HA-D3R, β-arrestin 2 and empty pCMV5 vector (mock). The HA-D3R was probed in immunocomplexes using anti-HA

antibodies to confirm HA-D3R was immunoprecipitated (Fig. 27B). On the other hand, β-arrestin 2 was detected in HA immunocomplexes in cells transfected with HA-D3Ser9 or HA-D3Gly9, β-arrestin 2 with and without Flag-AC5 under basal and DA exposure (Fig. 26C). To confirm the expression of Flag-AC5 and β-arrestin 2, and the amount of protein loaded, inputs of lysates were probed with anti-Flag-HRP, anti-β-arrestin 2, and anti-α-tubulin antibodies (Fig. 26 D, E, F). Radioligand binding assays were used to determine D3R expression. The results suggest that both HA-D3R polymorphic variants interact with β-arrestin 2 in the presence and absence of Flag-AC5 under both basal and DA stimulated conditions. Furthermore, densitometric analysis showed that activation of D3R by DA does not significantly modify the interaction between HA-D3R•Flag-AC5 and β-arrestin 2.

Overall, the results suggest that there is a complex formation between D3R, AC5 and, β-arrestin 2 under basal and DA stimulation conditions.

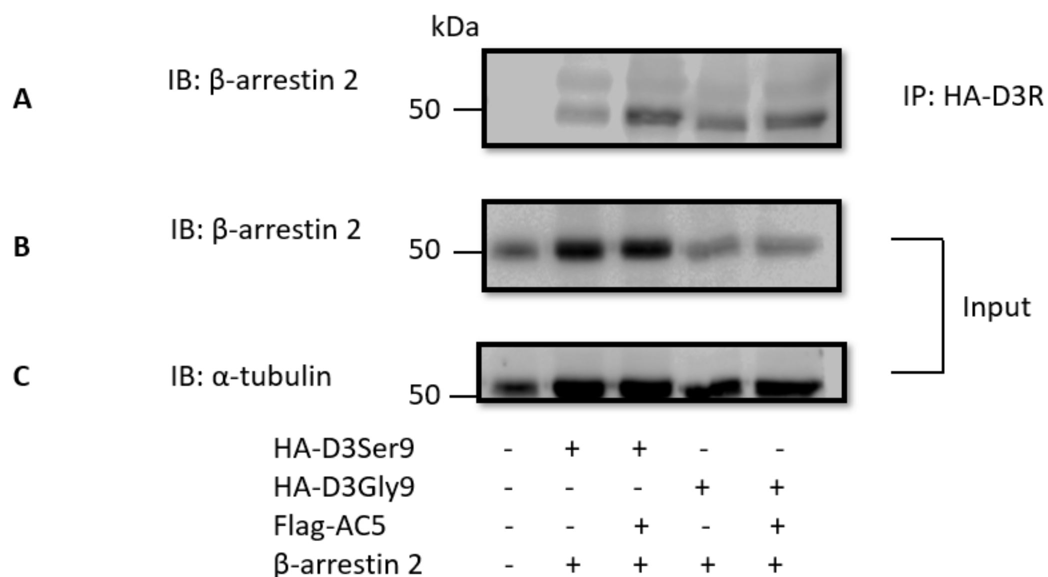

Figure 25. Complex Formation between HA-D3Ser9 or HA-D3Gly9 and β-arrestin 2 in Cells with and without Flag-AC5.

A representative example of a co-IP/IB experiment using HEK293 cells transfected with HA-D3R and β-arrestin 2 with and without Flag-AC5. A) D3R was immunoprecipitated using HA-conjugated beads and β-arrestin 2 was probed with anti-β-arrestin 2 antibodies. B, C) β-arrestin 2 and α-tubulin inputs were probed using anti-β-arrestin 2 and anti α-tubulin antibodies, respectively. B_{max} values (pmol/mg of membrane proteins) for [N-metyl-^{3}H]-spiperone in cells transfected with HA-D3Ser9+β-arrestin 2, HA-D3Ser9+Flag-AC5+β-arrestin 2, HA-D3Gly9+β-arrestin 2 and HA-D3Gly9+Flag-AC5+β-arrestin 2 were 2.66, 2.54, 2.42, 3.03, respectively. IP, immunoprecipitation; IB, immunoblot

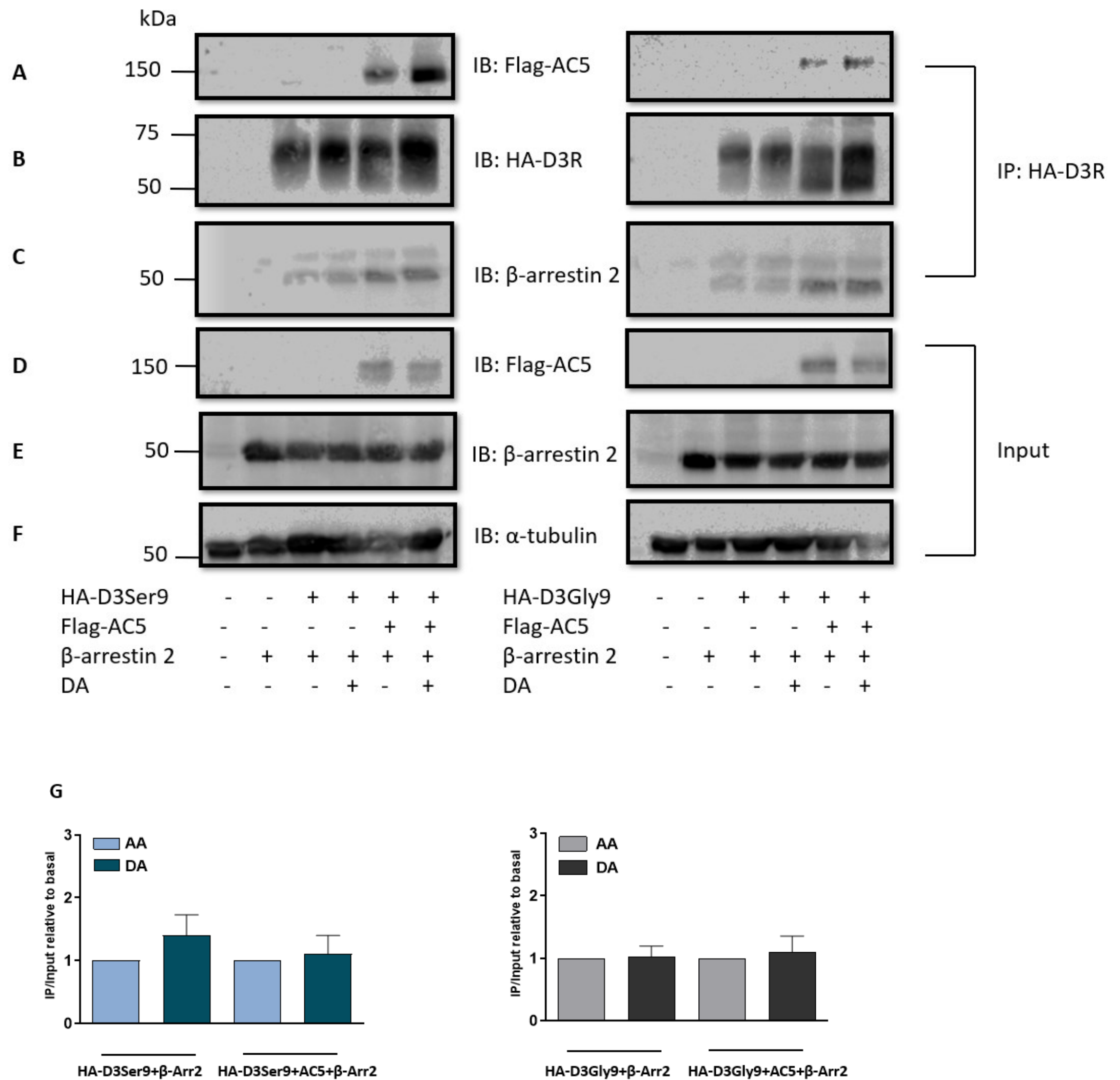

Figure 26. Complex Formation between HA-D3Ser9 or HA-D3Gly9 and β-arrestin 2 in Cells with and without Flag-AC5 under Basal and DA Stimulation.

A representative example of a co-IP/IB experiment using HEK293 cells transfected with HA-D3Ser9 or D3Gly9 and β-arrestin 2 with and without Flag-AC5. A, B and C) D3R was immunoprecipitated using HA-conjugated beads and Flag-AC5, HA-D3R and β-arrestin 2 were probed with anti-FLAG-HRP, anti-HA and anti-β-arrestin 2 antibodies. D, E and F) Flag-AC5, β-arrestin 2 and α-tubulin inputs were probed using anti-Flag-HRP, anti-β-arrestin 2 and anti α-tubulin antibodies. G) Densitometric analysis of the immunoreactive bands in HA immunocomplexes were corrected from β-arrestin 2 inputs and then normalized relative to basal. Bars represent means ± SEM of five independent experiments. Statistical analysis was done using a one-sample *t*-test. B_{max} values (pmol/mg of membrane proteins) for [^{3}H]-spiperone or [N-metyl-^{3}H]-spiperone in means ± SEM were as follows: HA-D3Ser9+β-arrestin 2, 2.65 ± 0.85; HA-D3Ser9+Flag-AC5+β-arrestin 2, 2.86 ± 0.74; HA-D3Gly9+β-arrestin 2, 2.41 ± 0.74; HA-D3Gly9+Flag-AC5+ β-arrestin 2, 3.50 ± 0.49. DA, dopamine; IP, immunoprecipitation; IB, immunoblot

Discussion

My thesis studies have demonstrated the formation of protein complex between D3R and AC5 *in vivo* and *in vitro*. Reciprocal co-IP revealed that activation of D3R by DA significantly increases the abundance of D3R•AC5 protein complex. However, interaction between D3R and AC5 does not change ligand binding properties and expression of the D3R as determined using radioligand binding studies. On the other hand, ELISA and GloSensore studies showed that AC5 can modulate D3R surface expression, internalization, and cAMP production. Indeed, a potential tripartite interaction between D3R, AC5 and β-arrestin 2 has been shown under basal and DA stimulation conditions.

Herein, I discuss possible mechanisms underlying the interaction between D3R and AC5, and the effect it may play on D3R trafficking and signaling as well as future studies to expand the findings presented in my thesis.

1. Possible Models of Interaction Between D3R and AC5

Data obtained from co-IP assay revealed the constitutive interaction between D3R and AC5. Two potential models can explain D3R•AC5 interaction. First, a hetero-dimerization could occur either in ER where they are synthesized, folded, and assembled or in late endosomes/multivesicular bodies, since these organelles are important for transport and sorting of proteins coming from the Golgi apparatus. Thus, the D3R•AC5 heterodimer could be transported from the Golgi apparatus through sorting in the trans Golgi network and vesicles to the plasma membrane. The second model is based on the proximity of proteins without pre-existing interactions. This suggests that D3R and AC5 form a protein complex on the plasma membrane (Fig. 27).

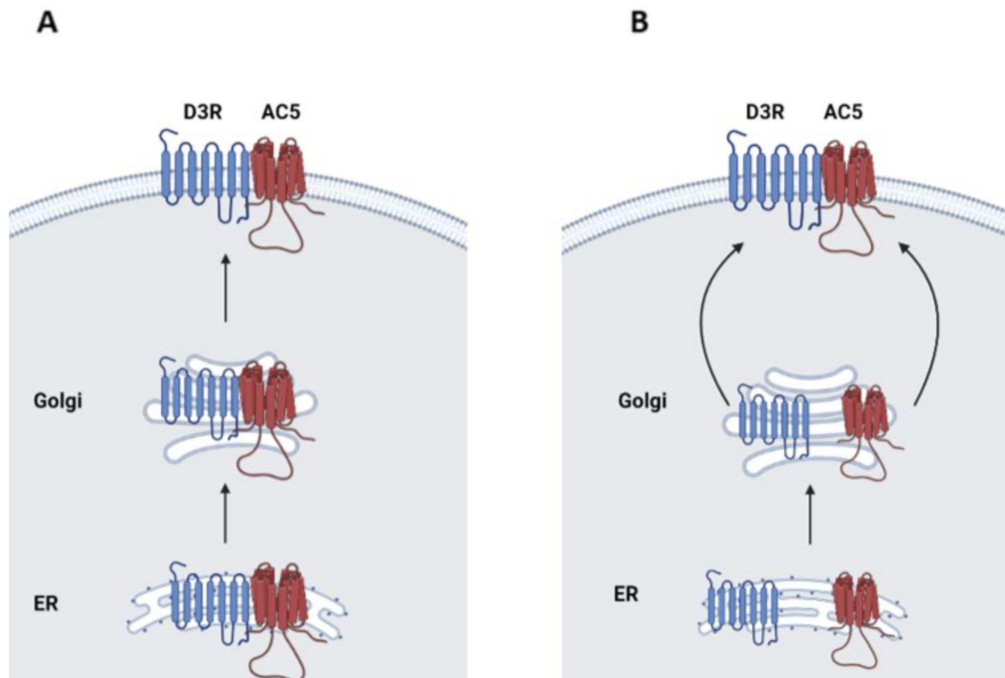

Figure 27. Hypothetical Models of Constitutive Interaction Between D3R and AC5.
A) Model 1: D3R•AC5 complex formation occurs in ER or Golgi apparatus. Thus, the D3R and AC5 protein complex is transported together as a heterodimer to the plasma membrane. B) Model 2: D3R and AC5 are transported independently from Golgi to the cell surface, where they form a protein complex. Created with BioRender.com.

The results also showed that DA significantly increases the abundance of complex formation between D3R and AC5. Two hypothetical models can be suggested to explain the effect of DA on D3R•AC5 heterodimerization. In model 1, the constitutive interaction may not be stable enough meaning that D3R and AC5 associate and dissociate quickly, but DA can stabilize this dimer. Thus, DA treatment helps the complex to become more stable or reduces the dynamic dissociation of the complex. In the second model, I hypothesize the presence of two intracellular pools of the D3R in the cells. The first D3R pool is associated with AC5 under basal condition and the second pool interacts with AC5 under DA exposure. It is not clear which of these possible models can explain the heterodimerization of D3R and AC5. Indeed, further investigation is required to identify the dynamic interaction between D3R and AC5. Moreover, further studies can be done to address the interaction sites between D3R and AC5. The involvement of intracellular domains of D3R (specificality IL3 and C-terminal domain) and AC5 to interact with other proteins highlights the importance of intracellular regions as the most possible interacting sites between D3R and AC5. However, the involvement of TMs of D3R and AC5 can not be ignored since the functional pre-coupled complexes of heteromers of $A_{2A}R$ and D2R homodimers coupled to their cognate $G\alpha_s$ and $G\alpha_i$ proteins and to AC5 have been shown through TMs from the receptors and the AC (Navarro et al., 2018).

2. The Impacts of D3R•AC5 Complex Formation on cAMP Production, Surface Expression, and Internalization

2.1. Potential Mechanisms of Selective Inhibition of AC5 by D3R

Interestingly, radioligand binding assays revealed that D3R•AC5 complex formation does not alter neither the affinity of the D3R for [^{3}H]-spiperone nor D3R expression level (B_{max} of radioligand).

Inhibition of ACs by D3R is weak and often undetectable in comparison to other D2-class subtypes. However, D3R specifically inhibits AC5 activity (Robinson & Caron, 1997; Zaworski et al., 1999). The results obtained from GloSensor assays showed that activation of D3Ser9 and D3Gly9 by DA or quinpirole leads to a significant reduction in the FSK and ISO-stimulated AC5 activity in cells transfected with D3Ser9 or D3Gly9 and AC5 in comparison to the cells transfected with D3Ser9 or D3Gly9 alone or with Flag-AC6. An interaction between D3R and AC5 may explain the specific inhibition of AC5 activity by D3R, which means that physical interaction between D3R and AC5 is necessary to inhibit cAMP production. It means that D3R alone is not able to couple $G\alpha_{i/o}$ and subsequently inhibits ACs activity as does other D2-class receptors. On the other hand, D3R needs to interact with AC5 initially. It has been shown that $G\alpha_i$ interacts with catalytic C1 domain of AC5 (Dessauer et al., 1998). Therefore, it is possible that AC5 acts as a scaffold for $G\alpha_{i/o}$ and helps D3R to couple to $G\alpha_{i/o}$ and induce inhibition of AC5. Studies also have shown that D3R can couple to $G\alpha_z$, and, albeit poorly, to $G\alpha_i$, but unlike D2R and D4R, it also couples to the stimulatory G protein, $G\alpha_s$ (Ilani et al., 2002; Obadiah et al., 1999; Robinson & Caron, 1997). Coupling of the D3R to both stimulatory and inhibitory G proteins leads to a minimal net effect. Therefore, the D3R•AC5 protein complex potentially helps D3R to adopt an active conformation to rather coupling to $G\alpha_i$ than $G\alpha_s$ and induce inhibition of cAMP production through $G\alpha_i$. However, it is also possible that $G\alpha_{i/o}$ acts as a driving force for D3R•AC5 complex formation and promotes D3R•AC5 interaction. Further investigation is required to clarify whether D3R•AC5 interaction is dependent on $G\alpha_{i/o}$.

2.2. Possible Mechanisms of the Effect of AC5 on Surface Expression of D3R

In addition, ELISA results showed that AC5 increases the D3R on the cell surface. Previous studies have suggested that GPCR association with ER chaperone proteins such as calnexin, calreticulin regulates GPCR export by stabilizing receptor conformation and promoting their

delivery to the plasma membrane. Moreover, several studies have indicated that the transport of some GPCRs from the ER to the cell surface requires dimerization (Duvernay et al., 2005). Namely, α1B/α1D-adrenergic receptors heterodimers promote surface expression of α1D-adrenergic receptor (Hague et al., 2004). It has also been shown that homodimerization of the β2AR is intimately linked to its ER export and cell surface trafficking (Salahpour et al., 2004). In addition, specific conserved motifs may modulate GPCR export from the ER. Finally, GPCRs migration to the Golgi and the cell surface after exiting from the ER is mediated through distinct pathways, in which Ras-like Rab GTPases and glycosylation may play an important role (Duvernay et al., 2005). Previous studies have highlighted the important role of protein dimerization with D3R in cell surface expression of the D3R. Namely, an interaction of D3R with Filamin A or protein 4.1N is required for localization of D3R on plasma membrane (Lin et al., 2001, Binda et al., 2002). In contrast, D3R dimerization with paralemmin or D3R splice variant D3nf reduces the surface expression of the D3R, leading to mislocalization of D3R from the plasma membrane to an intracellular compartment (Basile et al., 2006; Karpa et al., 2000).

Promoting surface expression of the D3R in the presence of AC5 can be explained by the following possibilities. First, the D3R and AC5 could be in a pre-existing complex when they are transferred from the Golgi. Thus, D3R•AC5 heterodimerization is required for D3R migration from Golgi to the cell surface. On the other hand, AC5 could act as a shuttle to increase D3R transport to the surface of the cells. Another possibility is that AC5 acts as a scaffold for other proteins involved in D3R migration to the cell surface. Finally, transmembrane AC5 could induce more recruitment of D3R to the cell surface, meaning that AC5 modifies the subcellular localization of D3R. This could happen whether by accelerating the D3R maturation processes, such as improvement of glycosylation or palmitoylation or by

acting as a scaffold for other proteins involved in surface expression of the D3R such as Filamin A.

It is possible that the D3R•AC5 interaction induces a signal transduction through AC5 promoting D3R incorporation at the cell surface, thus increasing D3R surface abundance and selective D3R-mediated inhibition of AC5 activity. Additionally, AC5 could stabilize D3R conformation through the interaction and promote their delivery to the plasma membrane, enabling D3R induced inhibition of cAMP production through coupling to $G\alpha_{i/o}$ subunits upon activation with DA and quinpirole.

Considering the same levels of receptor expression in the presence and absence of AC5 obtained from saturation studies and same intracellular levels of receptors under basal condition observed in ELISA studies, the enhancement in total expression could be related to higher surface expression of the receptor in the presence of AC5. It is possible that a pool of D3R is presented at the cell surface, which cannot bind spiperone properly in binding assays.

2.3. The Biased effect of AC5 on D3Ser9 Internalization Versus D3Gly9

It has been shown that DA-induced a subtle D3R homologous internalization in the presence of overexpressed GRK or β-arrestin 2, while D3R mainly undergoes heterologous internalization (Cho et al., 2007; Kim et al., 2001; Min et al., 2013; Xu et al., 2019). Results obtained from ELISA for D3Ser9, D3Gly9 and D3Gly9+AC5 are in line with previous studies. However, my results suggest that AC5 plays a biased effect on D3Ser9 internalization in comparison to D3Gly9 upon DA stimulation. Interestingly, this process is dependent on dynamin and β-arrestin 2 (Fig. 21-23). Dynamin is a GTPas that is required for the detachment of newly formed vesicles from the plasma membrane and regulate both clathrin- and caveolin-dependent internalization of GPCRs (Moo et al., 2021). As mentioned in the Introduction, AC5 is located in caveolae regions of the plasma membrane. It is not clear if D3Ser9 association

with AC5 promotes colocalization of AC5 and D3Ser9 to the plasma membrane region away from caveolae and facilitates clathrin-mediated endocytosis or AC5 facilitated DA-induced D3Ser9 internalization is through caveolin-mediated endocytosis. On the other hand, PMA significantly induces D3R heterologous internalization following 15 min exposure, a finding that is in agreement with previous reports on PMA-induced heterologous D3R internalization in HEK293 cells (Cho et al., 2007). However, AC5 attenuates heterologous internalization of D3Ser9, while it does not have any effect on D3Gly9 heterologous internalization (Fig. 24). As discussed in the Introduction, previous studies on D3Ser9 have revealed that PKCβII and PKCδ, but not PKCα or PKCζ significantly increase PMA-induced internalization of D3Ser9. It is worth mentioning that D3Ser9 interacts with PKCβII and PKCδ. Moreover, it has been shown that serine residues at positions 229 and 257 on the IL3 of D3Ser9 are putative phosphorylation sites in PMA-induced D3Ser9 phosphorylation, desensitization, internalization, and degradation (Cho et al., 2007; Zhang et al., 2016). In contrast, it has been shown that PKCα and PKCζ isoforms can directly phosphorylate and activate AC5 (Kawabe et al., 1996). Three scenarios can be suggested based on these data. The first scenario is that the interaction between D3Ser9 and AC5 recruits more PKCα and PKCζ to this complex rather than PKCβII and PKCδ which are important for D3R heterologous internalization. In the second scenario, the interaction between AC5 and PKCα and PKCζ isoforms leads to dissociation of D3R from PKCβII and PKCδ. Based on these two scenarios, PKCα and PKCζ are dominant PKC isoforms in D3R•AC5 complex and reduce heterologous internalization. The third scenario is based on possible interaction site between AC5 and D3Ser9. It could be possible that AC5 interacts with IL3 of D3Ser9. Thus, the interaction of AC5 with IL3 of D3Ser9 impairs phosphorylation of putative phosphorylation sites on the IL3 of D3Ser9 in PMA-induced internalization. However, the interaction sites between D3R and AC5 needs to be clarified. All three scenarios could explain the attenuation of PMA-induced internalization

of D3Ser9 in the presence of AC5. In contrast, no effect was observed for PMA-induced internalization of D3Gly9. One possible explanation is that the interaction between D3Gly9 and AC5 does not affect neither recruitment nor stability of the interaction between D3Gly9 and PKCβII and PKCδ. However, it is not clear, whether D3Gly9 also interacts with PKCβII and PKCδ or if this interaction is specific for D3Ser9. Thus, the interaction of AC5 and PKCα and PKCζ can not impair the interaction of D3Gly9 and PKCβII and PKCδ leading to PMA-induced internalization. Moreover, it is possible that AC5 interacts with different region of D3Gly9 rather than IL3. Thus, AC5 interaction with D3Gly9 would not impair putative phosphorylation sites on the IL3 of D3Gly9 involved in PMA-induced internalization. Since Cho et al. (2007) used D3Ser9 variant for this study, further investigation needs to be done using D3Gly9 variant to clarify if D3Gly9 also interacts with PKCβII and PKCδ isoforms and to identify putative phosphorylation sites.

Overall, AC5 plays an opposite effect on DA-induced internalization and PMA-induced internalization of D3Ser9. This contradictory effect could be explained by AC5 being rather involved in homologues internalization than heterologous internalization, thus switching the D3Ser9 internalization from heterologous to homologous internalization, while not changing D3Gly9 heterologous and being unable to promote homologous internalization. Another possibility is that following PKC activation, AC5 retains D3Ser9 on the plasma membrane leading to a reduction of the heterologous internalization, while being unable to prevent D3Gly9 heterologous internalization. In contrast, AC5 promotes D3Ser9 internalization upon DA stimulation, while it keeps D3Gly9 on the surface. It is worth mentioning that although the Ser9Gly variation is located in the extracellular N terminus of the D3R, a region that is glycosylated and predicted to play a role in receptor conformation, maturation, and trafficking, both variants displayed similar glycosylation and anterograde trafficking (Jeanneteau et al., 2006).

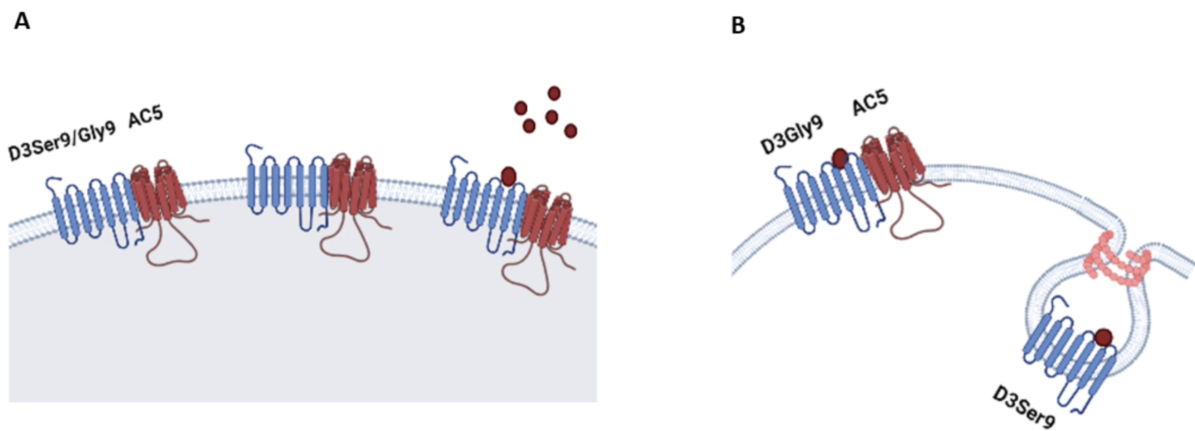

Figure 28. The Effect of AC5 on D3R Cell Surface Expression and DA-induced Internalization.

A) AC5 promotes cell surface expression of D3Ser9 and D3Gly9. B) Upon DA stimulation, AC5 induces D3Ser9 internalization in a process dependent on dynamin and β-arrestin 2, while D3Gly9 internalization does not happen even in the presence of overexpression of β-arrestin 2. Created with BioRender.com.

However, AC5 could modulate possible cis and/or trans interactions between N-terminus of D3R poly morphic variants and other proteins. This could potentially lead to keep one polymorphic variant on the cell surface, while the other one could undergo agonist/PMA-induced internalization. This suggests an important regulatory role of AC5 in D3R endocytosis. This could be an explanation for differences observed between D3Ser9 and D3Gly9 in diseases as well as signal transmission that were discussed in the Introduction.

Taken together, D3R undergoes various desensitization processes varying from PMA-induced heterologous internalization, agonist-induced pharmacological sequestration, and agonists-induced homologous internalization. These processes may play an important role in the regulation of D3R as autoreceptors. Thus, some of these mechanisms may render D3R autoreceptors more resistant to removal from the cell surface than other receptors. This means that D3R requires dynamic and versatile regulation according to cellular needs. Therefore, D3R employs unique regulation mechanism to perform highly flexible regulatory functions as an autoreceptor.

2.4. The Potential Role of β-arrestin 2 in D3R•AC5•β-arrestin 2 Protein Complex

Co-IP/IB results showed the interaction between D3R•AC5 and β-arrestin 2 under basal state. This result is in agreement with previous studies that showed the interaction between D3R and β-arrestin 2 (Kim et al., 2005; Min et al., 2013). The constitutive interaction between D3R and β-arrestin 2 can compensate the lack of β-arrestin 2 translocation to D3R after agonist stimulation. It has been suggested that an interaction between β-arrestin 2 and D3R could be involved in the pharmacological sequestration of the D3R (Min et al., 2013). To explain constitutive interaction between D3R•AC5•β-arrestin 2 two possibilities should be considered. First, although visual arrestin binding to rhodopsin is highly dependent on receptor phosphorylation, β-arrestin binding to GPCRs is less phosphorylation-dependent and can also

bind to unphosphorylated receptors (Peterson & Luttrell, 2017; Vishnivetskiy et al., 2011). The other possibility could be related to constitutive activity of D3R (Burstein et al., 2005; Griffon et al., 1996; Malmberg et al., 1998; B. Zhang et al., 2014). Data obtained from whole-cell phosphorylation assays in HEK293 cells showed that overexpression of GRK3 phosphorylates D3R in the basal state and only a subtle increase in the level of D3R phosphorylation occurs after DA exposure (Kim et al., 2001). Unpublished data from our lab obtained from whole cell phosphorylation assays also showed that D3R is constitutively phosphorylated. The extent of the D3R phosphorylation is further increased following DA exposure. Thus, β-arrestin 2 can bind to phosphorylated D3R under basal state with high affinity. More investigations on D3R phosphorylation status are required to address which described possibilities explain the constitutive interaction between D3R and β-arrestin 2.

Interestingly, DA stimulation does not significantly change the association of the D3R•AC5•β-arrestin 2 complex. Thus, the association of β-arrestin 2 with D3Ser9 and AC5 rather than β-arrestin 2 recruitment to the D3R after DA stimulation could be important for AC5-induced D3Ser9 internalization, pharmacological sequestration and β-arrestin 2 related signal transduction. On the other hand, the role of β-arrestin 2 in D3Gly9•AC5•β-arrestin 2 complex could be related to pharmacological desensitization or signal transduction. Moreover, this interaction may promote or inhibit other signaling pathways involving β-arrestin 2. As discussed in the Introduction, it has been suggested that GPCRs promote $G\alpha_i$ and β-arrestins interaction, regardless of their canonical $G\alpha_i$ subtype coupling. Moreover, different GPCRs ligands could induce complex formation between $G\alpha_i$/β-arrestin (Smith et al., 2021). Based on these findings, it could be a constitutive interaction between D3R, AC5 and β-arrestin 2 and DA evokes $G\alpha_i/_o$ to this complex, which initiates inhibition of AC5 activity. Taken together, D3R alone is not able to inhibit ACs. On the other hand, there is a constitutive interaction

between D3R•AC5•β-arrestin 2 and DA promotes the stability of this complex. Following stimulation with DA, $G\alpha_i/_o$ will be added to this complex through interaction with β-arrestin 2.

Another possibility is that AC5 helps D3R to couple to $G\alpha_i/_o$ and inhibits cAMP production as discussed earlier (Fig. 29). However, further investigation needs to be done to identify the role of $G\alpha_i/_o$ in this process.

Ser9Gly substitution is located in the extracellular N terminus of the D3R. Interestingly, AC5 can interact with both D3Ser9 and D3Gly9, although the interaction sites are still unidentified. Nevertheless, both variants can inhibit AC5 activity. Indeed, AC5 promotes both variants to reach the plasma membrane. Surprisingly, AC5 regulates both homologous and heterologous internalization of D3Ser9, while it does not play a role in D3Gly9 homologous or heterologous internalization. Considering the differences between D3Ser9 and D3Gly9 in diseases and signal transduction as discussed in the Introduction as well as data obtained from my thesis, the N-terminal region may therefore provide an unexpected structural determinant to D3R function.

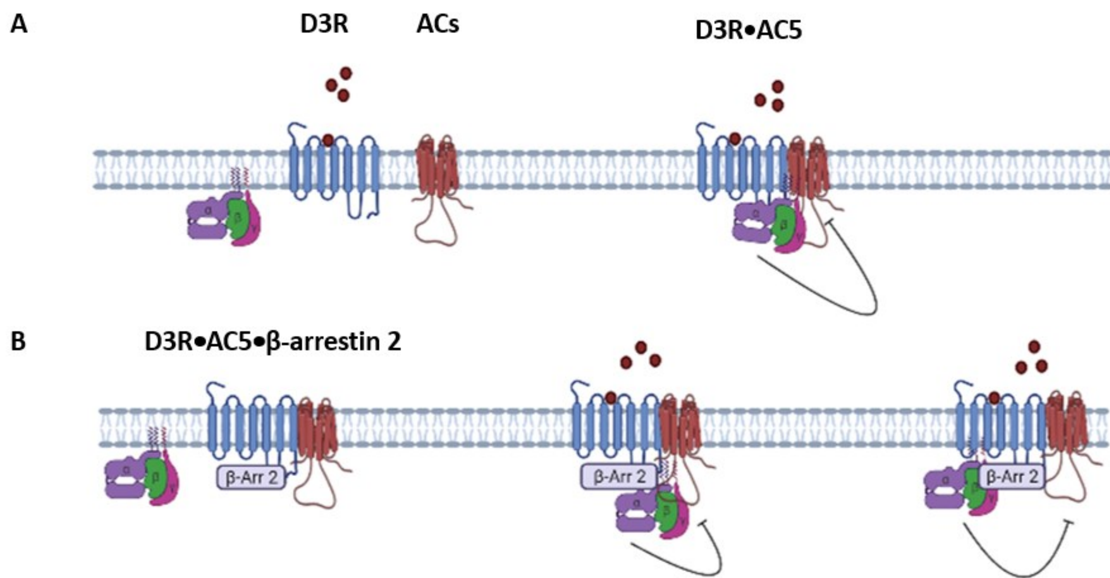

Figure 29. Hypothetical Models of Inhibition of cAMP Production.

A) left, D3R alone is not able to inhibit ACs activity. Right, D3R•AC5 interaction leads to coupling to $G\alpha_{i/o}$ either through interaction between AC5 and $G\alpha_{i/o}$ or potentially helps D3R to adopt an active conformation to couple to $G\alpha_i$ and inhibit of cAMP production. B) left, there is a constitutive interaction between D3R•AC5•β-arrestin 2. Middle, DA stimulation promotes the stability of the complex and recruits $G\alpha_{i/o}$ to this complex based on two possibilities that discussed in A. Then, D3R inhibits AC5 activity through coupling to $G\alpha_{i/o}$. Right, following stimulation with DA, $G\alpha_i/_o$ will be added to this complex through interaction with β-arrestin 2. Then, D3R inhibits AC5 activity through coupling to $G\alpha_{i/o}$. Created with BioRender.com.

3. Conclusions

Overall, my results highlighted two main findings. First, the interaction between D3R and AC5 has been identified. Secondly, the impact of this interaction on surface expression, internalization and cAMP production has been demonstrated. Taken together, a novel regulatory mechanism for D3R is suggested here. AC5 association with D3R and the role it may play in the modulation of D3R trafficking, signaling and internalization could have profound implications for the treatment of neuropsychiatric disorders. First, either D3R•AC5 complex or D3R•AC5•β-arrestin 2 could be important to understand the diseases for which D3R impairment has been observed. Moreover, this interaction could be meaningful for development of novel drugs to reduce presumptive side effects. Data obtained from internalization of the receptor and specifically the biased effect of AC5 on D3Ser9 versus D3Gly9 could also have profound implications for the treatment of neuropsychiatric disorders. This is important because alterations in the levels of D3R have been identified in several different pathological states as discussed in the Introduction.

4. Future Studies

My master studies have suggested a novel regulatory mechanism of D3R by AC5. However, more experiments are required to further explore the D3R•AC5 interaction and the role it may play in signal transmission. Here, I suggested some important experiments that can be done to further investigate the interaction of D3R•AC5 and its impact on other signaling cascades. To assess the dynamic interaction between D3R and AC5, nanoluciferase binary technology (NanoBiT), BRET or FRET assays could be performed. Moreover, taking advantages of GST fusion pull down assays using intracellular domains of D3R will be beneficial to assess the interaction sites of D3R•AC5. Furthermore, considering the important role of IL3 of D3R in interacting with other proteins, the role of proline-X-X-proline interaction motif in this region

in the complex formation between D3R and AC5 can be tested by performing alanine mutations of all proline residues in IL3 of D3R. To identify critical residues in D3R which are involved in D3R•AC5 hetero-oligomerization and regulation of D3R•AC5 signaling, mutations in Ser, Thr and Tyr residues of D3R could be tested. Tyr-to-Phe, Ser-to-Ala, and Thr-to-Val mutations can be done followed by co-IP/IB approaches to find out whether putative phosphorylation sites play a role in the interaction between D3R and AC5. Moreover, to determine the effect of $G\alpha_{i/o}$ activity on D3R•AC5 complex formation, pre-treatment with PTX followed by co-IP/IB assays can be used. For future studies, it can be important to test whether $G\alpha_{i/o}$ also interacts with D3R and AC5 or D3R, AC5 and β-arrestin 2 using co-IP/IB assays or confocal microscopy methods to visualize localization of D3R•AC5•β-arrestin 2•$G\alpha_{i/o}$. In addition, it is important to assess whether AC5 and β-arrestin 2 form a complex as well. To investigate more effects of AC5 on D3R regulation, the effect of AC5 on other D3R signaling pathways could be evaluated (e.g., ERK pathway) or the effect of AC5 on D3Ser9 recycling upon activation by DA or PMA. To determine whether AC5 promotes DA-induced D3Ser9 internalization involved the clathrin or caveolin pathways either the caveolin-1 or CHC could be knocked down. Cells could be also treated with sucrose, which can block both clathrin and caveolin-dependent endocytic pathways. Alternatively, studies could be performed using Pitstop 2 or MβCD, which inhibits clathrin-mediated internalization and caveolin-dependent endocytic routes, respectively.

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