Investigating the effects of palmitoylation on the dopamine 1 receptor (D_1)

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Abstract

The dopamine D_1 receptor (D_1) is a G protein-coupled receptor (GPCR) which regulates various key brain functions like attention, movement, reward, and memory. Understanding D_1 signalling may open the horizon for novel treatments for neurological disorders.

Upon agonist activation, the heterotrimeric G proteins $G\alpha_s$ activate adenylyl cyclase to increase cAMP/PKA signalling. D_1 also engages β-arrestin proteins leading to β-arrestin dependent signalling. The D_1 has two palmitoylation sites on cysteines 347&351 in its C-tail domain. However, the distinct roles and implications of palmitoylation on the D_1 signalling, trafficking and β-arrestins recruitment are still largely unexplored. A palmitoylation D_1 mutant was generated and luminescent based techniques such as BRET and split-Nanoluc complementation assay were employed, to delineate D_1 palmitoylation effects on its pharmacology and signalling. The D_1 agonists induced 50% less cAMP production in the mutant compared to wildtype (WT) and WT showed a more efficient dissociation of its $G\alpha_s$. Moreover, the mutant receptor failed to recruit β-arrestin1&2, induced less ERK1/2 activation and internalises in an agonist-independent process while showing an altered intracellular Golgi trafficking. Also, in β-arrestin 1&2 KO HEK 293 cells similar cAMP production levels were reported for D_1 WT and palmitoylation mutant. β-arrestin 1&2 KO blocked agonist-induced WT D_1 plasma membrane trafficking, indicating that these β-arrestins are driving the differences between WT and the palmitoylation mutant D_1. Taken together, our studies indicate that $G\alpha_s$ is the main transducer for D_1 cAMP and ERK1/2 signalling and that palmitoylation is essential for its β-arrestin 1&2 interactions and modulating D_1 signalling cascades in a drug-dependant process.

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Glossary

2-AG	2-Arachidonoyl Glycerol	**CMV**	Cytomegalovirus
$A_{2A}R$	Adenosine $_{2A}$ receptor	**CNS**	Central Nervous System
AADC	Aromatic L-Amino Acid decarboxylase	**Co-IP**	Co-Immunoprecipitation
AC	Adenylyl cyclase	**CREB**	cAMP Response Element Binding protein
Ach	Acetylcholine	**CXCR**	C-X-C chemokine receptor type
ADHD	Attention-Deficit Hyperactivity Disorder	**D_1**	Dopamine D_1 receptor
ATP	Adenosine triphosphate	**DA**	Dopamine
β_1AR	β_1 adrenoceptor	**DAT**	Dopamine Active Transporter
$\beta_{2A}R$	β_2 adrenoceptor	**DOI**	2,5-dimethoxy-4-iodoamphetamine
BBB	Blood-Brain Barrier	**ECL/ICL**	Extracellular/Intracellular Loop
BRET	Bioluminescent Resonance Energy Transfer	**ECS**	Endocannabinoid system
CAM	Constitutive Active Mutant	**EEs**	Early Endosomes
cAMP	Cyclic adenosine monophosphate	**EM**	Electron Microscopy
CART	Cocaine and Amphetamine Regulated Transcript	**ER**	Endoplasmic Reticulum
CaSR	Calcium-Sensing Receptor	**ERK**	Extracellular signal–Regulated Kinases
CCPs	Clathrin-Coated Pits	**EYFP**	Enhanced Yellow Fluorescent Protein
FK	Forskolin	**FCS**	Fluorescence Correlation Spectroscopy
FRET	Förster/Fluorescent Resonance Energy Transfer	**FDA**	Food and Drug Administration
GABA	γ-aminobutyric acid	**MAPK**	Mitogen-Activated Protein Kinase
GAPs	GTPase-Accelerating Proteins	**MD**	Molecular Dynamics
GASP-1	GPCR-Associated Binding Protein-1	**mHTT**	MDL 100907
GI	Gastrointestinal tract	**MW**	Molecular Weight
GIPC	GAIP Interacting Protein C terminus	**Nac**	Nucleus accumbens
GIPR	Glucose-dependent Insulinotropic Polypeptide Receptor	**NLuc**	Nanoluciferase
GIRKs	G protein coupled Inwardly Rectifying K^+ Channel	**GDP**	Guanosine diphosphate
GPCRs	G Protein-Coupled Receptors	**GEFs**	Guanine nucleotide Exchange Factors
GRK	G protein-coupled receptor kinase	**Rluc**	*Renilla reniformis* luciferase
STAT	Signal Transducer and Activator of Transcription	**SNc**	Substantia Nigra pars compacta
RET	Resonance Energy Transfer	**SNr**	Substantia Nigra pars reticulata
RGSR	RGS proteins of the R7 family	**TM**	Transmembrane domain
RGSs	Regulators of G protein Signalling	**TR-FRET**	Time-Resolved FRET
YFP	Yellow Fluorescent Protein	**WHO**	World Health Organisation

GRP	Gastrin Releasing Peptide	**PCR**	Polymerase Chain Reaction
GTP	Guanosine triphosphate	**FCS**	Fluorescence Correlation Spectroscopy
HC	High Content	**PDEs**	Phosphodiesterases
HD	Huntington's Disease	**PDS-95**	Postsynaptic Density Protein 95
HDAC	Histone deacetylase	**PDZ**	Postsynaptic Density 95/Discs large/Zona occludens-1
HSV-TK	Herpes Simplex Virus Thymidine Kinase	**PI_3K**	Phosphoinositide 3-kinase
HTRF	Homogeneous Time Resolved Fluorescence	**PIP_2**	Phosphatidylinositol 4,5 bisphosphate
HTT	Huntingtin protein	**PKA**	Protein Kinase A
IF	Immunofluorescence	**PDZ**	Postsynaptic density 95/Discs large/Zona occludens-1
ILVs	Intraluminal vesicles	**PKB**	protein kinase B
IP_3	Inositol 1,4,5 trisphosphate	**PLA**	Proximity Ligation Assay
IP_3R	IP_3 receptor	**PLC**	Phospholipase C
JAK	Janus Kinase	**PNS**	Peripheral Nervous System
JNK	c-Jun N-terminal Kinase	**PPIs**	Protein-Protein Interactions
Kir	Inwardly rectifying K^+ channels	**ACE**	Acetyl
L-DOPA	l-3,4-dihydroxyphenylalanine	**AUC**	Area Under the Curve
LBD	Ligand-Binding Domain	**BiLC**	Bimolecular Luminescence Complementation
LPA_2R	Lysophosphatidic acid receptor isoform 2	**Ca^{2+}**	Calcium
LSD	Lysergic acid dimethylamine	**CMV**	Cytomegalovirus
LTM	Long-Term Memory	**(D)MEM**	(Dulbecco's) Modified Eagle Medium
SSRI	Selective Serotonin Reuptake Inhibitor	**DMSO**	Dimethyl Sulfoxide
DRC	Dose Response Curve	**(D)PBS**	(Dulbecco's) Phosphate Buffered Saline
ECL	Extracellular Loop	**EDTA**	Ethylenediaminetetraacetic acid
GEF	Guanine Nucleotide Exchange Factor	**ECL**	extracellular loop
SDM	Site-Directed Mutagenesis	**SEM**	Standard Error of the Mean
SOC	Super Optimal broth with Catabolite repression	**SOB**	Super Optimal Broth

Chapter 1: Introduction to Post translational Modifications and palmitoylation of G protein coupled receptors

I. Introduction

In recent decades, scientists have discovered that the human proteome is much more complex than the human genome. While the human genome has between 20,000 and 25,000 genes (Collins et al., 2004), the human proteome is estimated to be over 1 million. This means that a single gene can encode for different proteins. Different mRNA transcripts can be generated from a single gene due to genomic recombination, transcription initiation with different promoters, various transcription termination, and splicing (Ayoubi & Van De Ven, 1996). This complexity is further mediated by protein post-translational modifications (PTMs) (O. N. Jensen, 2004) **(Fig.1)**. PTMs are chemical modifications that play critical roles in functional proteomics because they impact localisation and activity and could modify protein/protein interactions and protein interaction with nucleic acids, cofactors, and lipids. The human proteome has proven itself to be very dynamic and capable of modifying itself in response to stimuli, and PTMs are a tool to regulate cell activity (A. B. Ross et al., 2021). PTMs occur at specific amino acid residues of a protein and are generally mediated by enzymatic activity. It is estimated that 5% of total proteins are enzymes responsible for more than 200 types of PTMs (Wood, 2006). PTMs play a central role in various biological processes; they affect the structure and dynamics of proteins (Mann & Jensen, 2003; Y. Xu & Chou, 2016). PTMs change the properties of a protein by proteolytic cleavage and adding a modifying group such as acetyl, phosphoryl, glycosyl, and methyl to one or more amino acids (Ramazi et al., 2020). Generally, PTMs can be reversible or irreversible (Y.-C. Wang et al., 2014). Reversible modifications are covalent modifications, while irreversible ones are proteolytic modifications involving specific hydrolysis of peptide bonds (Blom et al., 2004). PTMs can occur in a single type of

amino acid or multiple amino acids and introduce changes in the chemical properties of the modified sites (K.-Y. Huang et al., 2019). These modifications affect protein behaviours and characteristics, such as enzyme function and assembly (Ryšlavá et al., 2013), protein half-life, protein-protein interactions (Marshall, 1993), cell-cell and cell-matrix interactions, trafficking, receptor activation, protein solubility (Caragea et al., 2007; Cundy et al., 2002; Goulabchand et al., 2014; Haltiwanger & Lowe, 2004; Karve & Cheema, 2011; Ohtsubo & Marth, 2006), protein folding (Del Monte & Agnetti, 2014) and protein localisation (Audagnotto & Dal Peraro, 2017). Therefore, these modifications are at the centre of various biological processes, such as signal transduction, gene expression regulation, DNA quality control, and cell division control (Strumillo & Beltrao, 2015; M. Wang et al., 2015). PTMs occur in cellular organelles around membranes such as the nucleus, cytoplasm, endoplasmic reticulum, and Golgi apparatus (Blom et al., 2004).

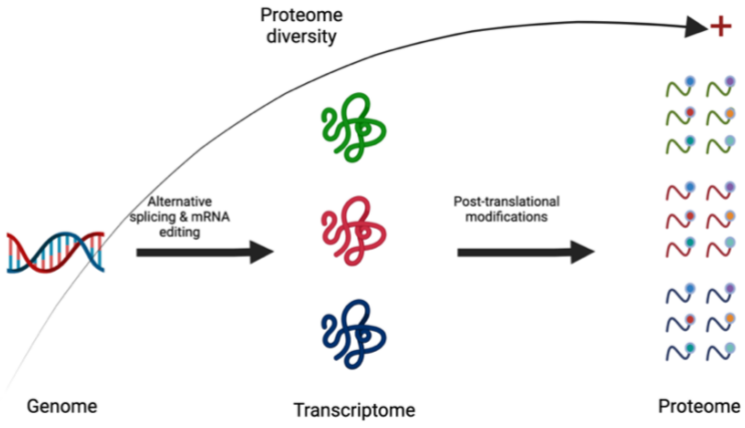


Figure 1- post-translational modifications and diversity

Figure showing that while the genome comprises around 25,000 genes, the proteome is estimated to be over 1 million proteins. Changes at the transcriptional and mRNA levels increase the size of the transcriptome relative to the genome, and the myriad of different post-translational modifications exponentially increase the complexity of the proteome relative to both the transcriptome and genome.

II. Lipidations and their enzymatic regulators

Protein lipidation is a PTM where there is a covalent addition of a variety of lipids, such as fatty acids, isoprenoids, and cholesterol, to the target proteins (**Table. 1**). Protein lipidation can be segregated into two types depending on the localisation of the targeted proteins: those subject to glycosylphosphatidylinositol (GPI) anchor and cholesterylation, in the endoplasmic reticulum (ER) lumen and secreted, and those that are targeted for N-myristoylation, acylation, and prenylation in the cytoplasm (Nadolski & Linder, 2007).

GPI anchors were first observed in a parasite the *Trypanosoma brucei,* where the highly expressed variant surface glycoprotein is attached to the cell surface via a glycolipid containing phosphatidylinositol (Ferguson et al., 1985; Ferguson & Williams, 1988). Since then, various proteins in mammalian organisms and lower eukaryotes have been shown to contain GPI anchors with diverse structures. These anchors typically consist of an ethanolamine linked to the protein's carboxyl terminus, a glycan core, inositol, and lipid moieties (Paulick & Bertozzi, 2008; Thomas et al., 1990). Proteins with GPI anchors often have a removable N-terminal signal sequence that guides the peptide to the endoplasmic reticulum lumen, as well as a hydrophobic C-terminal sequence that is cleaved during the addition of the GPI anchor (Caras et al., 1987; Englund, 1993; Takeda & Kinoshita, 1995). GPI anchors play a crucial role in tethering proteins to the extracellular side of the plasma membrane, contributing to various cellular functions such as adhesion, membrane trafficking, and immune system signalling (Chatterjee & Mayor, 2001; Fujita & Kinoshita, 2010).

The precursor of GPI, synthesised in the ER lumen, is passed on the target proteins by GPI transamidase, a multi-subunit membrane-bound enzyme (Benghezal et al., 1995; Fraering et al., 2001). GPI transamidase cleaves the C-terminal signal peptide of target

proteins. It catalytically attacks the carbonyl group of the amino acid at the ω site, forming a carbonyl intermediate between the precursor protein and the enzyme. The GPI moiety is then transferred to this intermediate, where the amino group of the terminal ethanolamine attacks the intermediate, completing the transamidation reaction (R. Chen et al., 2003).

Cholesterylation is a unique feature of the mammalian Hedgehog family proteins, which are secreted signalling proteins that regulate the embryonic patterning of many tissues and structures (Nüsslein-Volhard & Wieschaus, 1980; Porter et al., 1996). The Hedgehog protein undergoes an autocatalytic processing that internally cleaves between the conserved Gly257 and Cys258 at the GCF motif and yields a ~20kD N-terminal signalling domain and a ~25kD C-terminal catalytic domain (J. J. Lee et al., 1994; Porter et al., 1995). The N-terminal domain receives a cholesterol moiety and is active in signalling (Fan et al., 1995; Porter et al., 1995, 1996). Interestingly, various studies have detected other potentially cholesterylated proteins (Porter et al., 1996; Xiao et al., 2017). However, the identification and characterisation of these targets remain poorly understood. Cholesterylation of the N-terminal signalling domain of Hedgehog proteins appears to be dependent solely on the presence of the C-terminal catalytic domain, suggesting an autocatalytic process (Porter et al., 1996).

N-myristoylation is the attachment of the 14-carbon myristic acid to an N-terminal Gly residue via an amide bond (Towler et al., 1987). Initially, it was described as a modification that blocks N-terminal degradation during Edman degradation of the catalytic subunit of cyclic AMP-dependent protein kinase and the calcium-binding β-subunit of calcineurin (Aitken et al., 1982; Carr et al., 1982). Many other proteins regulating key signalling pathways, including the Src family non-receptor protein tyrosine kinases (Buss & Sefton, 1985; Linder & Burr, 1988) and Gα proteins (Buss

et al., 1987; Mumby et al., 1990) were shown to be myristoylated. These proteins typically possess an N-terminal sequence of Met-Gly- and frequently have a Ser/Thr/Cys residue at position 6 (Maurer-Stroh et al., 2002; Towler et al., 1987). Myristoylation can happen co-translationally following the removal of the initiator methionine residue (Wilcox et al., 1987). Although myristoylation is necessary for membrane targeting of many proteins, its weak hydrophobic nature is insufficient for stable membrane anchoring, often requiring additional lipid modifications (Peitzsch & McLaughlin, 1993; Seykora et al., 1996). Additionally, myristoylation can occur post-translationally during apoptosis when caspase cleavage exposes an internal glycine in substrate proteins. Many apoptotic proteins, such as Bid, gelsolin, and p21-activated kinase 2, require post-translational myristoylation following caspase cleavage for proper subcellular localisation and subsequent functions (Sakurai & Utsumi, 2006; Utsumi et al., 2003; Zha et al., 2000).

N-myristoylation is catalysed by N-myristoyltransferases (NMTs) (Duronio et al., 1989; Giang & Cravatt, 1998; Ntwasa et al., 1997). NMTs bind first to myristoyl and then to the peptide, followed by a direct nucleophilic addition-elimination reaction and subsequent release of CoA and the myristoylated peptide (Rudnick et al., 1991). Studies in multiple tissues and cell types have demonstrated that the enzymatic activity of NMTs is mainly distributed in the cytosolic fraction (Boutin et al., 1993; McIlhinney et al., 1993; McIlhinney & McGlone, 1996; Raju et al., 1994). Some studies have shown that low levels of myristoyl-CoA may be rate-limiting for NMT activity (Colombo et al., 2005; van der Vusse et al., 2002). However, the transcriptional up-regulation of NMTs under pathological conditions suggests this might not always be the case (Selvakumar et al., 2007).

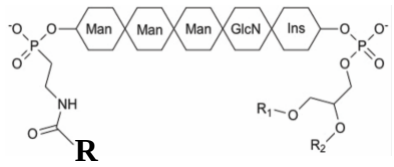
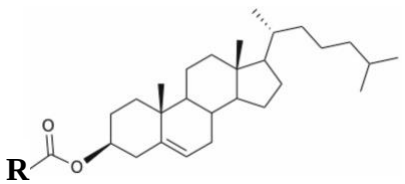
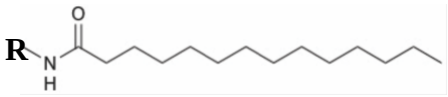
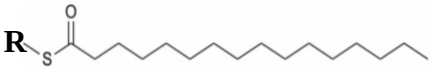
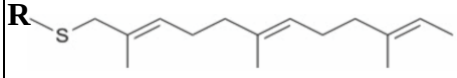
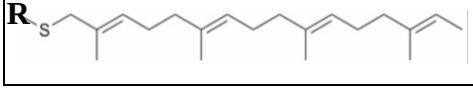
Prenylation involves the addition of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid lipid to cysteine residues through stable thioether bonds (Farnsworth et al., 1990; Kamiya et al., 1978). It requires a C-terminal CAAX motif, where C is a cysteine, A is an aliphatic amino acid, and X can be any amino acid. Prenylation at the CAAX motif is found in multiple proteins, including mammalian Ras proteins (Casey et al., 1989; Hancock et al., 1989). In addition to its role in membrane association, prenylation can regulate protein-protein interactions and subcellular distribution of the modified targets (Berg et al., 2010; Hoffman et al., 2000). Prenylation is catalysed by the enzymes farnesyltransferase (FTase), geranylgeranyltransferase I (GGTase 1), and Rab geranylgeranyltransferase (GGTase 2) (Gibbs, 1991; Maurer-Stroh et al., 2007). These prenylating enzymes are located in the cytosol and transfer isoprenoids, generated from the mevalonate/HMG-CoA reductase pathway to the target proteins. Unlike FTase and GGTase 1, geranylgeranyl transfer by GGTase 2 requires the co-factor REP (Rab escort protein) (Andres et al., 1993). GGTase 1 and FTase generally have high specificity for the protein targets, depending on the X residue (Hartman et al., 2005; Taylor et al., 2003; Zverina et al., 2012). However, they can act on each other's substrates. For example, K-Ras and N-Ras, which are typically targets of FTase, can be geranylgeranylated in Ras-mutant human cancer cells treated with FTase inhibitors (Ahearn et al., 2011; Rao et al., 2004).

Acylation is the addition of various fatty acids, such as palmitic acid, oleic acid, and stearic acid, on different amino acid residues (X. Liang et al., 2002; Magee et al., 1984; Takada et al., 2006). One of the most studied and better understood types of acylation is S-palmitoylation, which is characterised by the reversible addition of the 16-carbon saturated palmitic acid to a Cys residues *via* a thioester bond (Magee et al., 1984).

Despite the assistance of multiple algorithms in predicting palmitoylation sites, there is no validated consensus sequence for palmitoylation (Y.-X. Li et al., 2011; Ren et al., 2008; Xue et al., 2006; Y. Zhang et al., 2021).

G protein-coupled receptors are a protein superfamily that regulates numerous cellular and physiologic responses and are subject to Post-translational modifications that modulate there abundance and/or activity (B. Zhang et al., 2022).

Table 1- Summary of Different Types of Lipidation

Lipid structure	Role
GPI anchor 	Plasma membrane anchoring Incorporation into specific membrane micro-domains Protein-protein interaction
Cholesterylation 	Hedgehog signalling activation
Myristoylation 	Membrane localisation and autoinhibition
Palmitoylation 	Plasma membrane targeting Partitioning into lipid rafts Protein maturation and quality control
Farnesylation 	Membrane localization Conformational change Protein-protein interaction
Geranylgeranylation 	Membrane localisation Protein-protein interaction

III. Cellular signalling via transmembrane Proteins

For the survival of any living organism, it is crucial to respond to environmental changes effectively. Throughout evolution, organisms have developed various control and regulatory systems to address this need. In multicellular systems, coordinating and harmonising the response across different cells within the organism presents a complex challenge. Consequently, disruptions and malfunctions in intercellular signalling pathways often underlie various disorders and diseases.

Receptors, existing both on the cell surface and within the cell, play a vital role in these processes. Traditionally, receptors are viewed as recognition sites for endogenous hormones, neurotransmitters, neuromodulators, and potential drugs. To initiate a cellular response, the information from a stimulus must be transmitted across the cell membrane. While certain molecules can directly cross the membrane to trigger a response, most extracellular stimuli are detected by transmembrane receptors.

Transmembrane receptors span the cell membrane, featuring an extracellular and intracellular portion. The extracellular domain detects the specific stimulus relevant to the receptor. The region spanning the membrane relays this information to the intracellular domain, which then communicates with intracellular molecules to initiate a cellular response. This elucidates why proteins involved in signal transduction are often the primary targets for drug interventions.

To interpret and respond to extracellular stimuli, cells possess a variety of surface receptors that respond to specific and distinct stimuli. Transmembrane receptors are broadly classified into three major families based on the mechanism by which they generate an intracellular signal. When activated by a stimulus, Ligand-gated ion

channels open or close a pore unit, thereby altering the membrane's permeability to specific ions. This process often forms the basis for neuronal signalling. The second class comprises enzyme-linked receptors that contain a catalytic domain at their C-terminus. Upon agonist binding, these receptors dimerize, activating the catalytic domain. A well-known example of these receptors is the receptor tyrosine kinases, including the insulin receptor. The final class, which will be the focus of this thesis, consists of G protein-coupled Receptors (GPCRs). These receptors mediate a wide range of physiological responses by interacting with heterotrimeric G-proteins, activating appropriate second messengers.

1. Classification of GPCRs

The human genome has approximately 800 GPCRs, making it the largest family of membrane proteins (Ghosh et al., 2015). Various classification systems have grouped GPCRs based on the location of the ligand binding pocket, while some have utilised both the structural and physiological properties (Bockaert & Philippe Pin, 1999; Schiöth & Fredriksson, 2005). The A–F classification system was the first to be introduced (Davies et al., 2007). This was first proposed in 1994 as A-F and O by Kolakowski as the GCRDb system (Kolakowski, 1994) and was further developed, leading to the GPCRDB database by Horn *et al.* (Horn et al., 2003) with the rhodopsin family (class A) being the largest. All GPCRs comprise seven transmembrane domain helices alongside an eight helix and a palmitoylated cysteine at the C terminal tail (G. M. Hu et al., 2017). The diversity of GPCRs made it challenging to develop a comprehensive classification system. The A–F system orders the GPCRs into six classes based on their sequence homology and functional similarity:

-Family A (rhodopsin-like receptors)

-Family B (secretin receptor family)

-Family C (metabotropic glutamate receptors)

-Family D (parasitic mating pheromone receptors)

-Family E (cyclic AMP receptors)

-Family F (frizzled and smoothened receptors).

Based on phylogenetic studies, human GPCRs have been classified under a system called “GRAFS”. This system comprises five main families:

-Glutamate (G)

-Rhodopsin (R)

-Adhesion (A)

-Frizzled/taste2 (F)

-Secretin (S)

The major difference between the two systems is the additional division of family B into the adhesion and secretin families within GRAFS (G. M. Hu et al., 2017). This division was based on early findings highlighting the distinctive evolutionary history between the two families.

2. Structural properties of GPCRs

The G-protein-coupled receptors (GPCRs) are the superfamily of proteins that can recruit and regulate the activity of an intracellular heterotrimeric GTP binding proteins (G-proteins). Also, GPCRs can signal in a G protein independent manner most notably through beta arrestins (Hilger, 2018). GPCRs control the reactions to a wide variety of signals, like odours (Gaillard et al., 2004), taste (Hoon et al., 1999; Y. Zhang et al., 2003), light (Filipek et al., 2003), hormones and neurotransmitters. GPCRs are one of the biggest protein families in the human genome (Foord et al., 2005; Lander et al., 2001) and the largest family of membrane receptors. To date, 826 genes have been found to code for GPCRs in the human genome, which is equivalent

to approximately 4 % of the protein-coding sequences (Lagerström & Schiöth, 2008; D. Yang et al., 2021). These receptors are found to regulate various biological functions and represent one of the most important targets for therapeutic treatment. Around 30% of FDA-approved drugs target GPCRs. However, estimates identify between 88 and 157 GPCRs as drug targets, less than 20% of the total family, and roughly one-third of GPCRs with known endogenous ligands (Sriram & Insel, 2018). All GPCRs possess seven transmembrane-spanning α-helical domains (7TM) linked by alternating intracellular (ICL1-ICL3) and extracellular (ECL1-ECL3) loops, a cysteine bridge between ECL1 and ECL2 that is highly conserved among many class A GPCRs (Naranjo et al., 2015), and numerous studies indicate that this bridge is critically important for the structural stability, expression, and function of GPCRs as it was demonstrated by mutagenesis that the conserved cysteines between ECL1 and ECL2 are critical for maintaining the high-affinity ligand-binding conformation of rhodopsin (Karnik & Khorana, 1990), β_2 adrenergic receptor (Noda et al., 1994), and A_1adenosine receptor (Scholl & Wells, 2000). The amino terminus end points out on the extracellular side and the carboxyl terminus end on the intracellular side often containing a helical region, which lies parallel to the membrane (**Fig.2**). The extracellular N-terminus and loops, with transmembrane domains, are known to be central to the interaction of the receptor with its ligand (Gether, 2000).

a. The N-Terminal Extracellular Domain

It is the most diverse among all the GPCR structural regions, it varies in length across the different families. This variation is directly linked with the different agonist binding modes employed by the various classes of GPCRs. The region is commonly shorter in family A as it is less involved in ligand binding. Family A ligands

bind within the transmembrane bundle, and family B1 ligands bind both a transmembrane binding pocket and the extracellular domain, in a two-state binding model (Hoare, 2005). The high-affinity interaction between the peptide and extracellular domain enables higher potency signalling from Class B1 GPCRs than observed at Class A. The N-terminus and loops, with the transmembrane domains, are known to be central to the interaction of the receptor with its ligand. The intracellular parts of the GPCR, including its C-terminal domain and the ILs (especially ICL2 and the ICL3), were identified to be primordial for the G-protein recognition and activation (Kobilka, 1992; Peeters et al., 2011). Recent advances in GPCR structural biology have helped to resolve the structure of transmembrane domains of several GPCRs. However, the interconnecting loops and the N- and C-terminal extra membranous regions remain largely unresolved as the high flexibility of these looping regions makes it challenging to resolve their conformational states, but at the same time gives them a functional significance (Pal & Chattopadhyay, 2019).

b. Transmembrane Domains

The defining 7TM domains of a GPCR form the receptor nucleus and transduce the binding of an agonist into an intracellular response mediated by conformational change. On the extracellular surface of class A GPCRs, the transmembrane domains form the ligand binding pocket. This region confers a degree of ligand specificity, particularly for class A GPCRs that do not have an extracellular binding domain. For class B1 GPCRs, binding to the transmembrane domains bundle is primordial for the receptor activation but shows less agonist specificity than the extracellular domain.

Despite being in a similar position within the extracellular portion of the transmembrane domain bundle, the orthostatic binding site varies significantly across class A; this reflects the wide variety of agonists targeting this receptor family. For example, the mutation of specific residues within the core region can influence the binding of selective antagonists of the A3 receptor without effect on non-selective agonists (Barkan et al., 2020). After agonist binding in the transmembrane bundle, a part of the N-terminus folds over the binding pocket, restricting it and providing additional stability between the receptor and agonist (S. Liu et al., 2022).

The transmembrane domains also host allosteric binding sites. Allosteric ligands of GPCRs bind to the receptor and promote their signalling or influence the activity of an orthosteric agonist. Class A GPCRs contain a conserved motif in the 2nd transmembrane domain (Parker et al., 2008), found to bind sodium ions (W. Liu et al., 2012). Allosteric modulators can influence binding to the orthosteric site. For example, Cannabidiol was shown to be a negative allosteric modulator of the cannabinoid CB_1 receptor, thus inhibiting its agonist biding (Laprairie et al., 2015). They can also influence pathway selectivity of orthosteric agonists, as in the preferential promotion of 3',5'-cyclic adenosine monophosphate (cAMP) accumulation pathway, but not the recruitment of a non-G protein signalling molecule, β-arrestin, by LUF6000 at the A3R (Z.-G. Gao et al., 2011).

c. The C-Terminal Domain

Extending from the seventh transmembrane domain is the cytoplasmic C-terminal tail, often including an eight α helix, which is parallel to the membrane. The C-terminal tail is less conserved compared to other structural regions of the GPCR. However, its role is often conserved across various receptors. It has been demonstrated

that residues within the C-terminal tail are involved in receptor trafficking and, therefore, influence plasma membrane expression. Mutations of hydrophobic residues in the C-terminal domain of CB_1 greatly reduced maximal ligand binding and receptor activity (Ahn et al., 2010). This mutation affected the mutant receptor subcellular distribution when compared to the wild-type receptor, co-localising with markers of the endoplasmic reticulum (ER), suggesting impaired membrane trafficking.

It is suggested that phenylalanine residues within the helix eight (H8) form π-stacking interactions with the tyrosine residue of the $NP^{7.50}xxY$ motif, highlighting the importance of this region for correct receptor folding and expression (Fritze et al., 2003). Helix 8 is also suggested to interact with intracellular loop 1 (ICL1), forming interactions crucial for the stabilisation of both the inactive and active receptor conformations (Winfield et al., 2022).

The current understanding of the role played by each region of the receptor primarily stems from studies focused on class A receptors, as they have been the most extensively studied, and their structures have been well resolved. By examining how the various domains within the receptor interact, we can gain insights into how modifications in the receptor sequence, such as the introduction of nucleotide modifications, can impact the signalling process.

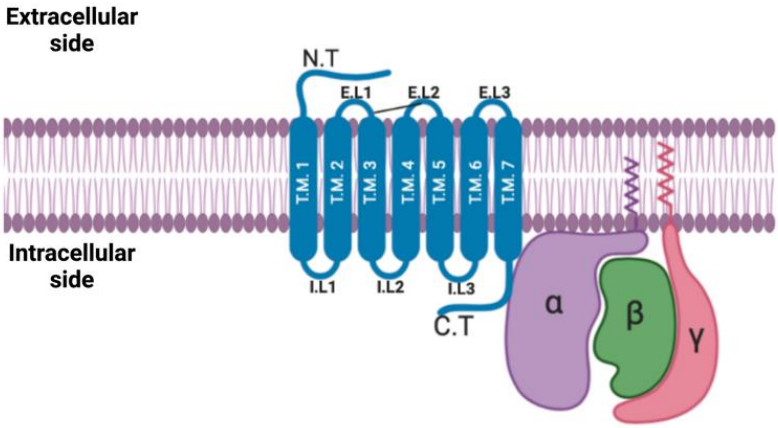


Figure 2- General structure of a G protein-coupled receptor (GPCR)

The schematic highlights the various intracellular domains (I.L: intracellular loop and C.T: cytoplasmic tail). Extracellular domains (E.L: extracellular loop and N.T: amino-terminal). Transmembrane (T.M) domains 1 to 7 sitting in the plasma membrane. The 7 T.M receptor is functional as a complex with its heterotrimeric associated G proteins. The disulfide bridge connecting E.L1 and E.L2 is a common feature for nearly all GPCRs. some receptors have a palmitoylated cysteine in CT creating a putative IL4.

3. Structural properties of the largest GPCR families

a. Family A (rhodopsin-like receptors)

The rhodopsin receptor family is the largest GPCR family, consisting of 719 members, and accounts for 80% of receptors in humans. It regroups aminergic, peptide, protein, lipid, melatonin, nucleotide, steroid, alicarboxylic acid, sensory, and orphan receptors (Foster et al., 2019; Gether, 2000). Family A has numerous characteristics (**Fig.3**) which relate to common ancestry (Gacasan et al., 2017; Schiöth & Fredriksson, 2005). These characteristics include a DRY motif located at the border between TM3 and intracellular loop (IL) 2 and NSxxNPxxY motif in TM7, their short extracellular amino terminus. Adding to that, many of the receptors from this family have one or multiple palmitoylated cysteines in their carboxyl-terminal tail (Basith et al., 2018; G. M. Hu et al., 2017; Qanbar & Bouvier, 2003), promoting the formation

of a putative fourth intracellular loop (Palczewski et al., 2000). Family A GPCRs exhibit significant variability in their preference for ligand binding. While there is consistency in the N-termini of family A GPCRs, there is diversity within the transmembrane domain (TMD) regions. However, certain sequence motifs within the TMD region are shared among some family A GPCRs. The ligand binding site is situated in the extracellular region of the TMD bundle, and the transmembrane domains of family A receptors play a crucial role in ligand binding (Gether, 2000). The binding pocket for small ligands is exclusively composed of the amino acid residues of the transmembrane domains of the receptor (Tota & Strader, 1990). On the other hand, mutational mapping of the ligand-binding sites in many of the peptide family A receptors has demonstrated critical involvement of the N-terminus and the extracellular loops for binding of the larger peptide ligands along with the pocket formed by the transmembrane domains (Gether, 2000). Having control over numerous physiological functions (cardiovascular, pulmonary, mental, CNS functions, etc…), class A receptors are the most attractive targets for the development of therapeutics among all other classes with over 500 drugs (Wishart et al., 2018).

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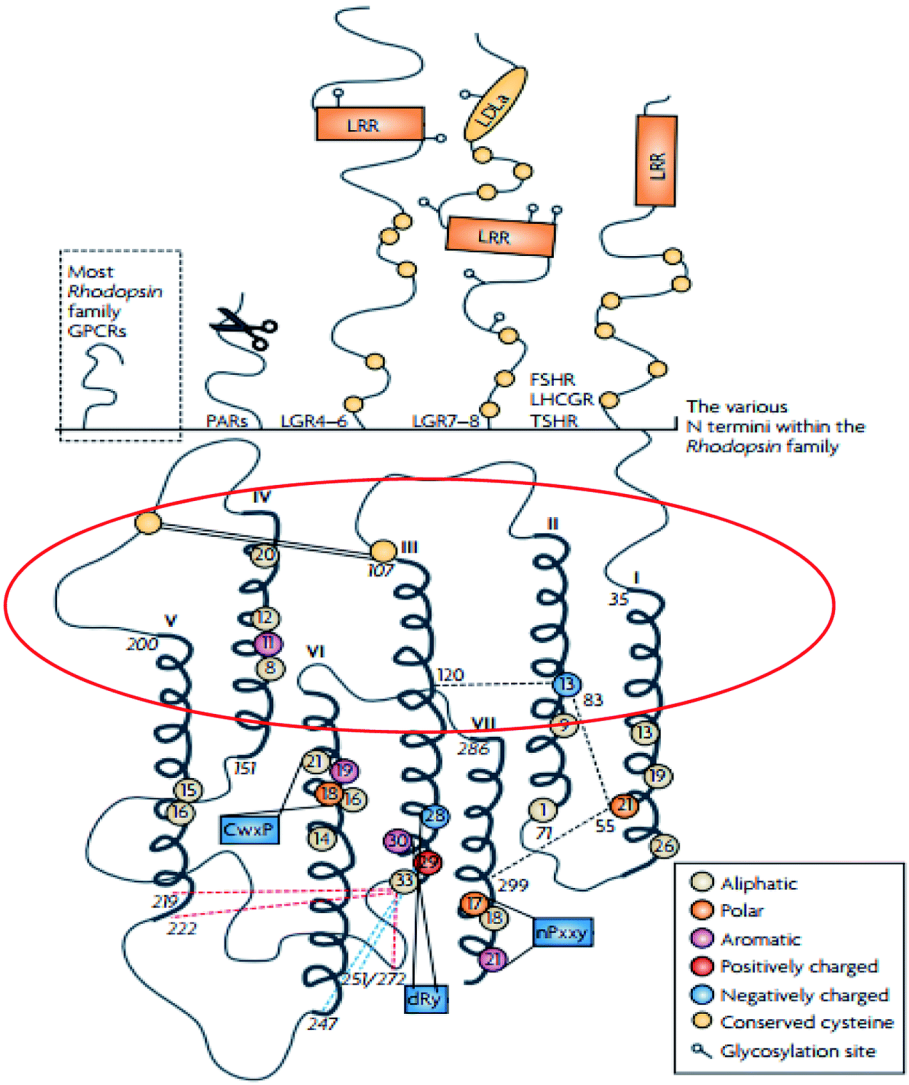


Figure 3- Structural properties of Family A GPCRs

The upper part of the figure illustrates the differences within the secondary structure of the N termini of the Rhodopsin receptors. The scissor indicates the cleavage site for the protease-activated receptors (PARs). In the lower part of the figure, the schematic transmembrane (TM) regions display the consensus of an alignment generated in ClustalW of eight diverse human Rhodopsin receptors. Residues conserved in all eight sequences are displayed as circles in which conserved aliphatic residues are shown in beige, polar in orange, aromatic in purple, positively charged in red and negatively charged in blue. The positions of the residues are calculated from the TM boundary starting with 1 in the N- to C-terminal direction. Numbers in italic correspond to the first position in each TM region of rhodopsin. Conserved sequence motifs found in the TM regions of the Rhodopsin receptor family are surrounded by blue boxes. Uppercase letters indicate completely conserved positions, lowercase letters indicate well-conserved positions (>50%), whereas x indicates variable positions. Conserved cysteine residues are pictured as yellow circles and the cysteine bridge between the extracellular loop 1 and 2, which is common to most G protein-coupled receptor (GPCR) families, is indicated by two straight lines. (Lagerström & Schiöth, 2008)

b. Family B (secretin receptor family)

The family B GPCRs (**Fig.4**) form a small group and corresponds to group B of the A–F system of classification, and with an extracellular hormone-binding site, they bind to large peptide receptors. The family name "secretin" derives from the secretin receptor, the first cloned in this family (Fredriksson et al., 2003). It includes 48 different receptors classed in two subfamilies: secretin (B1), for example, the vasoactive intestinal peptide (VIP), the calcitonin, the glucagon and others, and adhesion and migration (B2), such as epidermal growth factor, cadherin (S. P. H. Alexander et al., 2019; Hauser et al., 2017). The common feature of the (B1) receptors group is a large extracellular N-terminus domain, whereas the (B2) group of receptors possess a long N-terminal tail with unique motifs. The main characteristics of the (B2) subfamily is their autoproteolytic activity, a two-step activation model, the ligand N-terminal fragment interaction and the Stachel signalling/basal activity (Vizurraga et al., 2020), where the N- and C-terminal regions of the peptides interact with the J- and N-domains of the receptors respectively, in other words, the C terminus of the peptide initiates a peptide recognition with the extracellular domain, therefor allowing the peptide N terminus to bind the transmembrane domain ligand-binding pocket activating the receptor and initiating a downstream signalling cascade (Parthier et al., 2009; Wu et al., 2020). In addition, most of the receptors in this family have conserved cysteine residues that form a cluster of cysteine bridges in the N-terminus (Lagerström & Schiöth, 2008). Furthermore, they are characterised by the presence of a relatively large extracellular N-terminal domain of 120–160 residues, three intracellular and extracellular loops interconnect seven TMD (TM1-TM7) of 310–420 residues that are structurally similar and are thus members of the family B GPCR (de Graaf et al., 2017; Karageorgos et al., 2018). The presence of a conserved extracellular domain structure

and the ‘two-domain’ binding mode across the family B suggest a similar receptor activation across this GPCR family. In family B, the receptors of glucagon family peptides are major targets for therapeutic intervention, with obesity and mental health being the two hot subjects for drug research (Müller et al., 2019; Sekar et al., 2016; Williams et al., 2020).

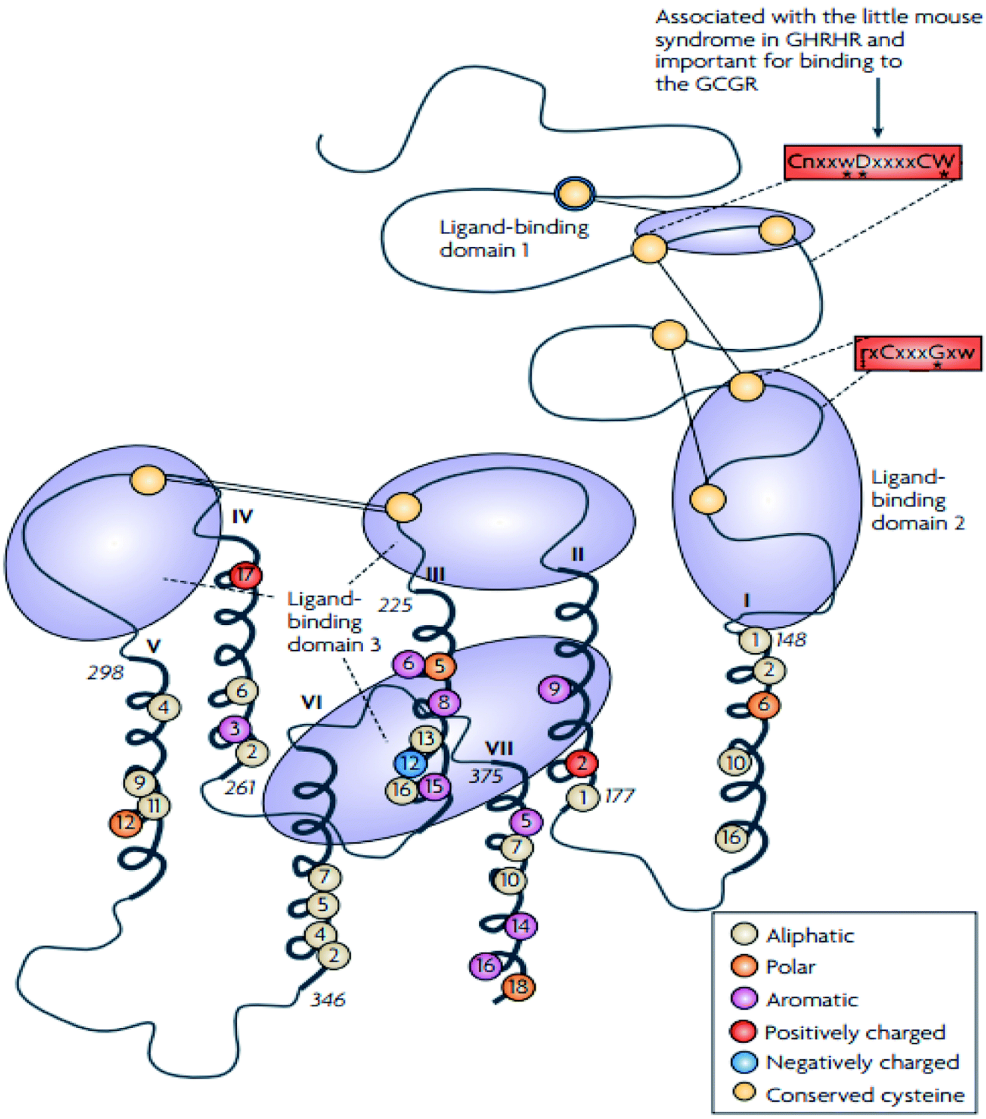


Figure 4- Structural properties of Family B GPCRs

The schematic transmembrane (TM) regions display the consensus of an alignment generated in ClustalW of 15 Secretin receptors from the human genome. The positions of the residues are calculated from the TM boundary starting with 1 in the N- to C-terminal direction. Numbers in italic correspond to the first position in each TM region of the human secretin receptor (SCTR). Uppercase letters indicate completely

conserved positions, lowercase letters indicate well-conserved positions (>50%), whereas x indicates variable positions. Residues conserved in all 15 sequences are displayed as circles in which conserved aliphatic residues are shown in beige, polar in orange, aromatic in purple, positively charged in red and negatively charged in blue. Conserved sequence motifs found in the TM regions of the Secretin family are surrounded by red boxes. Conserved cysteine residues are pictured as yellow circles, the N-terminal cysteine bridges are drawn as lines and the cysteine bridge between extracellular loops 1 and 2, which is common to most G protein-coupled receptor (GPCR) families, as two straight lines. (Lagerström & Schiöth, 2008)

c. Family C (metabotropic glutamate receptors)

The family C GPCRs is formed of the two γ-aminobutyric acid receptors, odorant receptors in fish, 8 metabotropic glutamate receptors, pheromone receptors, calcium-sensing receptors, sweet and umami taste receptors, GPCR class C Group 6 Member A (GPRC6A) and seven orphan receptors. These receptors possess a very long N-terminus domain and a short and highly conserved third intracellular loop. The large amino terminus forms a ligand-binding site with a conserved Venus fly trap and cysteine-rich domain on the ligand-binding site (**Fig.5**). Also, to be active, these receptors need to be organised in dimers (Pin et al., 2005). The latest updated information accounts for 16 FDA-approved drugs targeting 8 family C receptors, such as the metabotropic GABA receptors, which are implicated in various diseases, including cancer, schizophrenia, depression, and movement disorders (Messa et al., 2008; Shaye et al., 2020).

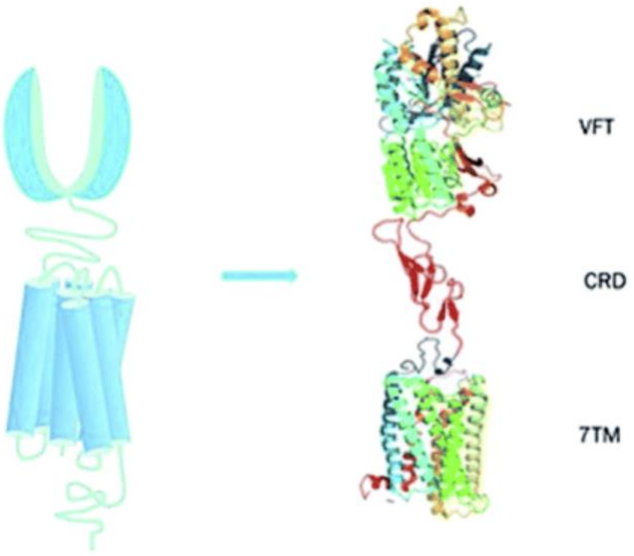


Figure 5- Structural properties of family C GPCRs

Graphical illustration of family C GPCR structure. Represents the structural organisation of family C GPCRs. Family C GPCRs have a peculiar structure which comprises of Venus flytrap domain (VFT) with two lobes separated by an orthosteric binding pocket, a cysteine rich domain (CRD) and a TMD except for $GABA_B$ receptor. (Chun et al., 2012)

IV. The heterotrimeric G-protein as Signalling Mediators of GPCRs

GPCRs signal primarily via heterotrimeric G proteins, comprised of Gα, Gβ, and Gγ subunits (**Fig.6**). In the human genome, 16 genes encode for 21 Gα subunits, 5 genes encode for 5 Gβ subunits, and 12 genes encode for 12 Gγ subunits. The Gα subunit is composed of two domains: a highly conserved GTPase/Ras-like domain and a unique helical domain. The Ras-like domain has three flexible switch regions (SI-SIII) that can undergo consequent reorientations when the complex is activated. The helical domain has six α-helix bundles, with the N-terminal helix being parallel to the plasma membrane, forming a lid shape over the Gβγ subunit and contributing to stabilising this interaction (D. E. Coleman et al., 1994). In addition, all Gα subunits except $G\alpha_t$, undergo post-translational modifications

(palmitoylation/myristoylation) at their N-terminus, enhancing membrane trafficking and their interaction with Gβγ (Wedegaertner, 1998). To date, all known Gα-proteins are divided into four main classes according to their subunits' amino acid sequence similarity (Downes & Gautam, 1999; Oldham & Hamm, 2008).

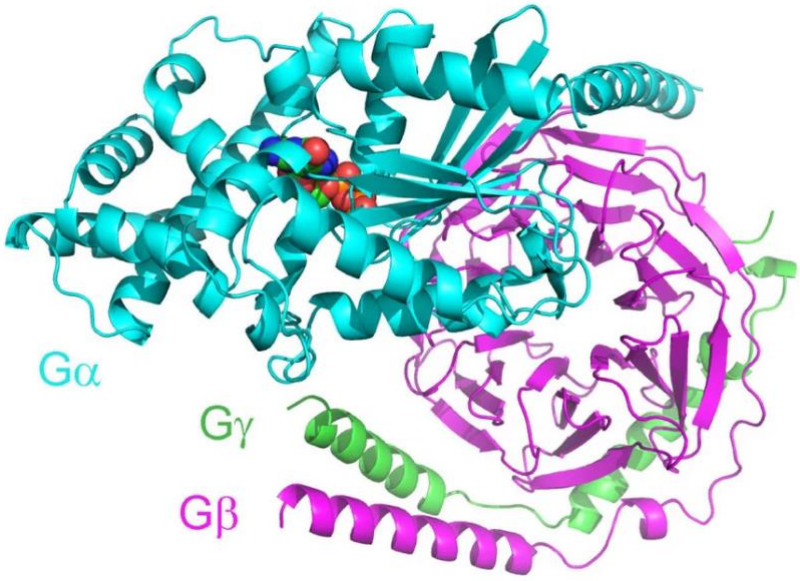


Figure 6- Heterotrimeric G protein structure

Structural basis of G protein. The structure of the heterotrimeric G protein, with GDP bound to Gα (cyan) and Gβ (magenta) linking Gα to Gγ (green). (Protein data bank)

1. Gα structure and mediated intracellular signalling.

The **G_S protein** class includes $G\alpha_s$ and olfactory $G\alpha_{olf}$. $G\alpha_s$ is known to stimulate the adenylate cyclase (AC), leading to an increase in cAMP concentrations **(Fig.7)**. This class consists of the Gα Stimulatory (S) group whose α subunits mediate signals from a variety of surface receptors to promote the activity of the adenylyl cyclase, thus generating cAMP. The $G\alpha_s$ subunit is encoded by two genes that have various promoters, thus transcribed as various products in a tissue-specific way (Weinstein et al., 2004). Additionally, the $G\alpha_s$ proteins can stimulate L-type calcium channels (Mattera et al., 1989) and inhibit voltage-dependent sodium channels in the

heart (Schubert et al., 1989). The G_{olf} protein is exclusive for the olfactory neuroepithelium and links odorant receptors with the olfactory-specific form of the adenylate cyclase.

The class of **$G_{q/11}$ proteins** includes the ubiquitously expressed $G\alpha_q$ and $G\alpha_{11}$ proteins (Mizuno & Itoh, 2009) that share a high degree of sequence similarity, with over 90% identity (UniProt), the $G\alpha_{14}$ (expressed in the lungs, kidneys, and liver tissues), and the murine $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ subunits expressed in the myeloid cells and the lymphocyte cells (Simon et al., 1991). The $G\alpha_{q/11}$ family activate phospholipase Cβ (PLCβ), which produces inositol-1,4,5-trisphosphate (IP3). IP3 activates its receptor on the endoplasmic reticulum, mobilising $(Ca^{2+})i$ from intracellular stores. There are further downstream signalling effects of $G\alpha_{q/11}$ activation, mediated through other actions of IP3, or the co-product of its production diacylglycerol (DAG) **(Fig.7)**. Also, G_q directly stimulates tyrosine kinase activity in lymphoma cells (Bence et al., 1997). In addition, the $G\alpha_{q/11}$ proteins inhibit neuronal inwardly rectifying potassium channels (Firth & Jones, 2001).

The class of **$G_{i/o}$ proteins** includes the ubiquitously expressed $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ proteins, as well as $G\alpha_z$ which is specifically expressed in the brain and the adrenal platelets. The class also includes $G\alpha_t$ and the $G\alpha_g$ specific to the retina and the taste buds, respectively. The common property of the $G_{i/o}$ protein family is their ability to inhibit the adenylate cyclase activity **(Fig.7)**. They were identified to mediate the inhibition of the enzyme adenylyl cyclase that generates cyclic adenosine monophosphate (Greif et al., 2017). Moreover, the G_i and the G_o proteins have been shown to activate the G protein coupled “inwardly rectifying potassium channels” (GIRK) (Yatani et al., 1988) and deactivate the L, N and P/Q subtypes of calcium

channels. The $G\alpha_t$ proteins stimulate the eye-located cGMP-specific phosphodiesterase in the retinal rods and cones (Stryer, 1986).

The **G_{12} protein** class is composed of the G_{12} and the G_{13} proteins, which are known to show relatively low sequence homology with the other classes of G-proteins. These proteins are implicated in the modulation of small GTPases activity and, therefore, contribute to of cell morphology regulation (Buhl et al., 1995; Kozasa et al., 1998; Suzuki et al., 2003). The $G_{12/13}$ proteins are also known to activate an extracellular signal-regulated activated kinase (T. A. Voyno-Yasenetskaya et al., 1996) and the Na^+/H^+ exchange (Yasenetskaya et al., 1994).

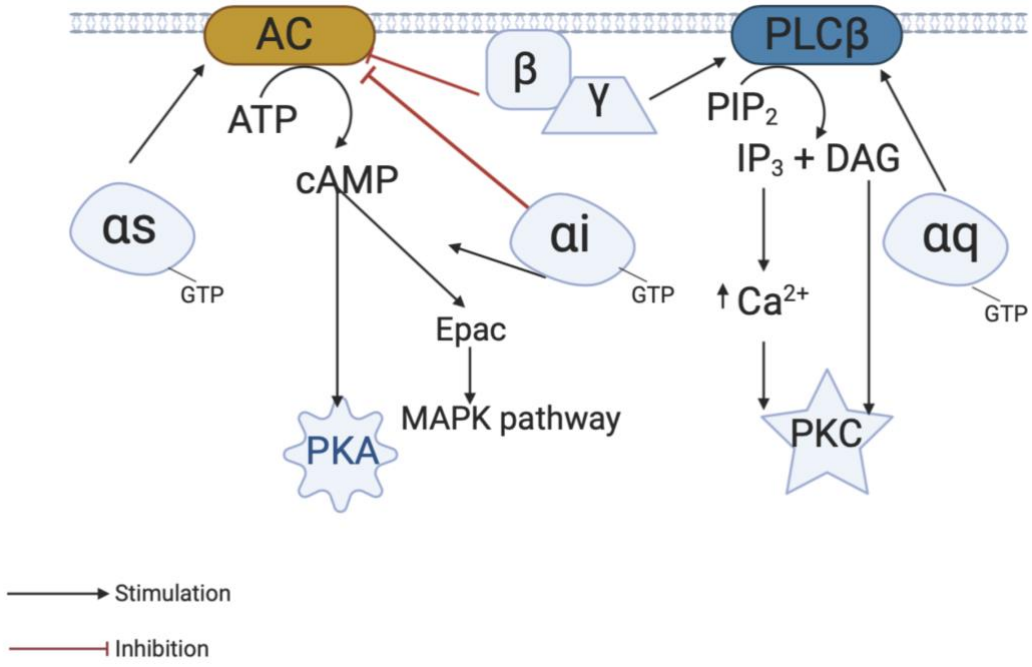


Figure 7- G protein mediated pathways

After agonist exposure, $G\alpha_S$-coupled receptors activate adenylyl cyclase (AC) thus promoting an increase of intracellular cyclic adenosine monophosphate (cAMP). This triggers the activation of cAMP-dependent protein kinase (PKA) and the mitogen-activated protein kinase (MAPK) through stimulation of the exchange protein directly activated by cAMP (Epac). $G\alpha_i$-coupled receptors inhibit AC and its downstream pathways. GTP-bound $G\alpha_q$ subunit or free $G\beta\gamma$ dimers (from $G\alpha_q$- or $G\alpha_i$- coupled receptors) activate phospholipase Cβ (PLCβ). Free $G\beta\gamma$ dimers also stimulate or inhibit AC activity in an AC isoform-dependent manner. Stimulation of PLCβ activity leads to the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3)

through phosphatidylinositol-4,5-biphosphate (PIP_2) hydrolysis. DAG activates protein kinase C (PKC) while the IP_3-mediated elevation of intracellular calcium concentration also activates some PKC isoforms.

2. Signalling capacity and structure of the Gβγ subunit

The **β and γ subunits** of the trimeric G protein are considered to be functional monomers due to their continuous tight interaction. The β subunit assumes a barrel-shaped beta-propeller structure consisting of an *N*-terminal alpha-helix followed by WD-40 repeats that are a repetitive sequence of about 43a.a (Fong et al., 2006). The WD-40 domain plays the role of an adaptor in protein complexes in various cellular processes (Rutherford & Daggett, 2010). Each WD40 repeat comprises a four-stranded antiparallel β-sheet. The γ subunit is related to the G proteins gamma-like (GGL) domain superfamily (Sondek & Siderovski, 2001); they are characterised by a sequence similarity that is translated in an extended alpha-helical polypeptide. The γ subunit is found in the form of a stable dimer with the beta subunit, and it contacts the opposite face of the G-beta subunit. The Gβγ complex has been shown to activate ion channels, like the muscarinic-dependent ACh-induced K^+ channel activated downstream of Muscarinic acetylcholine receptors activation in the heart (Logothetis et al., 1987), and inwardly rectifying K^+ channels (GIRK) which are found in neuronal membranes, inducing membrane hyperpolarisation and therefore reducing the excitability of neurons (Lüscher & Slesinger, 2010).

It is also important to highlight the ability of the Gβγ complex to mediate intracellular (Ca^{2+}) mobilisation following activation of $G\alpha_{i/o}$-coupled receptors. Like $G\alpha_{q/11}$, Gβγ complexes directly activate PLCβ (Park et al., 1993). There is evidence that different isoforms of PLCβ are preferentially activated by $G\alpha_q$ or Gβγ, with all PLCβ isoforms

activated by $G\alpha_q$ but only PLCβ2 and 3 by Gβγ (C. W. Lee et al., 1994; Smrcka & Sternweis, 1993). Both $G\alpha_q$ and Gβγ directly bind PLCβ and induce conformational changes, primarily in the autoinhibitory C-terminal domain (Fisher et al., 2020). However, it has since been shown that, whilst mediated by Gβγ, $G\alpha_{i/o}$ dependent release of the intracellular (Ca^{2+}) is also dependent on $G\alpha_q$, with no intracellular (Ca^{2+}) mobilisation observed in cells without active $G\alpha_q$ (Pfeil et al., 2020).

3. Heterotrimeric G protein activation

GPCR signalling can be dissected into three components: the heterotrimeric G protein, the seven transmembrane-spanning receptors and the enzymatic effector. The signal transmission from the outside to the inside of the cell is mediated through the heterotrimeric G protein cycle. GPCRs signal primarily via heterotrimeric G proteins **(Fig.8**). In the inactive state, Gα binds GDP. When the receptor is activated, it is accompanied by the outward movement of transmembrane helices 5 and 6; this creates a cavity on the cytoplasmic side of the receptor (Farrens et al., 1996). Activation-induced conformational changes appear to be smaller in the case of Gi-coupled GPCRs, as compared to Gs-coupled (Koehl et al., 2018; Rasmussen et al., 2007; Van Eps et al., 2018). Recent structural studies showed that this cavity serves as a docking site for the heterotrimeric G proteins of all subtypes (Koehl et al., 2018; Liang et al., 2017; Van Eps et al., 2018). Agonist-activated GPCRs act as guanyl nucleotide exchange factors for heterotrimeric G proteins. In the receptor-bound G protein, its nucleotide-binding pocket opens (Mahoney & Sunahara, 2016; Oldham & Hamm, 2008), which results in the loss of GDP occupying this site in the inactive form, and binding of GTP, which is much more abundant in the cytoplasm (Traut, 1994).

The conformational change in Gα enables the dissociation of GDP and binding of GTP, corresponding to Gα activation. In this regard, the receptor acts as a GEF (guanine-nucleotide exchange factor), which is a large and diverse group of proteins that catalyse the release of GDP and the uptake of GTP by GPCRs. When bound to GTP, G proteins transition into the active conformation. Upon hydrolysis of this bound GTP, by virtue of their own intrinsic GTPase activities, G proteins are inactivated. GDP release is the rate-limiting step for the GTPase reaction in vitro; in vivo, this kinetic barrier is reduced by GEFs (Sprang, 2001).

The activation of the Gα subunit leads to its dissociation from the Gβγ complex and diffusion towards further signalling components. G proteins can also be activated in a receptor-independent manner via activators of G protein signalling (AGS). Members of the AGS family share little structural similarity and are classified via the mechanism by which they activate the G protein (Blumer & Lanier, 2014). Group I act as GEFs and was the first discovered; AGS1 is a member of the Ras superfamily known to initiate G protein signalling (Cismowski et al., 2000). Group II are the largest in number, with seven members; they have in common a G protein regulatory (GPR) motif that binds the Gα subunit and stabilises the GDP-bound conformation. Group III interact with the Gβγ subunit but otherwise represents possibly the most diverse of the AGS subfamilies.

The Gα subunit is a GTPase. Therefore, it can hydrolyse GTP back to GDP, thus inactivating the G protein. GTP hydrolysis and G protein inactivation can be enhanced by GTPase activating proteins (GAPs), which are antagonistic to GEFs. The GAPs most associated with heterotrimeric G proteins, as opposed to their monomeric counterparts, are known as regulators of G protein signalling (RGS). RGS proteins reduce signalling mediated by different Gα subunits, with different RGS proteins

showing selectivity for different G protein subfamilies (Ladds et al., 2007). The signal stops when the Gα subunit catalyses GTP hydrolysis into GDP (through Gα intrinsic GTPase activity) and then reassociates with the βγ, dimer forming the inactive heterotrimeric state. This enables the heterotrimeric G protein to undergo another activation cycle.

However, everything the cell does has an energetic cost. Therefore, as soon as the cell processes the message, it makes biological sense to end the signalling. In the case of GPCRs, rapid signal turnoff is accomplished by a conserved two-step mechanism: receptor phosphorylation by GRKs followed by arrestin binding (Carman & Benovic, 1998).

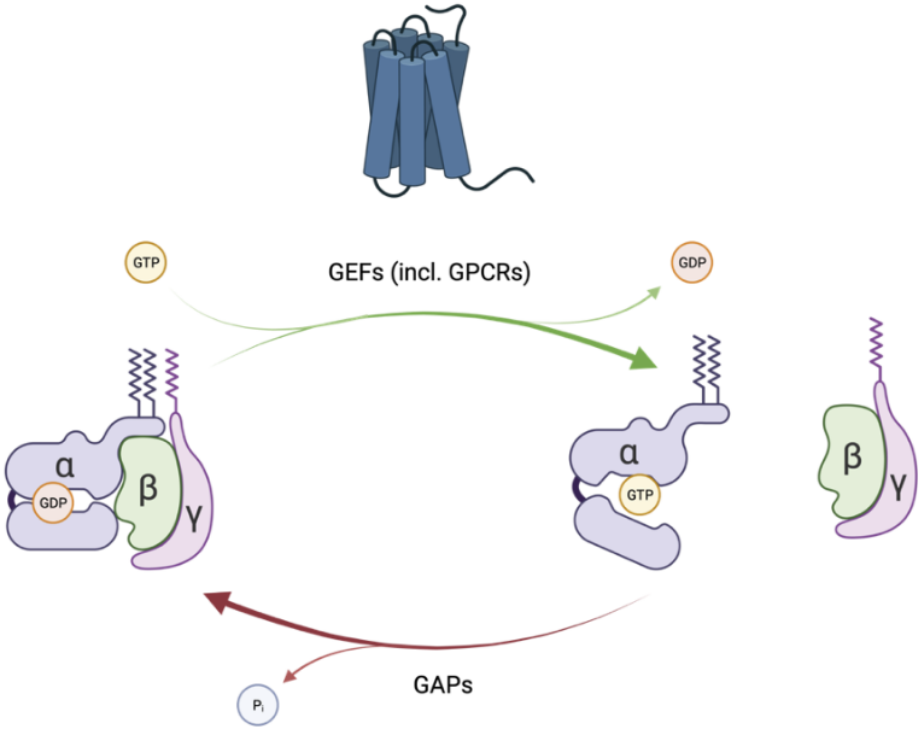


Figure 8- The Heterotrimeric G protein cycle

Activation cycle of a heterotrimeric G protein. At rest, the heterotrimeric G protein is associated, with GDP bound to the α subunit. Activation, through a GPCR or other GEF, mediates the exchange of GDP to GTP, giving an active α subunit. This then dissociates from the Gβγ complex. Inactivation occurs through hydrolysis of the GTP, back to GDP, which can be catalysed by GAPs.

4. Regulation of GPCRs responsiveness

Living organisms employ a variety of mechanisms to tightly control the responsiveness of GPCRs. As mentioned earlier, G protein activity can be regulated by AGS and RGS (Berman & Gilman, 1998; Ross & Wilkie, 2000). The activity of the effector is determined by the availability of free G protein subunits but can also be modulated by second messenger-dependent PKA and PKC. Additionally, the effector products (second messengers) can be enzymatically degraded by cAMP phosphodiesterases (PDE), phosphatidylinositol phosphatases, and diacylglycerol kinases. These enzymes can be recruited to GPCRs upon receptor activation, promoting signal termination (Baillie et al., 2003; Perry et al., 2002). The GPCR itself can also undergo negative regulation. Once the signal is transduced, the signalling machinery must be terminated. This is achieved through a common mechanism shared by the majority of GPCRs: receptor phosphorylation by a G protein-coupled receptor kinase (GRK), followed by the binding of an uncoupling protein called arrestin (Gurevich & Gurevich, 2019). While various pathways can contribute to the phosphorylation of the C-terminal tail, the two primary kinases involved are Protein Kinase A (PKA) and GRKs. PKA can phosphorylate the inactive receptor, leading to global desensitisation of different GPCRs upon activation (heterologous desensitisation), whereas GRKs primarily phosphorylate the active receptor (homologous desensitisation) (Carmona-Rosas et al., 2019).

a. Arrestin mediated regulation of GPCRs

The arrestin family is composed of members referred to as arrestin 1 and 4 being expressed in the retina (and therefore addressed as the visual arrestins), arrestin 2 and 3 are ubiquitously expressed and are referred to as β-arrestin1 and 2, due to their discovery in terminating β2 adrenoceptor signalling (Lohse et al., 1990). Arrestins are recruited to the phosphorylated C-terminal tail of the receptor, blocking G protein access. Arrestins exhibit preferential binding patterns to their associated receptors when they are active and phosphorylated at the same time (Krasel et al., 2005; Wilden et al., 1986).

The role of arrestins in terminating G protein-mediated GPCR signalling is well established (Carman & Benovic, 1998). Recent structural data showcased the molecular basis of the competition between G proteins and arrestins: both engage the receptor in the same inter-helical cavity on the cytoplasmic side of the receptor (Carpenter et al., 2016; Kang et al., 2015; Liang et al., 2017; Rasmussen et al., 2011; Zhou et al., 2017), and the binding of one prevents the binding of the other. In its GTP bound state, the G protein dissociates from its receptor, in contrast, arrestins do not. Arrestins bind with a higher affinity to phosphorylated GPCRs compared to G proteins (Gurevich & Gurevich, 2004), therefore easily winning in the competition. Moreover, in addition to the previously defined inter-helical cavity, which is a shared docking site of G protein and arrestins, Arrestins bind the phosphates attached to the receptor-attached phosphates that are arranged in patches of complete phosphorylation codes in their C-terminal tails of GPCRs (Zhou et al., 2017). This predicted dual-site binding opened the possibility that arrestin might engage the receptor via only one site. Indeed, this was confirmed, in the case of mutant GPCRs and/or arrestins, where arrestins can exclusively engage the phosphorylated receptor C-terminus, leaving the inter-helical

cavity accessible for the G protein (Cahill et al., 2017; Kumari et al., 2016; Thomsen et al., 2016). Under these conditions, we have the formation of a "super-complexes" that form a single GPCR simultaneously interacting with G protein and arrestin (Thomsen et al., 2016). Recent findings in studies of neuropeptide Y receptors proposed that this mechanism might operate in the case of at least some wild-type GPCRs (Wanka et al., 2018). However, the model of simultaneous arrestin interaction with both the inter-helical cavity and phosphorylated parts of the receptor, which hinders G protein binding, is the rule rather than an exception. This mode of arrestin binding is the basis of homologous GPCR desensitisation, ensuring direct competition of arrestins with G proteins (Krupnick et al., 1997; Wilden, 1995).

Moreover, non-visual arrestins inhibit receptor coupling to the G proteins and facilitate GPCR internalisation via coated pits, further reducing cell responsiveness (Gurevich & Gurevich, 2004) (**Fig.9**).

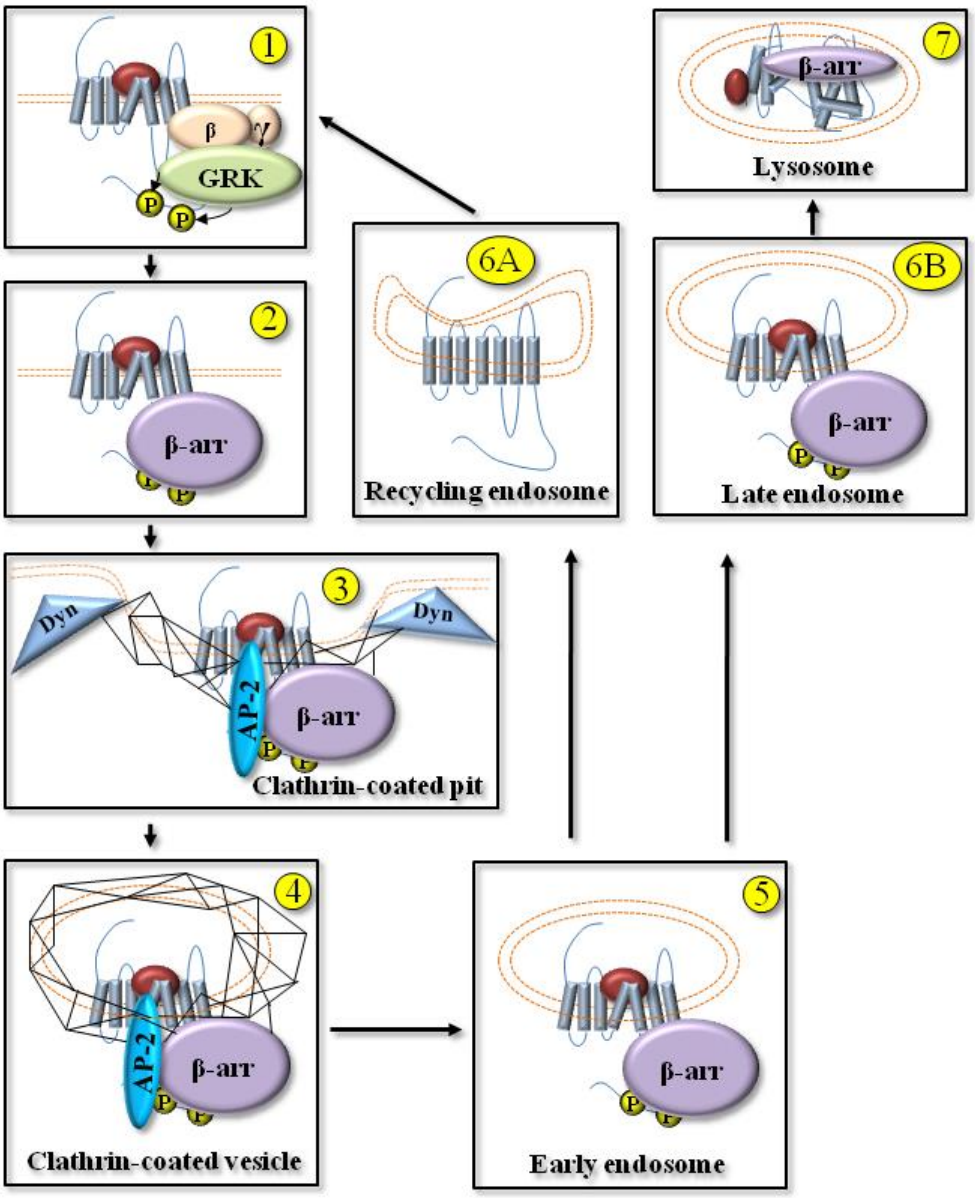


Figure 9-Signalling regulation of GPCRs mediated by GRKs and arrestins

Upon agonist binding to GPCRs, GRK is rapidly targeted to activated GPCR (1). GRK then phosphorylates the receptor. This phosphorylation triggers β-arrestin (β-arr) recruitment (2). β-arr binding to GPCR enables G protein coupling with the receptor and promotes the recruitment of clathrin and adaptor protein-2 (AP-2) (3). Once the clathrin-coated pit is completed, dynamin (Dyn) pinches the clathrin-coated vesicle off the cell membrane (4). In the early endosomes (5), a sorting occurs where class A receptors bind transiently to β-arr and are targeted into recycling endosomes to be recycled to the cell surface (6A), while class B receptors are tightly bound to β-arr in late endosomes (6B) and are mostly targeted toward lysosomes to be degraded (7).

b. GRKs and PKA mediated regulation of GPCRs

An activated receptor, through stimulation of second messenger-dependent kinases (PKA or PKC), can modulate the activity of a different receptor. In contrast to GRKs, PKA or PKC-mediated phosphorylation of a GPCR directly inhibits G protein binding to the receptor (Pitcher et al., 1992). Second messenger dependent kinase-mediated phosphorylation mediates changes in GPCR charge distribution that can lead to alterations in the conformation of the phosphorylated region. This change in conformation is generally targeted to the key regions on the GPCR where the G protein binds and is responsible for the decreased ability of the receptor to couple to G proteins. It is this pathway that mainly regulates receptor internalisation and desensitisation. Any dysregulation in these processes, mostly due to mutations in the regulatory proteins or within the GPCRs, is implicated in pathophysiology states such as retinitis pigmentosa (Mallory et al., 2018). Therefore, targeting GPCRs desensitisation can also be useful in the development of therapeutics. An example includes non-catechol D_1-targeting drugs that show reduced internalisation and desensitisation and are, therefore, better able to induce long-term effects (Gray et al., 2018). Preferential signalling through one pathway over another is known as agonist bias but is also referred to as signalling bias or functional selectivity. Bias can take the form of selectivity between G proteins but also includes preference for G protein signalling over β-arrestin recruitment (and vice versa). Most GPCRs exhibit inherent bias, giving rise to their canonical G protein-coupling. However, agonist bias can be influenced by factors such as the stimulating agonist or interacting proteins. In general, GRKs and second messengers-dependent kinases phosphorylate GPCRs on different sites (Lefkowitz et al., 1990). Classically, homologous and heterologous desensitisations are two independent processes. However, many studies demonstrated

that PKC and PKA do not only phosphorylate GPCRs but have the aptitude to also phosphorylate GRKs, which is strictly required for the formation of the hanging complexes of arrestin and GPCR and, therefore, regulate homologous regulation (Haider et al., 2022). Additionally, numerous studies show that PKA and PKC can also regulate GPCR activity through effector phosphorylation. It was shown that PKA inhibits the activity of AC5 and AC6. Additionally, it was reported that phosphorylation of AC1, AC2, AC3, AC5 and AC7 by PKCα stimulates the activity of these AC isoforms but inhibits AC4 activity. Moreover, PKCδ and PKCε were reported to inhibit AC6 activity and PKCδ to stimulate AC5 (Sadana & Dessauer, 2009).

V. Dopaminergic system

Dopamine, the predominant catecholamine neurotransmitter in the mammalian brain, plays a crucial role in various physiological processes. It regulates cognition, emotions, food intake, locomotor activity, positive reinforcement, and endocrine regulation. In addition to its central functions, dopamine also has peripheral roles in renal function, vascular tone, cardiovascular functions, hormone secretion, and gastrointestinal motility (Channer et al., 2023; Missale, Russel Nash, et al., 1998). Dopaminergic neurotransmission dysfunctions are found to be implicated in several pathological conditions such as schizophrenia, Parkinson's disease, bipolar disease, depression, drug and alcohol addiction, Huntington's disease, Tourette's syndrome and attention-deficit/hyperactivity disorder (Le Foll et al., 2009; Lebel et al., 2006; Wong et al., 2000).

1. Dopaminergic synapse

To induce effects on the postsynaptic neuron, the presynaptic neuron must synthesise and release dopamine (**Fig.10**). The biosynthesis of dopamine takes place in the terminal part of a dopaminergic neuron and can be divided into two key steps (Callier et al., 2003). The first step is the rate-limiting step and it consists of transforming the L-tyrosine to L-dihydroxyphenylalanine (L- DOPA) by a tyrosine hydroxylase (An et al., 2013; Molinoff & Axelrod, 1971). The second step is when the L-DOPA is decarboxylated into dopamine by DOPA-decarboxylase (Zhu & Juorio, 1995). Once dopamine is produced, it is held in storage vesicles by the vesicular monoamine transporter 2 (VMAT2). If dopamine is present in a free state in the presynaptic terminal, it can be easily degraded in the neurone by the enzyme monoamine oxidase (Juárez Olguín et al., 2016).

Elevated calcium concentrations in presynaptic terminals can trigger the fusion of dopamine vesicles with the presynaptic membrane, leading to the release of dopamine into the synaptic cleft. Once in the synaptic cleft, dopamine can interact with dopamine receptors present on both the postsynaptic neuron and presynaptic autoreceptors. The binding of dopamine to these receptors activates specific signalling pathways. The specific pathways activated depend on the type of dopaminergic receptor being stimulated and the neurons expressing these receptors. Antipsychotic drugs like lurasidone, cariprazine, and brexpiprazole can target dopamine receptors to terminate dopaminergic receptor signalling (Frankel & Schwartz, 2017); these drugs are identified as antagonists or inverse agonists. An excess of dopamine in the synaptic cleft can be neutralised by the enzyme catechol-O-methyltransferase, which is located on the post-synaptic neuron (Montag et al., 2012). Furthermore, dopamine can also be shuttled into the presynaptic terminal by the dopamine transporter. Amphetamine and

cocaine are known as potent inhibitors of dopamine transporter functions (Zhu & Reith, 2008). The use of these drugs leads to a sharp increase in dopamine concentrations in the synaptic cleft, which is the main cause of psychosis, and it is associated with psychostimulant abuse and dependence. Moreover, amphetamine is known to stimulate dopamine release by inducing the reverse transport of dopamine from the presynaptic neuron to the synaptic cleft (Kahlig et al., 2005).

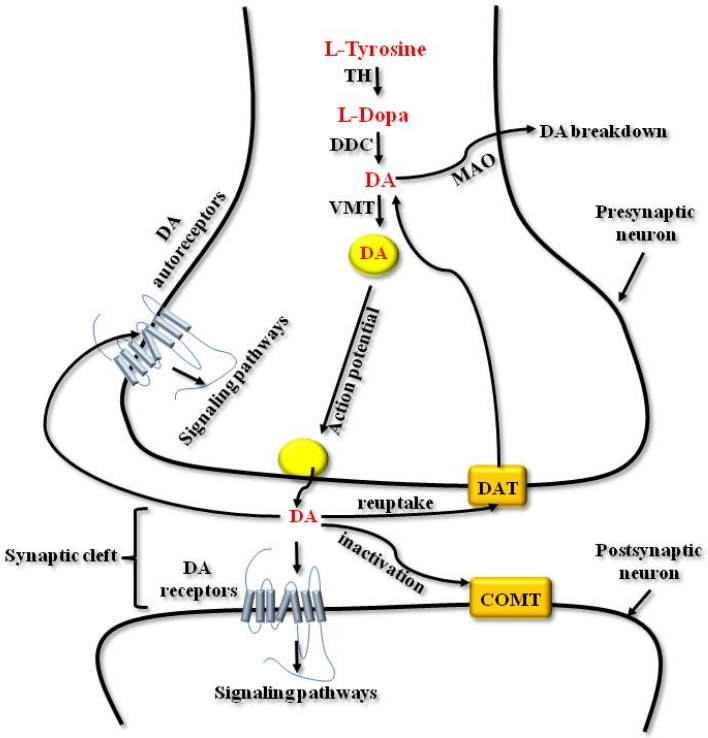


Figure 10- Dopaminergic synapse

Dopamine (DA) is synthesised in the presynaptic neuron. L-tyrosine is transformed into Levo-Dihydroxyphenylalanine (L-Dopa) by tyrosine hydroxylase (TH) and then converted into dopamine by dopamine decarboxylase (DDC). Free dopamine is stocked in vesicles by vesicular monoamine transporter (VMT), while the free dopamine can be degraded by monoamine oxidase (MAO). Upon an action potential, dopamine is released from vesicles to be in the synaptic cleft, where it can bind to postsynaptic DA receptors or presynaptic autoreceptors, generating activation of signalling pathways. Excess DA can be neutralised by catechol-O-methyltransferase (COMT) on postsynaptic cells or can be taken up by dopamine transporter (DAT) located in the presynaptic cells.

2. Dopaminergic neuronal pathways

Dopaminergic neurons are located in specific areas of the human brain. They are organised into four main dopaminergic neuronal tracts (Iversen & Iversen, 2007; Kienast & Heinz, 2006) (**Fig.11**). The first one is the nigrostriatal pathway. Some of these dopaminergic neurons can be found in the substantia nigra. The dopamine synthesised by these neurons is then released in the dorsal striatum. The dopaminergic transmission in this pathway is responsible for controlling motor behaviour. The destruction of these neurons is a well-characterised process which is associated with Parkinson's disease (Alexander, 2004). Any abnormalities in the dopaminergic system in the dorsal striatum have also been associated with motor tics in Tourette's syndrome (Leisman & Sheldon, 2022).

The mesolimbic pathway is another important dopaminergic pathway in the brain. Neurons in this pathway originate from the ventral tegmental area (VTA) and establish synaptic connections with neurons in the nucleus accumbens (NAc) situated in the ventral striatum. This specific neuronal pathway plays a crucial role in regulating motivation, anticipating rewards, and experiencing pleasure. Dysfunctions within this dopaminergic pathway are often associated with various negative symptoms observed in individuals with schizophrenia, such as social withdrawal, reduced motivation, decreased desire and pleasure, and blunted emotions (Brisch et al., 2014; McCutcheon et al., 2020). This neuronal dopaminergic pathway is also involved in the reinforcing effects of drug intake and depression (Nestler & Carlezon, 2006).

Another dopaminergic tract that originates from VTA and is also known to be involved in schizophrenia symptoms is the mesocortical pathway (McCutcheon et al., 2019). Neurons from this tract make synaptic contact with other neurons present in the prefrontal cortex (Luo & Huang, 2016). This pathway is critical for the working

memory and various cognitive functions. An abnormal prefrontal dopamine release is associated with the positive symptoms of schizophrenic patients (i.e., hallucinations and cognitive deficits) (Brisch et al., 2014).

The last dopaminergic tract is implicated in the neuroendocrine control (Juárez Olguín et al., 2016). Somas of neurons from the tuberoinfundibular tract are located in the arcuate nucleus of the hypothalamus. Dopamine release from these neurons occurs in the pituitary and causes the inhibition of prolactin secretion (Ben-Jonathan & Hnasko, 2001; Stagkourakis et al., 2019).

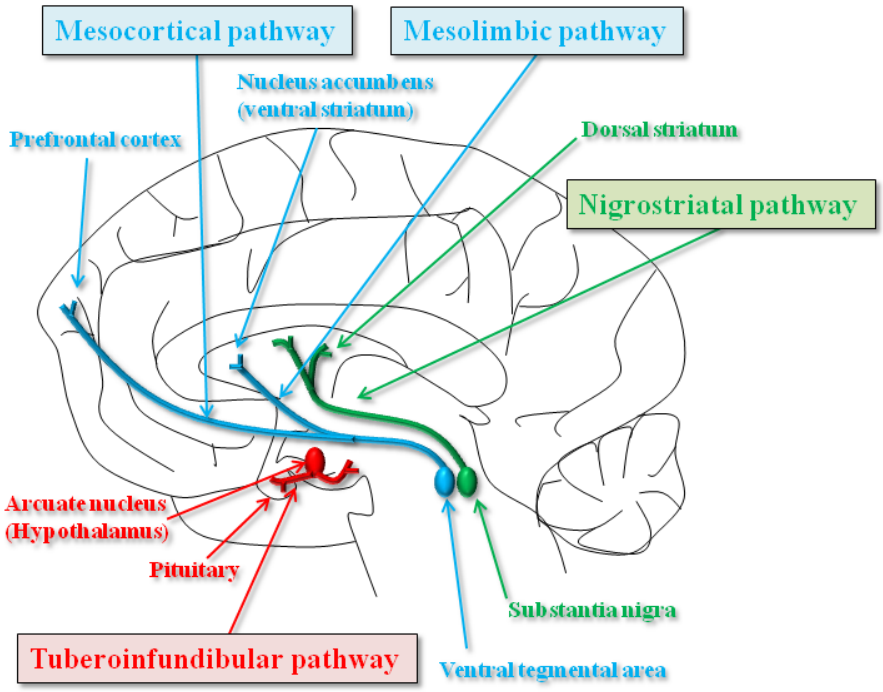


Figure 11- Dopaminergic neuronal pathways

There are four dopaminergic neuronal pathways: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular. Neurons located in the subtantia nigra and projecting to the dorsal striatum compose the nigrostriatal pathway. Neurons located in the ventral tegmental area that make synaptic contact with the nucleus accumbens in the ventral striatum form the mesolimbic pathway, while the one projecting to the prefrontal cortex represents the mesocortical pathway. Neurons located in the arcuate nucleus of the hypothalamus send projections to the pituitary from the tuberoinfundibular pathway.

3. Dopaminergic receptor classification

The dopamine receptors are members of the rhodopsin-like GPCR family and are widely expressed in the central nervous system (CNS). They are also expressed in the periphery, more prominently in the kidney and vasculature (Qaddumi & Jose, 2021). A dysfunction in the dopaminergic signalling or neurotransmission in the CNS is implicated in various neuropsychiatric disorders like social phobia (Schneier et al., 2000), Parkinson's disease (Fuxe et al., 2006), Tourette's syndrome (Kienast & Heinz, 2006), schizophrenia (Hong et al., 2010), attention-deficit hyperactivity disorder (ADHD) (Faraone & Khan, 2006), neuroleptic malignant syndrome (Mihara et al., 2003) and alcohol and drug dependence (Kienast & Heinz, 2006). This led the dopamine receptors to be an interesting, high-value target for drugs.

Five different subtypes of dopamine receptors have been identified to this day. Designated as D_1, D_2, D_3, D_4 and D_5 (Le Crom et al., 2003), they differ in ligand specificity, the nature of the G-protein coupled to the receptor, their tissue distribution and the physiological effect of their downstream signalling. These 5 subtypes are divided into two subfamilies: the D1-like family (D_1, D_5), which is characterised by its coupling to the $G\alpha_s$ subunit and induces an excitatory signal, and the D2-like family (D_2, D_3 and D_4) which couples to the $G_i/G_o/G_s$ G-protein alpha subunit and mediates an inhibitory signalling pathway. Even though dopamine receptors are highly expressed in the brain, they can be found in different locations with various densities. It has been proposed that there is a functional link between receptor density and the roles mediated (Howes & Kapur, 2009; Missale, Russel Nash, et al., 1998). D_1 and D_2 subtypes are found in greater density than the D_3, D_4, and D_5 subtypes (Hurley & Jenner, 2006).

a. D1-like receptors

D1-like receptors show 82% sequence homology and are characterised by the presence of a relatively short IL3, and a long serine and threonine rich CT (Xu et al., 2022). The short IL3 is characteristic of many receptors coupled to stimulatory G proteins (Beaulieu & Gainetdinov, 2011; Missale, Russel Nash, et al., 1998). D_1 and D_5 have very similar TM domains.

The D_1 gene is located on chromosome 5q35.1 and is the most abundant receptor subtype among all dopamine receptors (Wong et al., 2000). In vertebrate brains, D_1 is particularly abundant in the ventral and dorsal striatum, where it is mainly located on gamma-aminobutyric acid (GABA) medium-sized spiny neurons and co-localise with dynorphin and substance P (Aubert et al., 2000; De Keyser et al., 1988; Levey et al., 1993). Medium spiny neurons constitute 95% of the striatal neurons (Kemp & Powell, 1971). These neurons integrate inputs from glutamatergic cortical neurons and dopaminergic midbrain neurons. Abnormalities in these neurons to integrate these inputs strongly compromise striatum functions. Furthermore, D_1 is also highly expressed in the amygdaloid complex and the nucleus tractus solitaries involved in fear conditioning and coordinating the autonomic nervous system respectively (Callier et al., 2003).

The D5R gene is located on chromosome 4p15.1-p15.3 (Wong et al., 2000). The product of this gene is particularly abundant in the hippocampus, where D_1 expression is low (Callier et al., 2003). D5R is also highly expressed in the nucleus accumbens and thalamus and at a moderate level in the cortex, in cholinergic neurons of the dorsal striatum, substantia nigra, nucleus tractus solitaries, dorsal hypothalamus, amyloid complex and retina (Callier et al., 2003; Surmeier et al., 1996).

b. D2-like receptors

D2-like receptors have a long IL3, a common feature of GPCRs coupled to the inhibitory G protein (Martinez et al., 2020). Furthermore, the CT of D2-like receptors is approximately seven times shorter than the CT of D1-like receptors (Missale, Russel Nash, et al., 1998). While D1-like receptors possess two N-glycosylation sites (on NT and EL2 regions), D_2, D_3 and D_4 have four, three and one glycosylation sites respectively (Martinez et al., 2020), which affects both correct cell surface expression, signalling and internalisation (Min et al., 2015).

The D2R gene is located on chromosome 11q22-23 and is the most abundant D2-like receptor (Wong et al., 2000). D_2 exists as two alternatively spliced isoforms: $D_{2\ short}$ and $D_{2\ long}$ (Dal Toso et al., 1989; Giros et al., 1989). D_{2long} has a 29 amino acid insertion in IL3, which is absent in $D_{2\ short}$ (Ebersole et al., 2015). The distribution of D_2 in the brain is similar to that of D_1. Similarly to D_1, striatal D_2 are expressed in GABAergic medium spiny neurons, but co-localise with enkephalin (Aubert et al., 2000; Gerfen et al., 1995; Surmeier et al., 1996; Surmeier & Kitai, 1993). Striatal D_2 are also expressed in cholinergic interneurons (Tanimura et al., 2018).

D_3 gene is located on chromosome 3q13.3, and D_3 and D_2 share 75% identity within their TM domains (Missale, Russel Nash, et al., 1998; Wong et al., 2000). D_3 is mainly expressed in limbic areas. GABAergic medium spiny neurons of the nucleus accumbens (rostral and ventrolateral shell) and granule cells of the islands of Calleja have the largest D3R densities (Landwehrmeyer et al., 1993; Lévesque et al., 1992; Murray et al., 1994). Importantly, D_1 and D_3 co-localise in single neurons located in the nucleus accumbens (Solís et al., 2017). Indeed, their interaction at cellular and behavioural levels has been demonstrated (Schwartz et al., 1998). An interesting particularity of D_3 is its ability to inhibit AC5 activity but no other AC isoforms

(Missale, Russel Nash, et al., 1998). Furthermore, it is worth mentioning that D_3 dopamine affinity is about twenty times higher than that of D_2 (Sokoloff et al., 1990). This difference in affinity has been related to differences between IL3 of D_2 and D_3 (Missale, Russel Nash, et al., 1998). However, quinpirole, which acts as a selective D_2 and D_3 receptor agonist, is the most discriminating drug between D_2 and D_3, with over 100 times higher affinity for D_3 (Missale et al., 1998; Sokoloff et al., 1990).

Finally, D4R, located on chromosome 11p15.5, is mostly expressed in the same brain regions that D2R (Wong et al., 2000). The highest densities of D4R are located in the dorsomedial thalamus and the lateral septal nucleus. Comparison between amino acid sequences of D2-like receptors reveals that D4R is the most distantly related of the dopamine receptors coupled to inhibitory G proteins. Indeed, TM domains of D4R share only 53% identity with those of D2R (Missale et al., 1998). Notably, clozapine which is an antagonist of D4 receptors is particularly useful to distinguish D_4 from other D2-like receptors (Iijima & Van Tol, 1991). Indeed, clozapine is shown to have about 15 times higher affinity for D_4 than for D_2 and D_3.

4. Dopaminergic receptor signalling

D1-like and D2-like dopaminergic receptors have different structural features. These differences are suspected to determine their preferential coupling with stimulatory or inhibitory G proteins respectively (Missale et al., 1998). The nature of stimulated G proteins determines the signalling pathways activated downstream of the dopaminergic receptors upon the ligand binding to the receptor. The following section describes the most common signalling pathways associated with the activation of D1-like receptors, as in my research we focus on the dopamine D_1 receptor.

a. D1-like receptor signalling pathways

D1-like receptor signalling is mainly mediated by the production of intracellular cAMP through AC activation by the stimulatory G proteins $G\alpha_s$ or $G\alpha_{olf}$ (**Fig.12**). As previously elaborated, AC activation leads to disinhibition of regulatory subunits of PKA. This kinase phosphorylates several proteins involved in gene expression and important signal transduction signals (Neve et al., 2004). Furthermore, PKA activates dopamine and cyclic AMP-regulated phosphoprotein 32 kDa (DARPP-32). Phosphorylation of DARPP-32 by PKA occurs on Thr 34 (Lindskog et al., 2006). Phosphorylation of that DARPP-32 residue leads to inhibition of protein phosphatase 1 (PP1) (Hemmings et al., 1984). This phosphatase catalyses the dephosphorylation of several important proteins in the brain, such as voltage-gated ion channels and numerous neurotransmitter receptors (Greengard et al., 1999). Attenuated responses to antipsychotic drugs, psychostimulants and dopamine have been observed in DARPP-32 knockout mice (Fisone et al., 2011). However, when DARPP-32 is phosphorylated on Thr75 (by cyclin-dependent kinase 5), DARPP-32 inhibits PKA (Bibb et al., 1999). However, this DARPP-32-mediated inhibition of PKA is prevented by dephosphorylation of Thr75 on DARPP-32 by the PKA-stimulated protein phosphatase-2A (PP2A) (Nishi et al., 2000).

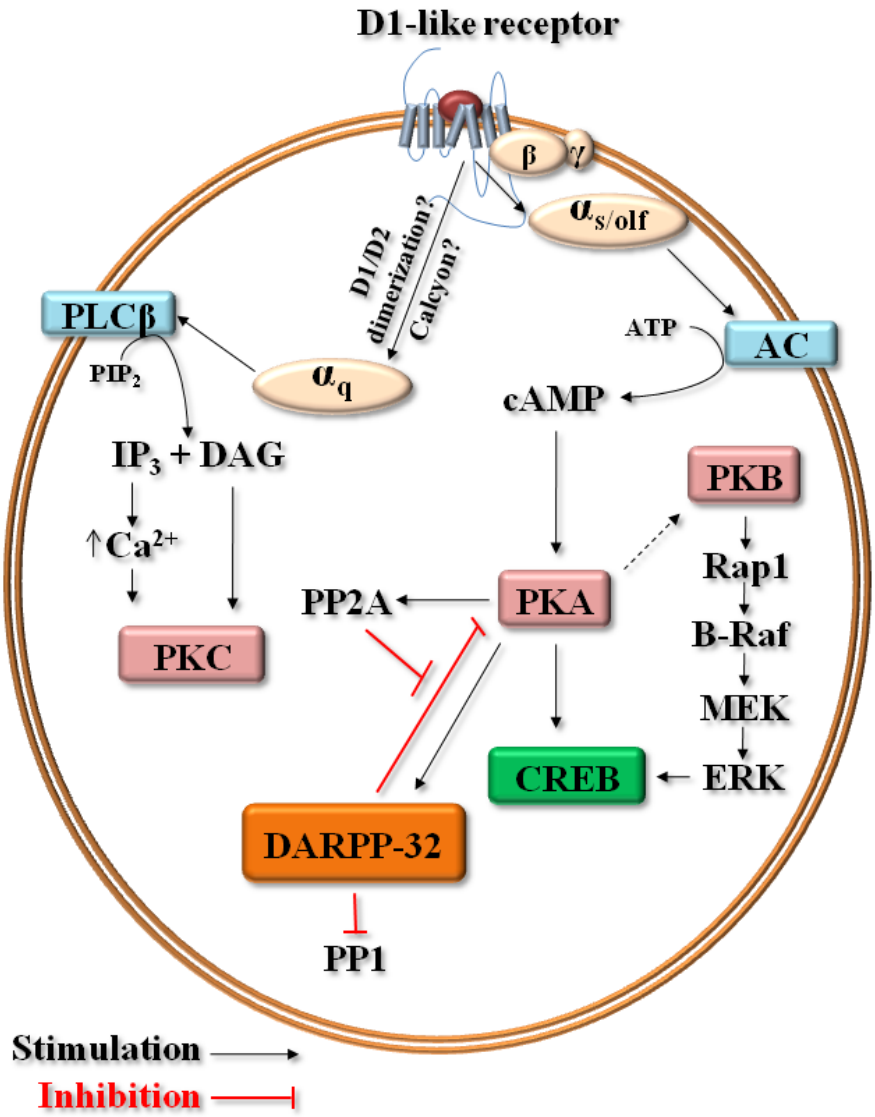


Figure 12- D1-like receptor signalling pathways

D1-like receptor signalling pathways are mediated by the production of intracellular cAMP via activation of adenylyl cyclase (AC) by stimulatory G proteins (α_S or α_{olf}). cAMP activates protein kinase A (PKA) activity, leading to dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32)-mediated inhibition of protein phosphatase 1 (PP1). Inhibition of PKA by DARPP-32 is prevented by PKA-mediated stimulation of PKA-stimulated protein phosphatase-2A (PP2A) activity. D1-like receptor-mediated activation of PKA also stimulates ERK activity via protein kinase B (PKB) activation by an unknown mechanism. Both PKA and ERK lead to the activation of a transcription factor called cAMP response element-binding protein (CREB), responsible for the transcription of several genes having a cAMP response element. $G\alpha_q$-coupling with D1-like receptors was also reported. This leads to the activation of phospholipase Cβ (PLCβ), resulting in an elevation of intracellular calcium concentration and activation of protein kinase C (PKC). The mechanism for which D1-like receptors couple with $G\alpha_q$ is not well understood but could be explained by the involvement of a protein called calcyon or through heterodimerisation of D_1 with D_2.

Another important PKA substrate is the transcription factor cAMP response element-binding protein (CREB) (Konradi et al., 1994). Phosphorylation of Ser133 of CREB by PKA allows the binding of CREB to CREB-binding proteins, leading to the transcription of genes with a cAMP response element (Cole et al., 1995). Activation of gene expression by phosphorylation of CREB has an important role in synaptic plasticity (Sakamoto et al., 2011). Gene transcription by CREB can also be mediated by activation of an extracellular signal-regulated kinase (ERK) (Brami-Cherrier et al., 2002). Activation of ERK via D1-like receptors is mediated by PKA-dependent activation of PKB (Sun et al., 2016).

Also, D1-like receptor signalling activates the PLCβ pathway, leading to the elevation of intracellular calcium concentration and PKC activation (Mahan et al., 1990). However, this pathway is controversial. An increase of intracellular calcium was reported in mouse LTK- and HEK293 cells transfected with human or goldfish D_1 upon receptor stimulation (Liu et al., 1992). Furthermore, evidence has been reported that D1-like receptors are coupled to PIP_2 hydrolysis (Cadet et al., 2010; Undieh, 2010). Intrarenal administration of a D1-like receptor agonist increases PLCβ activity (Jose et al., 1995). In addition, incubation with the D1-like receptor specific agonist SKF38393 increases IP_3 production and PIP_2 hydrolysis in rat brain striatal slices as well as post-mortem human prefrontal cortex membranes (Pacheco & Jope, 2002; Wang et al., 1995). SKF83959, a D1-like receptor agonist that does not stimulate AC activity, also stimulates PIP_2 hydrolysis or IP_3 production in membranes from the cerebellum and hippocampus from rat brain and in rat striatal and macaque caudate nucleus slices (Jin et al., 2003; Panchalingam & Undie, 2001). Some studies suggest that activation of this signalling pathway occurs through a D1-like receptor coupling

with the $G\alpha_q$ protein (Undieh, 2010). Surprisingly, incubation of rat frontal cortex and striatal membranes with SKF83959 or SKF38393 increases [^{35}S]GTPγS binding to $G\alpha_q$ but not to a lesser extent than $G\alpha_s$ (Jin et al., 2003; Panchalingam & Undie, 2001). Two possible mechanisms have been hypothesised. The first one is the involvement of a protein called calcyon (Lidow et al., 2001). The second mechanism is via heterodimerisation of D_1 with D_2 (Lee et al., 2004).

b. Modulation of ion channels by D1-like receptors

Activation of D1-like signalling pathways can also regulate the activity of several ion channels. Voltage-gated potassium channels are generally inhibited upon D1-like receptor activation via stimulation of the PKA/DARPP-32 pathway (Neve et al., 2004; Surmeier & Kitai, 1993). In a similar fashion to potassium channels, the conductance of N- and P/Q-types of calcium channels is also decreased upon D1-like receptor activation via the PKA/DARPP-32 pathway (Surmeier et al., 1995; Young & Yang, 2004). However, in contrast to N- and P/Q-type calcium channels, L-type calcium currents are increased by D1-like receptor activation. Sodium channels are also regulated by D1-like receptors by PKA activation and inhibition of PP1 via activation of DARPP-32. Moreover, dopaminergic modulation increasing PKA-mediated phosphorylation of specific serine residues of the pore-forming α-subunit of voltage-gated sodium channels leads to a reduction of sodium currents (Cantrell et al., 1997; Li et al., 1992; Murphy et al., 1993; Smith & Goldin, 1997; Surmeier et al., 1992).

c. Modulation of ligand-gated ion channels by D1-like receptors

In addition to non-ligand gated ion channels, D1-like receptor stimulation also modulates ligand-gated ion channel activity such as N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and γ-amino butyric acid type A ($GABA_A$) receptors.

NMDA receptor response is increased upon D1-like receptor activation by phosphorylation of the NR1 subunit of the NMDA receptor (Dudman et al., 2003). DARPP-32-mediated inhibition of PP1, activation of PKA, ERK, L-type calcium channels and PKC have been described to be involved in D1-like receptor-mediated NMDA receptor stimulation (Cepeda et al., 1998; Chergui & Lacey, 1999; Flores-Hernández et al., 2002; Sarantis et al., 2009; Snyder et al., 1998; Yang, 2000). It was also reported that D1-like receptor stimulation increases NMDA receptor trafficking to the membrane surface with increased expression of NMDA receptor subunits NR1 and NR2B (Dunah & Standaert, 2001; Hu et al., 2010). However, physical interaction between the CT of the D_1 and the NR2A subunit of the NMDA receptor has been shown to inhibit NMDA currents (Lee et al., 2002).

AMPA receptors are positively modulated by D1-like receptor activation. AMPA current amplitude is modestly enhanced through the activation of L-type calcium channels by D_1-like-mediated stimulation of the PKA/DARPP-32 pathway (Galarraga et al., 1997). In addition, D1-like receptor activation stabilises the AMPA receptor current through PKA-mediated phosphorylation of Ser845 of the AMPA receptor GluR1 subunit and by inhibition of PP1-mediated dephosphorylation of this serine residue (Chao et al., 2002; Snyder et al., 2000; Yan et al., 1999). In the hippocampus, it was reported that D1-like receptor stimulation increases AMPA receptor cell surface expression in a PKA-dependent manner (Gao et al., 2006). In a similar fashion to

NMDA receptors, the $GluR_1$ subunit of AMPA receptor surface expression is enhanced by D1-like receptors-mediated activation of AC (Chao et al., 2002; Gao et al., 2006).

Finally, $GABA_A$ receptors are differently regulated by D1-like receptors depending on the brain area and the cell type in which $GABA_A$ receptors are expressed. In the nucleus accumbens and the medium spiny neurons of the striatum, the activity of $GABA_A$ receptors is decreased via PKA/DARPP-32-mediated phosphorylation of β1/β3 subunits of $GABA_A$ receptor (Flores-hernandez et al., 2019; Nicola & Malenka, 1998). In contrast, in a subpopulation of zinc-sensitive $GABA_A$ receptors localised in large striatal cholinergic interneurons, $GABA_A$ currents are enhanced by D5R stimulation via activation of PKA and inhibition of PP1 (Yan et al., 1997). This opposite regulation of $GABA_A$ currents by D1-like receptor activation can be potentially explained by the presence of a variety of α, β and γ subunit isoforms of $GABA_A$ receptors (Neve et al., 2004). As it was described for the D_1 and NMDA receptor, direct physical interaction has been seen between CT of D5R and the γ_{2short} subunit of $GABA_A$ receptor (Liu et al., 2000). This interaction results in mutual inhibition of the D5R and the $GABA_A$ receptor.

5. D_1 related pathologies

The dopamine 1 receptors are abundant in the caudate-putamen, nucleus accumbens and olfactory tubercle, with lower levels in the frontal cortex, habenula, amygdala, hypothalamus, and thalamus. They are also found in the kidneys, heart, liver, and parathyroid glands. In humans, the pulmonary artery expresses D_1 receptors, which can be attributed to vasodilatory effects (Ricci et al., 2006). D_1 receptor knockout mice have been demonstrated to have reduced motivation for alcohol

consumption (El-Ghundi et al., 1998). D_1 is very important for locomotor activity, for example in Huntington's disease (HD), where we see a change in dopamine levels and receptor numbers, the patients develop movement and cognitive deficits (Peinemann et al., 2005; Wang et al., 2012). Also, the bradykinesia in hemiparkinsonian animal models was associated with a decrease in D_1 expression (Rangel-Barajas et al., 2008). Also, there are several drugs that target the D_1, mostly for the treatment of psychosis and schizophrenia. Schizophrenia is a neuropsychological disorder that research work has tightly linked with aberrant dopaminergic signalling (Grace, 2016; Lidow, 2003). There are numerous D_1-targeting drugs, and most of them have significant side effects. In the field of antipsychotics, drugs such as clozapine can lead to motor side effects, also known as extrapyramidal side effects (Kurz et al., 1995). These effects are caused by the activation of the nigrostriatal pathway which is central to motor control. It has been found that side effects are related to the occupancy of D2-like receptors (Farde et al., 1992). Beyond motor-related side effects, D_1 targeting antipsychotics have been shown to drive metabolic and endocrine aberrations. Especially in young patients, treatments with clozapine were found to be associated with excessive weight gain (Fleischhaker et al., 2007). This is probably related to the non-selective D2-like receptor activation by these drugs, although some evidence links other neurological systems, such as the histaminergic system (Hong et al., 2010; McIntyre et al., 2001). In the case of neurodegenerative diseases, treatments targeting the dopaminergic system, such as levodopa (a dopamine precursor) for Parkinson’s Disease treatment, have resulted in side effects. Levodopa usage was found to cause neuronal death through excessive stimulation and is now not recommended as an automatic go-to treatment (Brooks, 2000). The side effects commonly found with dopaminergic agonists as treatments are low tension, hallucinations, delusions and sleepiness, likely

through undesired effects on the mesolimbic and mesocortical pathways (Borovac, 2016; Gottwald et al., 1997).

Side effects can have serious effects on the patient's health and well-being. Additionally, side effects and patient perception of them are major driving factors in non-compliance and interruption of treatment, resulting in relapse (Haddad et al., 2014; McIntyre et al., 2001). The development of novel antipsychotics with limited side effects is a must to better serve patients. Therefore, understanding the pharmacology of the D_1 is very important as the most used pharmacological treatment for Parkinson disease is L-3,4-dihydroxyphenylalanine with studies showing that a chronic treatment may cause multiple side effects as L-3,4-dihydroxyphenylalanine induces dyskinesia, characterised by abnormal involuntary movements (Santini et al., 2007). The D1-like family mediate a signalling pathway that was related to a multitude of neuropsychiatric disorders through PLC activation and IP_3 accumulation, which induces intracellular calcium release. Ca^{2+} plays an important role in the activation of the protein kinase calcium-dependent (PKC) but also the modulation of neurotransmitter release by exocytosis (Koh et al., 2003; Loos et al., 2010).

VI. S-Palmitoylation

Palmitoylation is a lipid modification of proteins that was conserved during evolution (Hayashi, 2021; Tsukamoto et al., 2013). Palmitoylation is a reversible and dynamic phenom that influences many cellular properties of proteins ranging from protein stability, membrane domain organisation, protein trafficking and protein function. Recent improvements in chemical biology and proteomic techniques have helped discover more palmitoylated proteins in various species and tissues/cells and

revealed that the palmitoylation of proteins happens in membrane-bound organelles and specific membrane compartments. Adding to that, the identification and characterisation of membrane proteins with a cysteine-rich domain that contains a conserved sequence, Asp–His–His–Cys DHHC proteins are protein acyltransferases (PATs DHHC)/substrate pairs have helped to understand the regulatory mechanisms and pathophysiological significance of protein palmitoylation.

Palmitoylation increases the hydrophobicity of proteins and is suspected to be responsible for their proper membrane localisation in the cell (Dennis & Heather, 2023). Palmitoylation occurs on specific cysteine residues either through thioester-linkage (S-palmitoylation) or an amide-linkage (N-palmitoylation). Unlike some other lipid modifications, such as myristoylation and prenylation, palmitoylation represents the advantage of being reversible, suggesting that palmitoylation dynamically regulates the subcellular localisation of proteins (Busquets-Hernández & Triola, 2021; Guan & Fierke, 2011).

The first palmitoylated proteins were discovered in 1979 in the Sindbis virus (Schmidt & Schlesinger, 1979) and the vesicular stomatitis virus (Schmidt & Schlesinger, 1979; Veit, 2012). In 1987, the reversibility of the protein palmitoylation process was reported for ankyrin in erythrocytes, a family of proteins that mediate the attachment of membrane proteins to the cytoskeleton (Staufenbiel, 1987). Palmitoylation occurs on soluble proteins, for example the GTP-binding proteins (G protein α subunit, H/N-Ras), SNARE proteins (SNAP-25), postsynaptic scaffolding proteins (PSD-95, GRIP), cell adhesion molecules (integrin, claudin, and NCAM), and integral membrane proteins such as GPCRs (Chamberlain & Shipston, 2015; El-Husseini & Bredt, 2002; Fukata & Fukata, 2010; Linder & Deschenes, 2007). Another important aspect is that specific extracellular signals can modify the palmitoylation-

depalmitoylation balance on certain proteins, such as $G\alpha_s$ and PSD-95 (El-Husseini et al., 2002). Altogether, protein palmitoylation has established itself as one of the key post-translational modifications that can impact the localisation and functions of proteins and have a great effect on cellular signalling.

Historically, it has been challenging to investigate protein palmitoylation due to the lack of efficient, specific, rapid, and highly sensitive detection methods to detect and identify palmitoylated proteins (Gao & Hannoush, 2018; Main & Fuller, 2022), palmitoyl acyltransferases (PATs) that are a family of enzymes that catalyse protein S-palmitoylation and the depalmitoylation enzymes palmitoyl-protein thioesterases (PPT); the lack of tools for the spatiotemporal visualisation of the palmitoylated state of proteins in cells have slowed the progress of research in the field of protein palmitoylation, compared with that of protein phosphorylation (Gao & Hannoush, 2018). The identification of enzyme-protein pairs completely relied on the hypothesis of investigators because the purification method of palmitoylated proteins was not established, and the consensus sequence for protein palmitoylation remained undetermined, unlike that for myristoylation (MGXXXS/T) or prenylation (cysteine in the C-terminal CAAX motif) (Xie et al., 2016). Therefore, for a long time, discovering new palmitoylated proteins in cells stagnated, and whether the reactions of palmitoylation and depalmitoylation are meditated by enzymes was difficult to establish until recent developments in proteomic methods and chemical biology approaches in parallel to the discovery of enzymes catalysing palmitoylation caused a paradigm shift in the field of palmitoylation.

1. Methods of Protein palmitoylation detection

Recent advances in protein lipidation detection by proteomics and direct approaches have revealed that lipidation of signalling proteins is essential for regulating a wide variety of pathways. One of the earliest studies on S-palmitoylation in mammalian cells used [H^3] radiolabelled palmitate, followed by fluorography with long exposure (Schlesinger et al., 1980). To detect palmitoylation of specific proteins of interest, purified proteins are needed for cell-free labelling with [H^3] palmitate (Bizzozero & Lees, 1986; Caron, 1997). These methods have been used to identify various palmitoylation targets, but they have many disadvantages. Other than the health hazard of radioactivity and the time-consuming autoradiography exposure step, the sensitivity and the efficiency of radiolabelling were extremely low for many proteins (Drisdel & Green, 2004). Therefore, click chemistry based chemical probes were developed as an alternative to enhance protein palmitoylation detection (**Fig.13A**) (Hannoush & Arenas-Ramirez, 2009). Synthetic fatty acid analogues with alkyne groups at the terminal end furthest away from the carboxyl group are incorporated into live cells (Hannoush & Sun, 2010). Cells are then fixed for imaging or lysed for biochemical assays, followed by a catalysed reaction with azide-conjugated fluorescent groups or biotin for detection (Wang et al., 2003). Moreover, various lengths of synthetic fatty acids are shown to be incorporated into proteins *via* thioester linkage, suggesting that not only S-acylation but also other lipid groups can be added (Hannoush & Arenas-Ramirez, 2009). Chemistry click based probes were shown to have the advantage of allowing scientists to distinguish different fatty acid groups (Greaves et al., 2017). Even if click chemistry has led to many discoveries in protein palmitoylation, the efficiency of incorporating alkyne conjugated probes onto

substrates wasn't the same for all proteins, and thus it was not suitable for unbiased proteomic studies in native tissues. Recently acyl-biotin exchange ABE (**Fig.13B**) has been developed to detect S-acylated proteins in various tissues and organisms (Dowal et al., 2011; Kang et al., 2008; Roth et al., 2006). The first step in ABE consists of blocking the free cysteine sites of the proteins using thioreactive compounds such as N-ethylmaleimide (NEM) or methyl methanethiosulphonate (MMTS). Then the thioester bonds at the palmitoylated cysteines are cleaved using hydroxylamine (HA, neutral PH), thus exposing the free thiol groups that can be subsequently biotinylated. After the biotinylation, proteins are pulled down with streptavidin beads and eluted. Standard Western blotting can be used to detect the palmitoylation levels of proteins of interest (Drisdel & Green, 2004), or mass spectrometry can be used for global proteomic analysis (Roth et al., 2006).

Acyl-resin-assisted capture (Acyl-RAC) (**Fig.13B**) is a recently developed approach similar to ABE. Instead of using biotin conjugation and streptavidin pull-down, thioreactive Sepharose resin was used after the HA cleavage step (Forrester et al., 2011). This method reduced the duration of the procedure and improved sensitivity for detecting some palmitoylated proteins that were not detectable with the earlier cited methods (Edmonds et al., 2017).

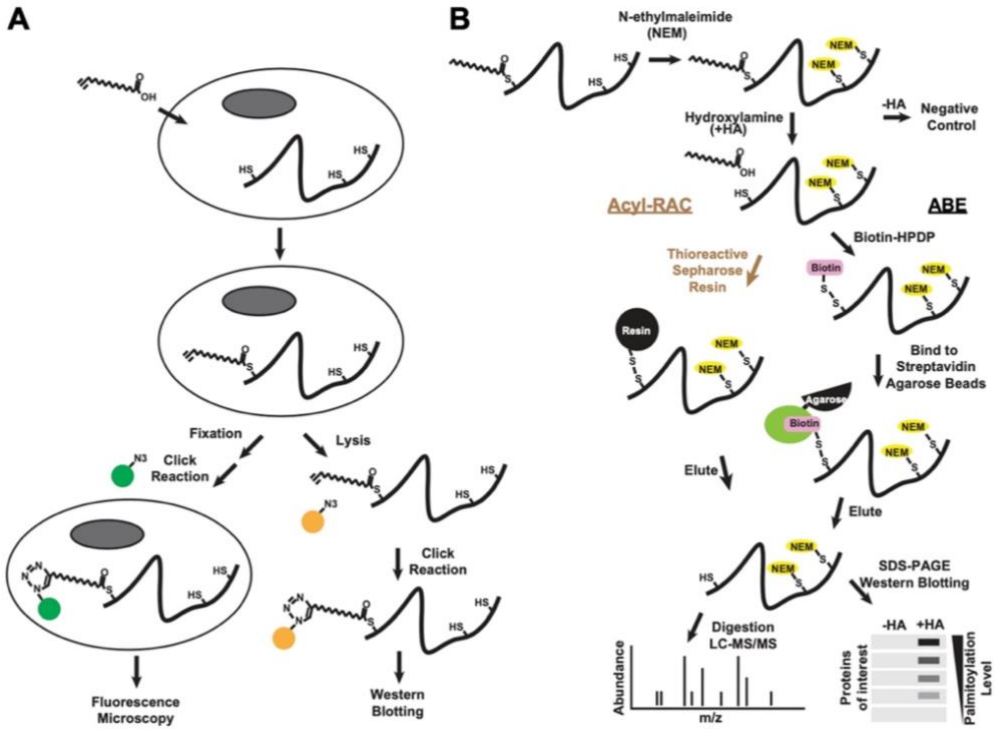


Figure 13- Schematics of methods to detect protein palmitoylation

(A) *fatty acid is incorporated into live cells, followed by click chemistry to detect palmitoylation using conjugated fluorophore (green) or biotin/streptavidin-HRP (yellow).* ***(B)*** *Procedures of using acyl-biotin exchange (ABE) and acyl-resin-assisted capture (Acyl-RAC).*

The development of purification methods for palmitoylated proteins helped speed the study and discovery of palmitoylated proteins. In 2004, Green and Drisdel established the acyl-biotinyl exchange method (ABE) for the purification of palmitoylated proteins (Drisdel & Green, 2004). Combining the ABE technique with quantitative mass spectrometry, a global analysis of protein palmitoylation in the yeast Saccharomyces cerevisiae (Roth et al., 2006). This approach was then applied to rat brain fractions (Kang et al., 2008). These experiments identified a substantial number of unexpected proteins that are subject to palmitoylation, such as SNAREs proteins, amino acid permeases, NMDA receptor subunits, and a brain-specific Cdc42 splice

variant (Kang et al., 2008; Roth et al., 2006). The ABE method has been modified since by several groups to enrich palmitoylated proteins/peptides more specifically and sensitively. Nonradioactive metabolic labelling methods of palmitoylated proteins by taking advantage of bio-orthogonal palmitate analogues were also developed (Charron et al., 2009; Martin & Cravatt, 2009). These two major methods contributed to the expansion of the knowledge about the number and types of palmitoylated proteins covering various species and their tissues (Fukata et al., 2016) (**Table. 2**).

Table 2-Protein S-palmitoylation studied in various species.

Species	Tested organism/tissue/cell-type	Number of candidate substrates	DHHC family members
Human	Jurkat T cells, DU145 prostate cancer cells, platelets, HEK293T cells, EA.hy926 endothelial cells, lymphoid B cells, and endothelial cells.	95-393	23 (ZDHHC 1-9, 11-24)
Mouse	DC2.4 dendritic cells, Raw2.4 macrophages, BW5147 T cells, neural stem cells, adipose tissue plus 3T3-L1	101-338	24 (ZDHHC1-9, 11-25)
Rat	Liver mitochondrial fraction and embryonic cortical neurons and brain	21-495	24 (ZDHHC1-9, 11-25)

For a long time, it was speculated that the majority of the palmitoylated proteins are located at the plasma membrane or the Golgi apparatus. Recent evidence surprisingly tells us that an important number of proteins, known to be localised at other specific organelles or subcellular compartments, are also modified by palmitoylation.

2. The Enzymes behind S-palmitoylation

Around 30% of eukaryotic proteins are membrane-associated, also many intracellular proteins can undergo modifications to delocalise to the phospholipid bilayer and enhance their association to the membrane (Guan & Fierke, 2011). Palmitoylation is a PTM, during which a fatty acid is attached to a protein (Pei et al., 2016). S-palmitoylation is the addition of a saturated 16-carbon fatty acid, for example, palmitic acid, to a specific cysteine thiol residue in the side chain of a target protein via a thioester bond (Han et al., 2015). S-palmitoylation is suspected to inhibit protein dissociation from the membrane, with the palmitate group acting as a hydrophobic membrane anchor (Greaves & Chamberlain, 2011; Yeste-Velasco et al., 2015).

Palmitate is the preferred substrate to attach during S-palmitoylation, but it is not the only one, other acyl chains can be used (stearate, oleate and arachidonate molecules) (Munday & López, 2007). This modification is reversible: S-palmitoylation is catalysed by enzymes called palmitoyltransferases (PATs) **(Fig.14)** that are expressed in various compartments of the cell (Philippe & Jenkins, 2019) **(Fig.15)**, while thioesterases (PTTs) catalyse depalmitoylation (Han et al., 2015). Not all palmitoylations are reversible which is the case of N-palmitoylation (Linder & Deschenes, 2007).

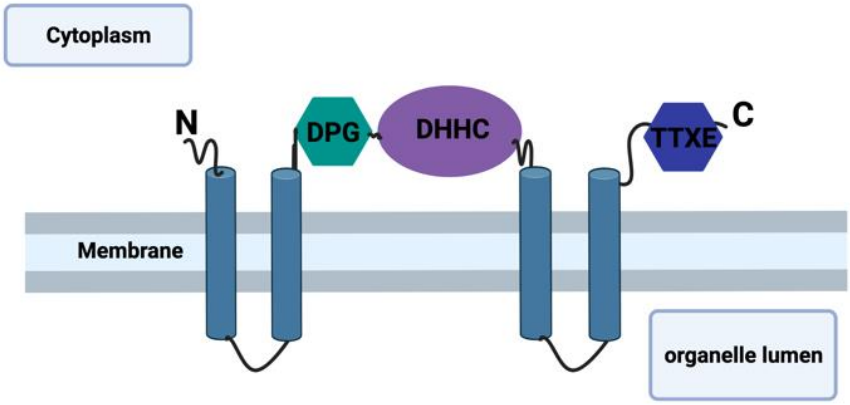


Figure 14- Schematic diagram of a DHHC domain-containing palmitoyltransferase

Schematic illustration of structures of mammalian and yeast DHHC protein family. DHHC proteins mostly have four transmembrane domains, a conserved cysteine-rich domain-containing DHHC motif in the cytoplasmic loop, and other conserved domains, DPG (Asp-Pro-Gly) and TTxE (Thr-Thr-X-Glu) motif adjacent to the C-terminus. (Chalhoub & McCormick, 2022)

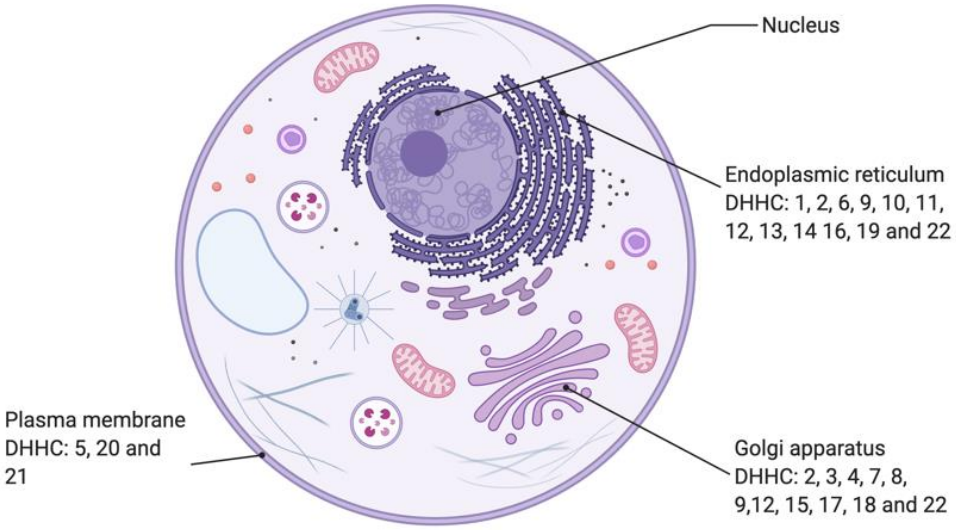


Figure 15- Subcellular localisation of various PATs DHHCs

The intracellular colocalisation analysis of all mammalian DHHCs exogenously expressed in vitro with endogenous intracellular organelle marker proteins revealed that the majority of DHHC proteins localise to the endoplasmic reticulum (ER) and Golgi, and a small number of DHHC proteins localise to post-Golgi membranes and Plasma membrane. (Chalhoub & McCormick, 2022)

d. Palmitoyltransferases (PATs)

PATs are characterised by a conserved DHHC (Asp-His-His-Cys) sequence essential for the enzyme catalytic activity (Fukata & Fukata, 2010), 23 PATs exhibiting a DHHC sequence have been discovered until today. These PATs function as follows **(Fig.16)**: They first use acyl-coenzyme A, a palmitate donor, to form a transient acyl-enzyme intermediate. The palmitoyl is then transferred from the enzyme to the substrate, the palmitoylation targets proteins specific cysteine residues (Jennings & Linder, 2012; Mitchell et al., 2010). Even though there are only 23 identified DHHCs, hundreds of S-palmitoylated proteins were found in the human body, therefore showing the importance of protein S-palmitoylation (Blaskovic et al., 2013).

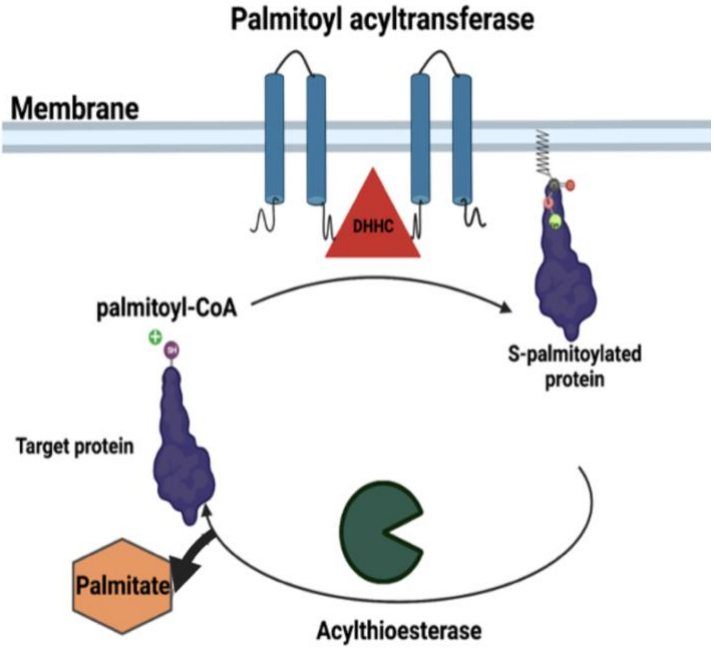


Figure 16- The PATs mechanism

PATs mechanism: They first use acyl-coenzyme A, a palmitate donor, to form a transient acyl-enzyme intermediate. The palmitoyl is then transferred from the enzyme to the substrate on specific cysteine residues. (Chalhoub & McCormick, 2022)

3. Role of protein palmitoylation

Palmitoylation is a crucial process for peripheral membrane proteins, such as PSD-95 and Gαq, as it plays a vital role in their trafficking and anchoring to the plasma membrane. By increasing the hydrophobicity of proteins, palmitoylation enhances their interaction with the membrane (Linder et al., 1993; Topinka & Bredt, 1998). Moreover, palmitoylation has the potential to influence the segregation of proteins into specialised membrane domains (Levental et al., 2010). thereby facilitating efficient signal transduction by partitioning proteins at distinct membrane compartments. Consequently, palmitoylation can be considered a common tag that assembles the appropriate compartments. For example, PSD-95, when palmitoylated, exhibits a restricted distribution in neurons, primarily localised to postsynaptic densities (PSDs) (Fukata et al., 2013). The GPCR signalling pathway also exploits palmitoylation for efficient and specific signal transduction. Multiple components involved in GPCR signalling, such as GPCRs themselves (Tobin & Wheatley, 2004), trimeric Gα subunits (Linder et al., 1993; Wedegaertner et al., 1993), RGS and its binding protein, R7BP (Rose et al., 2000), phosphodiesterase (Charych et al., 2010), undergo palmitoylation.

Palmitoylation of membrane proteins is essential for their proper conformation and protection from degradation via the ER-associated degradation (ERAD) pathway. Therefore, palmitoylation contributes to their stability and facilitates their trafficking to the plasma membrane (Blaskovic et al., 2013; Linder & Deschenes, 2007) (**Fig.17)**. The significance of palmitoylation in protein stability is evident from studies showing that palmitoylation-deficient transmembrane proteins have a shortened half-life and reduced cell-surface expression. Examples of such proteins include the A_1 adenosine receptor (Gao et al., 1999) and the chemokine receptor CCR5 (Blanpain et al., 2001;

Percherancier et al., 2001). Additionally, it is speculated that S-palmitoylation plays a broader role in modulating protein conformation (Ko & Dixon, 2018; Yeste-Velasco et al., 2015; Zhang et al., 2021).

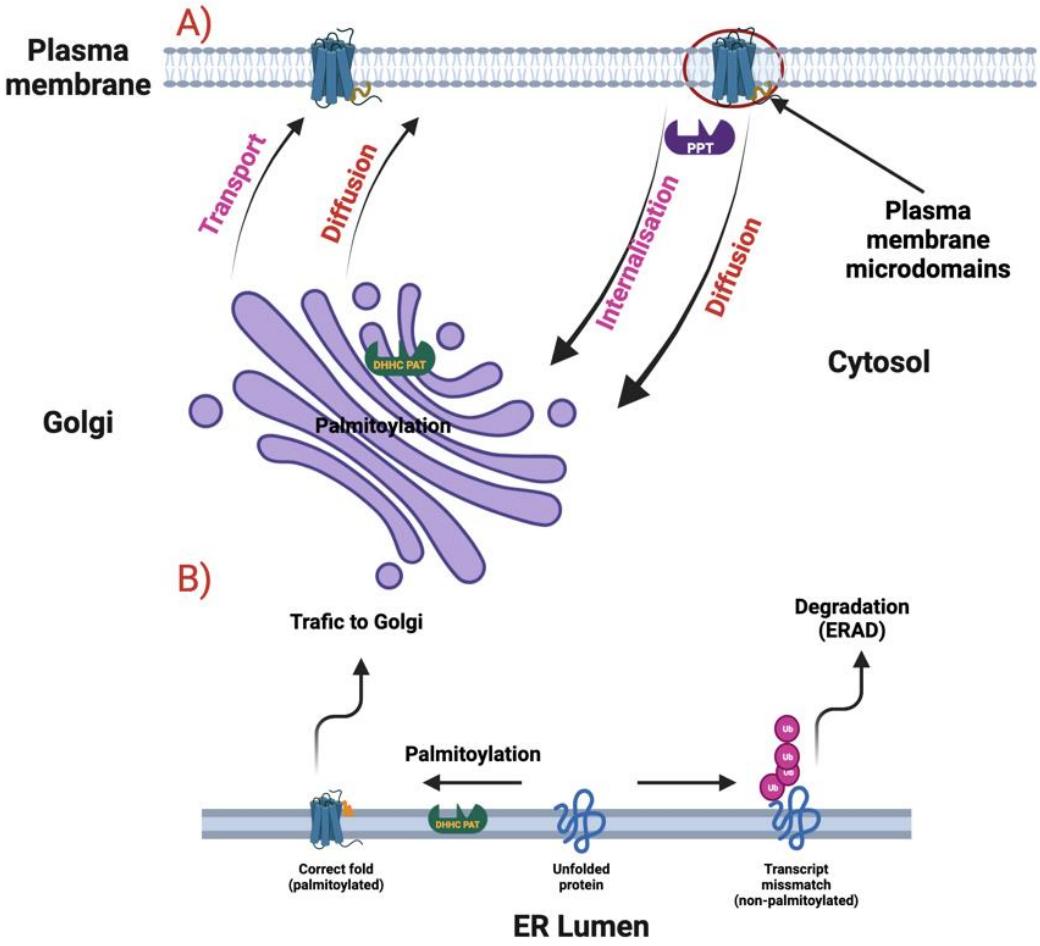


Figure 17- Roles of protein palmitoylation

(A)Palmitoylation modulates protein trafficking to the plasma membrane and protein incorporation into specialised membrane domains (e.g., detergent-insoluble lipid-enriched domains). In addition, palmitoylation and depalmitoylation cycles allow several proteins to traffic between the inter-membrane compartments. The shuttling route could be determined by specific DHHC PAT and PPT. (B) Palmitoylation of integral membrane proteins regulates their conformation and stability. In this case, non-palmitoylated proteins are recognised as misfolded by the ER quality control system, do not exit the ER, and finally enter the ERAD pathway for degradation. DHHC, aspartate-histidine-histidine-cysteine; ER, endoplasmic reticulum; PAT, palmitoyl acyltransferase; PPT, palmitoyl-protein thioesterases; ERAD, ER-associated degradation. (Chalhoub & McCormick, 2022)

VII. G proteins and G protein coupled receptors are subject to palmitoylation

When investigating protein/lipid and protein/protein interactions, it is crucial not to disregard post-translational modifications of signalling proteins, including receptors, G proteins, G protein-coupled effector enzymes, and receptor kinases. These modifications play a significant role in modulating the activity, localisation, and interaction of these signalling molecules. Thus, considering PTMs is essential for understanding the intricate mechanisms underlying cellular signalling pathways. (Buss et al., 1987; O'Brien & Zatz, 1984; Stoffel et al., 1994; Sugars et al., 1999). GPCRs are post-translationally modified in a multitude of ways, including glycosylation, phosphorylation and palmitoylation.

Palmitoylation occurs on one or more cysteines on the intracellular side of GPCRs (Goddard & Watts, 2012), usually on the cytoplasmic (C-terminal tail) of the receptor. Moreover, GPCR palmitoylation can occur in other sections, for instance, in the intracellular loops in the case of the vasopressin receptor (Hawtin et al., 2001). The thioester bond that links the palmitate to the cysteine is cleavable; a receptor's palmitoylation state can be a tool to regulate its activity (Gorinski et al., 2019). The effects of palmitoylation are unpredictable and GPCR-dependent (Shpakov, 2023) and depalmitoylation seems to be regulated by the bound state of the receptor, being accelerated upon agonist binding as demonstrated for certain receptors (dopamine D_1, serotonin 4A, delta opioid and adrenergic receptors for example) (Chini & Parenti, 2009). Palmitoylation exerts a comprehensive impact on all facets of GPCR signalling. The addition of palmitate to the cytoplasmic side of the plasma membrane can generate an additional loop, thereby influencing the structure of GPCRs and subsequently affecting their interactions with intracellular partner proteins. This

modification plays a pivotal role in shaping the functional properties of GPCRs and regulating their intracellular signalling events. (Chini & Parenti, 2009). Up to three palmitate groups can be found on GPCRs and different palmitoylation profiles can result in various conformations of the carboxy-terminal tail, which may select for certain G protein interactions (Baccouch et al., 2022). Palmitoylation can influence the phosphorylation of a receptor, modulating desensitisation and control internalisation independently of phosphorylation (Gauthier-Kemper et al., 2014; Zhang et al., 2022). It has been suggested that palmitate binding in the endoplasmic reticulum ensures correct processing and trafficking of receptors and, once at the cell membrane, may target GPCRs to lipid rafts and impact dimerization (Chini & Parenti, 2009; Qanbar & Bouvier, 2003). However, not all palmitoylated receptors associate with rafts and not all raft-associated GPCRs are palmitoylated (Chini & Parenti, 2009). In the case of OPRM1, it appears that palmitoylation and cholesterol association (and presumably raft interactions) are intrinsically linked (Zheng et al., 2012). In addition, palmitoylation plays an important role in receptor trafficking and localisation to the cell surface.

1. Effect of S-palmitoylation on GPCR surface expression

Palmitoylation plays a role in regulating GPCR surface expression. Mutation of three C-tail cysteine residues of the chemokine CCR5 receptor resulted in its retention in the ER and Golgi complex (Blanpain et al., 2001; Percherancier et al., 2001), they also showed that the non-palmitoylated CCR5 mutant presented impaired diffusion properties in the ER. Likewise, loss of palmitoylation caused a significantly reduced expression of endogenous protease-activated receptor 2 (PAR2) at the plasma membrane (Adams et al., 2011). This was also observed for the thyrotropin receptor,

vasopressin V_2 receptor, adenosine A_1 receptor, histamine H_2 receptor, and the dopamine D_1 and D_2 receptors (Ebersole et al., 2015; Fukushima et al., 2001; Sadeghi et al., 1997; Schülein et al., 1996; Tanaka et al., 1998). The mechanism by which altered palmitoylation reduces GPCR surface expression is attributed mainly to receptor misfolding, leading to proteasomal degradation (Patwardhan et al., 2021). This has been reported for deficiencies in palmitoylation of CCR5 and adenosine A_1 receptor, where the loss of palmitoylation enhanced receptor degradation (Gao et al., 1999; Percherancier et al., 2001). In the case of the Follicle-stimulating hormone receptor containing three cytosolic cysteine residues, the mutation of a single C269 was sufficient to compromise cell surface expression, likely due to protein misfolding and degradation (Uribe et al., 2008). These conclusions are coherent with an important role for palmitoylation in assisting the proper folding and maturation of GPCRs.

2. Effects of GPCR palmitoylation on dimerisation and lipid rafts

GPCRs incorporation into rich cholesterol lipid raft at the plasma membrane microdomains is regulated by palmitoylation (**Fig.18;19**) (Barnett-Norris et al., 2005; Villar et al., 2016). The palmitoylation mutant serotonin 5-HT_{1A} receptor showed a reduction in its association with lipid rafts (Papoucheva et al., 2004; Renner et al., 2007). Similarly, the dopamine D_1 receptor (Tiu et al., 2020) and cannabinoid receptor type 1 (CB1) (Oddi et al., 2012, 2018) palmitoylation mutants displayed an impaired lipid raft association.

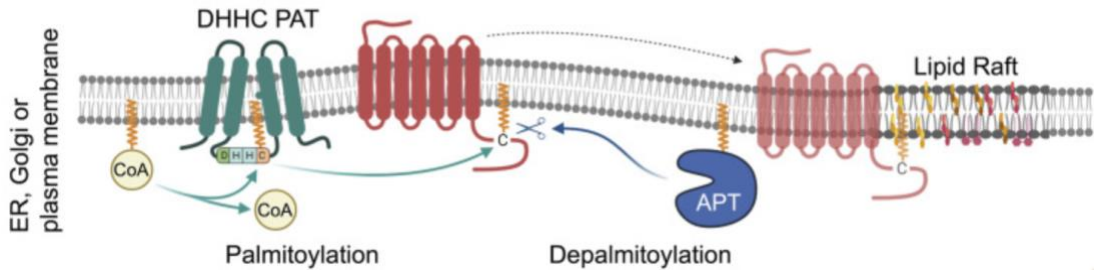


Figure 18- Effects of GPCR palmitoylation and membrane distribution

DHHC PATs are located in the endoplasmic reticulum (ER), Golgi, and plasma membrane. GPCR palmitoylation on the C-terminal tail cysteines creates a fourth IL and can facilitate GPCR localisation to lipid rafts and membrane localisation. (Pat*wardhan et al., 2021)*

Also, the crystal structure of the human β_2-adrenoceptor revealed a receptor dimer complex, where palmitic acid and cholesterol mediated the majority of the dimer interactions (Cherezov et al., 2007). Furthermore, palmitoylation of several other GPCRs has been shown to facilitate lipid raft association and dimerization, including the μ-opioid receptors (MOR) (Zheng et al., 2012), rhodopsin (Seno & Hayashi, 2017), and the serotonin 5-HT_{1A} receptor (Kobe et al., 2008). These results supported that for some GPCRs, palmitoylation facilitates receptor compartmentalisation in lipid rafts and dimerisation.

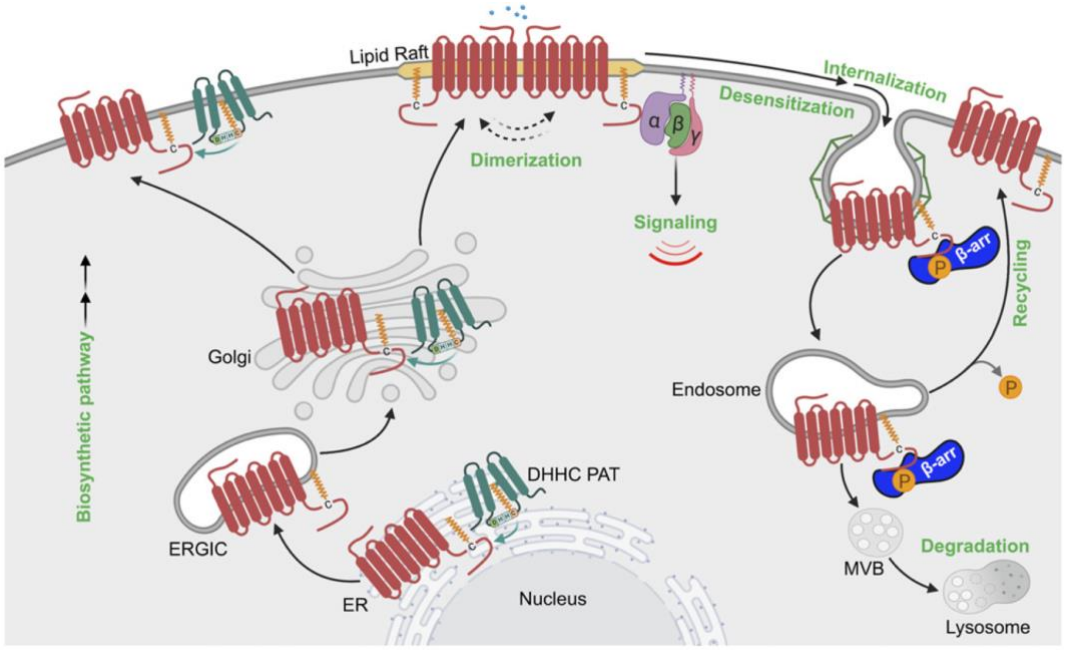


Figure 19- Model of GPCR regulation by palmitoylation

GPCRs are palmitoylated during their biosynthesis and can occur at the endoplasmic reticulum (ER), endoplasmic-reticulum-Golgi intermediate compartment (ERGIC), Golgi and the plasma membrane, where DHHC PATs are known to be localised. GPCR palmitoylation regulates partitioning into membrane microdomains such as lipid rafts and caveolae. GPCR palmitoylation has also been implicated in receptor dimerization and G protein coupling. Palmitoylation of GPCRs can influence β-arrestin recruitment and receptor internalisation. GPCR palmitoylation is also important for regulating receptor recycling and lysosomal degradation. (Patwardhan et al., 2021)

3. Effects of palmitoylation on GPCR internalisation, recycling, and degradation

In addition to GPCR plasma membrane localisation, palmitoylation is implicated in GPCR internalisation, recycling, and lysosomal degradation (**Fig.19**). Several studies have reported a role for palmitoylation in GPCR internalisation. Defects in CB_1 palmitoylation inhibited agonist-induced internalisation and association with caveolin 1 a scaffolding protein (Oddi et al., 2017). Similarly, defects in palmitoylation of the prostanoid thromboxane A_2 receptor (TxA2), PAR2, and

thyrotropin receptor affected agonist-induced β-arrestin recruitment and receptor internalisation (Adams et al., 2011; Reid & Kinsella, 2007; Tanaka et al., 1998). On the other hand, the vasopressin V_{1A} receptor palmitoylation deficient mutant displayed an increased rate of agonist-induced internalisation without it affecting intracellular signalling (Hawtin et al., 2001). Similarly, the dopamine D_1 receptor palmitoylation mutant showed an enhanced rate of plasma membrane internalisation compared to the wild-type and manifested a preference to internalise via a clathrin-dependent pathway over caveolae (Kong et al., 2011).

Once internalised, GPCRs either recycle back to the cell surface or are targeted to the lysosomes for degradation. Although palmitoylation of PAR2 is essential for its efficient internalisation and lysosomal degradation (Adams et al., 2011), palmitoylation has an opposing effect on PAR1, the palmitoylation-deficient PAR1 mutant presented an enhanced rate of internalisation and lysosomal degradation (Canto & Trejo, 2013). The altered trafficking caused by PAR1's absence of palmitoylation is due to the masking of C-tail tyrosine-based sorting motifs for endocytic adaptor proteins (Canto & Trejo, 2013). For instance, in the absence of palmitoylation, PAR1 sorting motifs are more accessible to the clathrin adaptor binding proteins (AP-2) and adaptor protein complex 3, which are implicated in accelerating the rate of internalisation from the plasma membrane as well as enhancing sorting from endosomes to lysosomes and degradation (Canto & Trejo, 2013). Similarly, a CCR5 palmitoylation mutant exhibits rapid lysosomal degradation and a reduced half-life (Percherancier et al., 2001).

4. Effects of Palmitoylation on GPCR Signalling

In addition to regulating GPCR trafficking, palmitoylation is important for modulating activated GPCR coupling to G protein signalling (**Fig.19**). In many cases, deficiencies in GPCR palmitoylation failed to affect ligand binding but affected G protein coupling or altered the specificity of coupling to certain G protein subtypes. Studies of a β2-adrenoceptor C341 palmitoylation mutant highlighted defects in coupling to the stimulatory guanine nucleotide binding protein (Gs) and impaired cAMP production (O'Dowd et al., 1989). In other studies, deficiencies in GPCR palmitoylation were shown to hinder the G protein coupling of the agonist-activated serotonin 5-HT_{1A} receptor, human somatostatin receptor type 5 (SSTR5), human endothelin ETA receptor, α2A-adrenoreceptor, dopamine D_1 receptor, human adenosine A1 receptor, and the human thyrotropin receptor (Albert et al., 1999; Hukovic et al., 1998). This is not surprising since GPCR localisation in lipid rafts is known to promote the assembly of signalling ensembles (Barnett-Norris et al., 2005; Villar et al., 2016). Actually, methyl-β-cyclodextrin, a cholesterol-chelating reagent that disrupts lipid rafts, reduced the localisation of the serotonin 5-HT_{1A} receptor to lipid rafts and its G protein coupling (Papoucheva et al., 2004; Renner et al., 2007). Thus, palmitoylation-driven lipid raft localisation of certain GPCRs is primordial for signalling regulation. However, some studies suggest that conformational changes induced by modulating lipid interaction of pre-existing dimers may alter G protein coupling preferences. Although the β_2-adrenoceptor couples to both G_s and G_i proteins, depletion of cholesterol resulted in preferential coupling to G_s proteins (Xiang et al., 2002). Moreover, β_2-adrenoceptor coupled to G_s protein was shown to occur with receptor monomers (Whorton et al., 2007), showcasing that dimers are not conditional for G_s coupling. In mice, treatment with palmostatin B, a cell-permeable

inhibitor of the acyl-protein thioesterases 1 (APT1), increased the melanocortin receptor 1 (MC_1) palmitoylation and enhanced its stimulated cAMP production, which protected against the progression of melanoma (Chen et al., 2017). Thus, the effects of palmitoylation are partly due to defects in compartmentalisation, receptor conformation, and receptor capacity to couple to G protein activation.

5. Dopamine receptors palmitoylation

Impairments in dopaminergic neurotransmission within the brain have been implicated in a range of neuropsychiatric disorders, including Parkinson's disease (Lavine et al., 2002), schizophrenia (Lee et al., 2001), attention-deficit hyperactivity disorder (Lefkowitz et al., 2002), and drug addiction (Lefkowitz & Shenoy, 2005; Li et al., 2000). Disruptions in the normal functioning of dopamine pathways contribute to the pathogenesis and manifestation of these conditions. Five subtypes of dopamine receptors have been reported (Sibley & Monsma, 1992), which can be classified into two subfamilies: D1-like and D2-like. The D1-like family consists of the D_1 and D_5 receptors, which couple to the Gαs subunit, whereas the D2-like family consists of the D_2, D_3, and D_4 receptors, which couple to the Gαi/o subunit. Each dopamine receptor subtype exhibits a different tissue expression pattern and density (Luttrell et al., 1997; Missale et al., 1998).

The palmitoylation of D_1 was demonstrated almost three decades ago using overexpression of recombinant receptors in the baculovirus system (Ng et al., 1994). In the same study, the authors identified that agonist activation of the receptor resulted in an increased level of [^{3}H]-palmitate incorporation into the receptor. Studies on the functional role of D_1 palmitoylation revealed that substitution of palmitoylated cysteines residues Cys347 and Cys351 (**fig.20)** by alanine failed to affect the receptor

affinity for agonists as well as receptor ability to stimulate AC (Jin et al., 1997, 1999). More recently, Kong et al. demonstrated that palmitoylation of D_1 plays an essential role in agonist-dependent receptor internalisation (Kong et al., 2011).

Both short and long isoforms of D_2 (D_{2S} and D_{2L}) have also been shown to be palmitoylated (Grünewald et al., 1996; Ng et al., 1994). The palmitoylation of D_{2L} occurs at the Cys443 residue and is involved in the regulation of receptor stability and trafficking to the plasma membrane (Ebersole et al., 2015). In the same study, palmitoyl acyltransferase (PAT) DHHC4 was identified as a D_{2L} interaction partner, suggesting that this PAT is responsible for its palmitoylation.

The D_3 receptor also undergoes PTMs, which were found to be involved in the regulation of various receptor functions, including cell surface expression, protein kinase C-mediated endocytosis, agonist affinity, and agonist-induced receptor tolerance (Zhang et al., 2016). The same study also noted that despite the C-terminal domains of D_2 and D_3 receptors having a high sequence homology, the D_3 receptor is palmitoylated more extensively. Based on these results, it was suggested that regulating palmitoylation may represent a new strategy for selective modulation of D_3. This assumption is highly important since D_2 and D_3 receptors are the main targets of currently used neuroleptic drugs. The most serious side effects of the currently used antipsychotics are disturbances in motor functions (Cho et al., 2010). Since D_2 and D_3 receptors are heavily expressed in the regions responsible for motor and emotion-related mental functions. Development of D_3 receptor-specific ligands or selective manipulation of the specific signalling pathways of D_3 receptors can be used as a strategy to separate the desired therapeutic antipsychotic activities from side effects on motor function.

Moreover, $D_{4\text{-}4}$, an alternatively spliced form of D_4R, undergo palmitoylation on its Cys467, the terminal amino acid residue of the receptor. When palmitoylation of D_4 was inhibited by mutation of the consensus site or by treatment with the palmitoylation inhibitor 2-bromopalmitate, D_4 plasma membrane expression, signalling, and endocytosis were all hindered (Zhang & Kim, 2016).

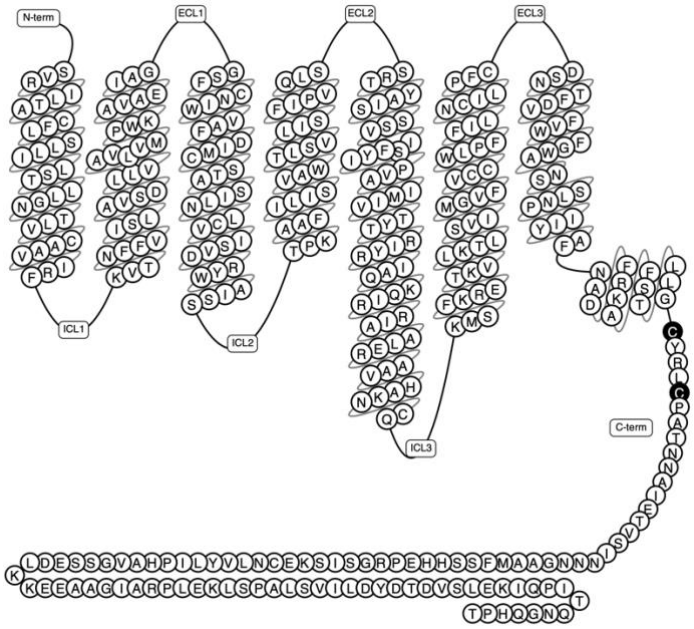


Figure 20- The human dopamine D_1 receptor palmitoylation sites in its carboxyl tail

The Snake plot of D_1 with its carboxyl tail containing two putative palmitoylation sites (Cys^{347} and Cys^{351}) are highlighted in black. (image generated with gpcrdb.org)

VIII. Hypothesis and Aims

Previous research findings have highlighted the significant role of palmitoylation in the function and signalling of G protein-coupled receptors (GPCRs). However, the identification of the enzymes responsible for catalysing the palmitoylation of GPCRs, namely palmitoyl acyltransferases (PATs) and palmitoyl

thioesterases (PTTs), has been largely overlooked in most studies. Given the vast number of GPCR family members, their functional diversity, and their importance as drug targets, investigating palmitoylation holds great potential for advancing our understanding of GPCRs. Therefore, gaining deeper insights into this modification and its functional effects on GPCRs, particularly focusing on the D_1 receptor in this thesis, will contribute to the development of novel therapeutic strategies.

As discussed earlier in this chapter, GPCRs are dynamic proteins that are regulated by their ligands and interacting proteins. This inherent flexibility is reflected in receptor post-translational modifications (PTMs), which enable 7-transmembrane receptors to control and modulate intracellular processes through diverse mechanisms such as differential trafficking, pharmacology, and protein interactions. However, the functional consequences of palmitoylation on D_1 receptor signalling, including its cAMP signalling pathway and downstream pathways such as ERK 1/2, as well as its interactions with effector proteins (e.g., arrestins and G proteins), remain understudied and poorly understood.

Therefore, the specific aims of my research were as follows:

- To understand the effects of palmitoylation on D_1 receptor signalling.
- To investigate the interactions of D_1 receptors with effector proteins, including arrestins and G proteins.
- To identify the specific DHHC enzymes responsible for the palmitoylation of the D_1 receptor.

Classically, GPCRs suffered from poor spatial and temporal resolution to differentiate various aspects of their trafficking and signalling. These limitations have been overcome by the application of fluorescence resonance energy transfer (FRET)-based sensors that allowed real-time observations of signalling and trafficking events in live

cells (Irannejad et al., 2013; Vilardaga et al., 2009), and enhanced bioluminescence resonance energy transfer (BRET) experiments that allowed measurement of GPCR activation and trafficking (Namkung et al., 2016b; Szalai et al., 2014).

Spatial and temporal resolution are crucial aspects when studying G protein-coupled receptors (GPCRs). Spatial resolution refers to the ability to determine the precise location and distribution of GPCRs within cells or tissues. This includes identifying the specific subcellular compartments where GPCRs are localised, such as plasma membrane domains or intracellular compartments. Techniques such as immunohistochemistry, fluorescence microscopy, and electron microscopy enable us to visualise and map the spatial distribution of GPCRs, providing valuable insights into their localisation patterns and potential functional implications. On the other hand, temporal resolution focuses on understanding the dynamic nature of GPCR signalling over time. GPCRs exhibit complex temporal dynamics, including ligand binding, receptor activation, desensitisation, and internalisation. Studying the temporal aspects requires techniques that can capture and analyse rapid changes in GPCR activity and downstream signalling events. By combining spatial and temporal resolution, we can gain a comprehensive understanding of GPCR function and signalling dynamics. This knowledge is crucial for unravelling the complex regulatory mechanisms underlying GPCR-mediated cellular responses and can aid in the development of novel therapeutic strategies targeting GPCR signalling pathways.

BRET has emerged as a valuable tool for investigating G protein-coupled receptor (GPCR) activation and trafficking. BRET allows the measurement of protein-protein interactions and conformational changes in real-time within live cells. In the context of GPCRs, BRET can be utilised to monitor receptor activation by detecting the proximity between the receptor and downstream signalling molecules, such as G

proteins, arrestins, or other interacting proteins. By fusing the GPCR of interest with a bioluminescent donor and the interacting protein with an acceptor fluorophore, BRET signals are generated upon receptor activation, enabling the dynamic monitoring of protein-protein interactions, trafficking and signalling events. Therefore, gaining insights into the spatiotemporal dynamics of GPCR activation and trafficking provides valuable information for understanding GPCR function and developing new therapeutic strategies. In the context of the above, we sought to re-examine the role of palmitoylation and explore its effects on the spatial and temporal aspects of GPCR function.

Chapter 2: Materials and methodology

I. Materials

1. Primary & Secondary Antibodies

Table 3- Primary & Secondary Antibodies

Antibody	*Host*	*Application& (Dilution; v: v)*	*Supplier*	*Catalogue no.*
Anti-mouse Alexa Fluor™ 488	Goat	IF (1:10000)	Thermo Fisher Scientific	A-11029
AlphaLISA® CaptSure™ Acceptor Beads	N.A.	(1:100)	Perkin Elmer®	ALSU-PERK-A10K
AlphaScreen® Streptavidin Donor Beads	N.A.	(1:100)	Perkin Elmer®	ALSU-PERK-A10K
Anti-rabbit Alexa Fluor™ 568	Goat	IF (1:10000)	Thermo Fisher Scientific	A-11011
Monoclonal Anti-FLAG	Mouse	IF (1:1000)	Sigma-Aldrich	F1804-50UG
Anti-beta Actin antibody	Mouse	IF (1:1000)	Abcam	ab8226
Monoclonal Anti-HA	Rabbit	IF (1:1600)	Sigma-Aldrich	3724S

2. Bacterial strains and mammalian cell lines

Table 4- Bacterial strains and mammalian cell lines

Strain	*Source*	*Catalogue no.*
DH5α	In-House	-
HEK293T	ATTC®	CRL-1573™
HEK293T β-arrestin 1/2 KO	Pr. Asuka INOUE	-

3. Commercial Kits

Table 5- Commercial Kits

Kit Name	*Supplier*	*Catalogue no.*
AlphaLISA SureFire Ultra p-ERK1/2 (Thr202/Tyr204) Assay Kit	Perkin Elmer®	ALSU-PERK
Dream Taq PCR Master Mix (2X)	Fisher Scientific	K 1071
Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit	Fisher Scientific	11992242
Macherey-Nagel™ NucleoSpin™ Plasmid Kit	Fisher Scientific	11932392
NanoBiT® PPI MCS Starter System	Promega	N 2014
NanoBRET™ PPI Flexi® Starter System	Promega	N 1821
NEBuilder® HiFi DNA Assembly Master Mix	New England Biolabs	E 2621S
Pierce™ BCA Protein Assay Kit	Thermo Scientific™	23225
PureYield™ Plasmid Maxiprep System	Promega	A 2393

4. Compounds and Ligands

Table 6- Compounds and Ligands

Name	*Supplier*	*Catalogue no.*
SKF 81297 hydrobromide	Tocris®	1447
Dopamine hydrochloride	Tocris®	*3548*
A-68930 hydrochloride	Tocris®	1534
Forskoline	HelloBio	HB1348
Dynasore	HelloBio	HB1245

5. Solutions, Enzymes and Other Reagents

Table 7- Solutions, Enzymes and Other Reagents

Solutions, Enzymes and Other Reagents	*Supplier*	*Catalogue no.*
50X Tris-Acetate EDTA (TAE)	Severn Biotech Ltd®	20-6001-10
1 Kb Plus DNA Ladder	Thermo Fisher Scientific	SM0331
Agar	Thermo Fisher Scientific	10548030
Acetic acid glacial	Thermo Fisher Scientific	12686657
Ampicillin	Thermo Fisher Scientific	10193433
Agarose	Sigma-Aldrich	A9539
β-Nicotinamide Adenine Dinucleotide (NAD^+)	New England Biolab	B9007S
$CaCl_2.2H_2O$	Thermo Fisher Scientific	10316313
Bovine Serum Albumin	Sigma-Aldrich	A4503
CH_3CO_2K	Thermo Fisher Scientific	10522955
Coelenterazine 400a	NanoLight Technology	340
DPN1	New England Biolab	R0176L
DMEM, High Glucose with sodium pyruvate	Sigma-Aldrich	D6429
DTT (Dithiothreitol)	Thermo Fisher Scientific	R0862
DMEM, High Glucose without sodium pyruvate	Sigma-Aldrich	D5796
DMSO	Sigma-Aldrich	276855
Dulbecco′S Phosphate Buffered Saline	Sigma-Aldrich	D8537
Ethanol	Thermo Fisher Scientific	10000652
dNTPs Mix	Thermo Fisher Scientific	18427013
Fetal Bovine Serum	PAN-Biotech	P40-37500
Firefly Luciferin Free Acid	NanoLight Technology	306

Freezing Medium Cryo-SFM	PromoCell®	C-29910
Glycerol (99.5%)	Thermo Fisher Scientific	10692372
HBSS 10X no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	14185052
HBSS 10X, calcium, magnesium, no phenol red	Thermo Fisher Scientific	14065056
HEPES	Sigma-Aldrich	H3375
Invitrogen Lipofectamine™ 3000	Thermo Fisher Scientific	15212475
Isopropanol	Thermo Fisher Scientific	10173240
KOH	Thermo Fisher Scientific	10366240
LB Broth (Lennox)	Sigma-Aldrich	L3022
Loading Dye (6x)	Thermo Fisher Scientific	R0611
$MgCl_2.6H_2O$	Thermo Fisher Scientific	10647032
$MgSO_4.7H_2O$	Thermo Fisher Scientific	10346190
MnCl2.4H2O	Thermo Fisher Scientific	11452844
MOPS	Sigma-Aldrich	M3183
NaCl	Thermo Fisher Scientific	S7400
$NaHCO_3$	Thermo Fisher Scientific	10244683
NanoFuel Solvent	NanoLight Technology	399
NanoFuel® Glow Assay Oplophorus Luciferases	NanoLight Technology	325
Nano-Glo® Luciferase Assay System	Promega	N1150
NaOH	Thermo Fisher Scientific	10396240
Opti-MEM™	Thermo Fisher Scientific	31985062
Penicillin-Streptomycin	Sigma-Aldrich	P4458
Phosphate Buffered Saline Tablets	Thermo Fisher Scientific	10209252
Phusion™ High-Fidelity DNA Polymerase	Thermo Fisher Scientific	F530S
Pierce™ 16% Formaldehyde (w/v)	Thermo Fisher Scientific	28908
Poly-D-Lysine Hydrobromide	Sigma-Aldrich	P0899

Poly(ethylene glycol) PEG8000	Sigma-Aldrich	P2139
RbCl	Thermo Fisher Scientific	10549390
T5 Exonuclease	New England Biolab	M0663S
TAQ 2X MM	New England Biolab	M0270L
TAQ DNA Ligase	New England Biolab	M0208L
Triton™ X-100	Fisher Scientific	BP151
Trypan Blue Solution (0.4%)	Thermo Fisher Scientific	11538886
Trypsin-EDTA 10X	Sigma-Aldrich	59418C
Tryptone	Thermo Fisher Scientific	11365982
Ultrapure™ DNAse/RNAse-Free Distilled Water	Thermo Fisher Scientific	10977035
UltraPure™ Tris Hydrochloride	Thermo Fisher Scientific	15506017
Yeast	Thermo Fisher Scientific	11385992
RIPA buffer	Thermo Fisher Scientific	89900

6. Homemade buffers and solutions

Table 8- Homemade buffers and solutions

Buffer	***Ingredient***	***Final Concentration***
Gibson Assembly Master Mix	• 2X ISO Buffer • Phusion DNA Polymerase • Taq DNA Ligase • T5 Exonuclease • H2O	• 66 % • 0.033 U/µl • 5.3 U/µl • 0.005 U/µl • *to a final volume
HBSS-based luminescent assay buffer	• HBSS 10X (with Ca2+/Mg2+) • HEPES 1M • $NaHCO_3$ 1M • $MgSO_4.7H_2O$ 1mM • $CaCl_2.2H_2O$ • BSA 10% • H_2O *Adjust to pH 7.4 with Sodium Hydroxide (1M)	• 1x • 24 mM • 3.96 mM • 1 mM • 1mM • 0.1% • *to a final volume
ISO buffer (2X)	• PEG 800 • Tris-HCl pH 7.5	• 10% • 200 mM

	• $MgCl_2.6H_2O$ • DTT • dNTP mix • NAD + • H_2O	• 20 mM • 20 mM • 0.4 mM • 2 mM • *to a final volume
LB Agar	• LB Broth • Agar • H2O	• 2% (w:v) • 1.5% (w:v) • *to a final volume
LB Broth Media	• LB Broth • H2O	• 5% (w:v) • *to a final volume
TRUPATH Buffer	• HBSS 10X (with Ca2+/Mg2+) • HEPES 1M • H2O * Adjust to pH 7.4 with Sodium Hydroxide (1M)	• 1x • 20 mM • *to a final volume
Transformation Buffer 1 (TFB1)	• CaCl2.2H2O • CH3CO2K • Glycerol • MnCl2.4H2O • RbCl • H2O * Adjust to pH 5.8 with Glacial Acetic Acid (2M)	• 10 mM • 30 mM • 15% (v:v) • 50 mM • 100 mM • *to a final volume
Transformation Buffer 2 (TFB2)	• CaCl2.2H2O • MOPS • Glycerol • RbCl • H2O * Adjust to pH 6.5 with Potassium Hydroxide (1M)	• 75 mM • 10 mM • 15% (v:v) • 100 mM • *to a final volume
Tris-ACETATE EDTA Buffer (TAE)	▪ TAE 50X ▪ H_2O	1x *to a final volume
2X Yeast (YT) Media	• Tryptone • Yeast • NaCl • H2O * Adjust to pH 7.0 with Sodium Hydroxide (1M)	• 1.6% (w:v) • 1% (w:v) • 0.5% (w:v) • *to a final volume

7. Plasmids

Table 9- Outsourced Plasmids

Name	Source
pcDNA5/FRT/TO-GAlphasS-RLuc8	*Addgene*
pcDNA3.1-Beta1	*Addgene*
pcDNA3.1-GGamma8-GFP2	*Addgene*
pcDNA5/FRT/TO-GAlphasL-RLuc8	*Addgene*
pcDNA3.1-Empty-Vector	*Addgene*
pcDNA3.1-SmBiT-b-arrestin 2	*Dr Joaquin Botta*
pcDNA3.1-LgBiT-b-arrestin 1	*Dr Joaquin Botta*
pcDNA3.1-LgBiT-CAAX	*Genscript*
pBiT1.1-C [TK/LgBiT]	*Promega*
pBiT1.1-N [TK/LgBiT]	*Promega*
pBiT2.1-C [TK/SmBiT]	*Promega*
pBiT2.1-N [TK/SmBiT]	*Promega*
pcDNA3.1-Flag-D_1	*Genscript*
pcDNA3.1-ARRB2	*cDNA resource centre*
pcDNA3.1-D_1	*cDNA resource centre*
pcDNA3.1-Galphai1	*Dr. Andy Chevigné*
pcDNA3.1-Galphai3	*Dr. Andy Chevigné*
pcDNA3.1-Galphaq	*Dr. Andy Chevigné*
pGAP43-CFP-Gαi1	*Dr. Josef Lazar*
HA-DHHC1	*Dr. Stephane Lefrancois*
HA-DHHC2	*Dr. Stephane Lefrancois*
HA-DHHC3	*Dr. Stephane Lefrancois*
HA-DHHC4	*Dr. Stephane Lefrancois*
HA-DHHC5	*Dr. Stephane Lefrancois*
HA-DHHC6	*Dr. Stephane Lefrancois*
HA-DHHC7	*Dr. Stephane Lefrancois*
HA-DHHC8	*Dr. Stephane Lefrancois*
HA-DHHC9	*Dr. Stephane Lefrancois*

HA-DHHC10	*Dr. Stephane Lefrancois*
HA-DHHC11	*Dr. Stephane Lefrancois*
HA-DHHC12	*Dr. Stephane Lefrancois*
HA-DHHC13	*Dr. Stephane Lefrancois*
HA-DHHC14	*Dr. Stephane Lefrancois*
HA-DHHC15	*Dr. Stephane Lefrancois*
HA-DHHC16	*Dr. Stephane Lefrancois*
HA-DHHC17	*Dr. Stephane Lefrancois*
HA-DHHC18	*Dr. Stephane Lefrancois*
HA-DHHC19	*Dr. Stephane Lefrancois*
HA-DHHC20	*Dr. Stephane Lefrancois*
HA-DHHC21	*Dr. Stephane Lefrancois*
HA-DHHC1-GFP	*Dr. Stephane Lefrancois*
HA-DHHC2-GFP	*Dr. Stephane Lefrancois*
HA-DHHC3-GFP	*Dr. Stephane Lefrancois*
HA-DHHC4-GFP	*Dr. Stephane Lefrancois*
HA-DHHC5-GFP	*Dr. Stephane Lefrancois*
HA-DHHC6-GFP	*Dr. Stephane Lefrancois*
HA-DHHC7-GFP	*Dr. Stephane Lefrancois*
HA-DHHC8-GFP	*Dr. Stephane Lefrancois*
HA-DHHC9-GFP	*Dr. Stephane Lefrancois*
HA-DHHC10-GFP	*Dr. Stephane Lefrancois*
HA-DHHC11-GFP	*Dr. Stephane Lefrancois*
HA-DHHC12-GFP	*Dr. Stephane Lefrancois*
HA-DHHC13-GFP	*Dr. Stephane Lefrancois*
HA-DHHC14-GFP	*Dr. Stephane Lefrancois*
HA-DHHC15-GFP	*Dr. Stephane Lefrancois*
HA-DHHC16-GFP	*Dr. Stephane Lefrancois*
HA-DHHC17-GFP	*Dr. Stephane Lefrancois*
HA-DHHC18-GFP	*Dr. Stephane Lefrancois*
HA-DHHC19-GFP	*Dr. Stephane Lefrancois*
HA-DHHC20-GFP	*Dr. Stephane Lefrancois*

HA-DHHC21-GFP	*Dr. Stephane Lefrancois*
pGβ1	*Dr. Josef Lazar*
pGγ2	*Dr. Josef Lazar*
pFN21A HaloTag® CMV Flexi® Vector	*Promega*
pGloSensor™-22F cAMP Plasmid	*Promega*
SmBiT-PRKACA	*Promega*
pcDNA3.1-GGamma1-GFP2	*Addgene*
pcDNA3.1-Beta1	*Addgene*
Venus K-Ras	*Dr. K.P. Flegger*
pcDNA5/FRT/TO-GAlphasL-RLuc8	*Addgene*
Venus-Rab1	*Dr. Kevin.P. Flegger*
Venus-Rab5	*Dr. Kevin.P. Flegger*
Venus-Rab7	*Dr. Kevin.P. Flegger*
Venus-Rab6	*Dr. Kevin.P. Flegger*
Venus-Rab4	*Dr. Kevin.P. Flegger*
Venus-Rab11a	*Dr. Kevin.P. Flegger*

Table 10- Plasmids generated in my thesis.

Construct	***primers***	***Tag***	***Backbone***
D_1 p.C347;351S palmitoylation mutant receptor	FW_C347S: GGC ATT TTC AAC CCT CTT AGG AAG CTA CAG REV_C347S: TCG CAG GGC AAA GTC TGT AGC TTC CTA AG FW_C351S: ACC CTC TTA GGA TGC TAC AGA CTT AGC CCT G REV_C351S: TCT ATG GCA TTA TTC GTC GCA GGG CTA AGT CTG	N-ter Flag tag	PCDNA3.1
D_1_p.C347;351S-Nluc	D_1_FWD: CGC ATT CTG GCG TAA CTC GAG TCT AGA GGG C D_1_REV: GCC GCT CGA GCC GAG GGT TGG GTG CTG ACC Nluc_FWD: GGT CAG CAC CCA ACC CTC GGC TCG AGC GGC Nluc_REV: CCC TCT AGA CTC GAG TTA CGC CAG AAT GCG TTC	N-ter Flag C-ter Nluc	PCDNA3.1
D_1-Nluc	D_1_FWD: CGC ATT CTG GCG TAA CTC GAG TCT AGA GGG C D_1_REV: GCC GCT CGA GCC GAG GGT TGG GTG CTG ACC Nluc_FWD: GGT CAG CAC CCA ACC CTC GGC TCG AGC GGC Nluc_REV: CCC TCT AGA CTC GAG TTA CGC CAG AAT GCG TTC	N-ter Flag C-ter Nluc	PCDNA3.1
D_1_p.C347;351S-Rluc8	D_1_FWD: TGC TGA AGA ACG AGC AGT AAC TCG AGT CTA GAG GGC CCG TTT AAA CG Rluc8_FWD: CTC TGA ACA CCT CTG CCA TGG A Rluc8_REV: GCC TTG GGG GTC ATC TTT CTC T	N-ter Flag C-ter Rluc8	PCDNA3.1

	D_1_REV: TCG TAC ACC TTG GAA GCC ATG CCG CTC GAG CCG AGG GTT G		
D_1-Rluc8	D1_FWD: TGC TGA AGA ACG AGC AGT AAC TCG AGT CTA GAG GGC CCG TTT AAA CG Rluc8_FWD: CTC TGA ACA CCT CTG CCA TGG A Rluc8_REV: GCC TTG GGG GTC ATC TTT CTC T D_1_REV: TCG TAC ACC TTG GAA GCC ATG CCG CTC GAG CCG AGG GTT G	N-ter Flag C-ter Rluc8	PCDNA3.1
D_1-LgBiT	D_1_FW: TCC GAG TAA CCA TCA ACA GTA TGG TCT TCA CAC TCG AAG ATT TCG TTG GGG ACT GGG AAC A LgBiT_FW: GGG AGT TCC GGT GGC GGG AGC GGA GGT GGA GGC TCG AGC GGT ATG GTC TTC ACA CTC GAA GAT TTC GTT GGG GAC TGG GAA CAG ACA D_1_REV: ACC GCT CGA GCC TCC ACC TCC GCT CCC GCC ACC GGA ACT CCC ACT GTT GAT GGT TAC TCG GAA CAG CAT GGA GCC GTC GGG GGT GAT LgBiT_REV: TCT TCG AGT GTG AAG ACC ATA CTG TTG ATG GTT ACT CGG AAC AGC ATG GAG CCG	N-ter Flag C-ter LgBiT	PCDNA3.1
D_1_p.C347;351S-LgBiT	D_1_FW: TCC GAG TAA CCA TCA ACA GTA TGG TCT TCA CAC TCG AAG ATT TCG TTG GGG ACT GGG AAC A LgBiT_FW: GGG AGT TCC GGT GGC GGG AGC GGA GGT GGA GGC TCG AGC GGT ATG GTC TTC ACA CTC GAA GAT TTC GTT GGG GAC TGG GAA CAG ACA D_1_REV: ACC GCT CGA GCC TCC ACC TCC GCT CCC GCC ACC GGA ACT CCC ACT GTT GAT GGT TAC TCG GAA CAG CAT GGA GCC GTC GGG GGT GAT LgBiT_REV: TCT TCG AGT GTG AAG ACC ATA CTG TTG ATG GTT ACT CGG AAC AGC ATG GAG CCG	N-ter Flag C-ter LgBiT	PCDNA3.1

II. Methods

1. Molecular Biology

a. Chemical Competency of *E.coli* bacteria preparation

The DH5α strain of *Escherichia coli* chemically competent cells commercially acquired was thawed on ice for 20 to 30 minutes. The thawed cells were streaked under sterile conditions on previously prepared agar plates and incubated at 37°C overnight. The next day, Lysogeny broth (LB) media (15 mL) was added to an Erlenmeyer flask, and a single colony was carefully picked from the agar plate. The flask is then placed in a shaking incubator at 37°C overnight. The next day, the flask was removed from the shaking incubator, and 2.5mL of this culture was added into a new Erlenmeyer flask containing 250mL of Yeast media (YT). This flask

was then placed back inside the shaking incubator for 2 hours; thereafter, 1mL of the growing bacterial culture was used to measure the optical density OD_{600} until it reached 0.5. Thereafter, the culture was centrifuged at 1800 x g for 15 minutes and 4 °C; the pellet was rinsed (25 mL) and then vortexed (40 mL) with TFB1 buffer to resuspend the pellet. This mixture was then centrifuged at 4 °C and 2500 xg, for 5 minutes. The supernatant was then removed and TFB2 (4 mL) was added to the cell pellet and resuspended on ice while Aliquots were made and stored at -80 °C.

b. Bacterial Transformation.

DH5α competent cells that are stored at -80°C were used to perform the transformation. Under sterile conditions, 5µL of DNA plasmid was added to *E.Coli* 50 µL of DH5α competent cells in a 1.5 mL microcentrifuge tube. The tube was gently flicked to allow a gentle mixing and then incubated on ice for 10 minutes. The tube containing the mixture was then heat-shocked by placing it into a water bath at 42°C for 30 seconds. Thereafter, the tube containing the transformed solution was placed back on ice for an additional 2 minutes. At this stage, previously prepared agar plates with the desired antibiotic resistance Ampicillin (100µg/mL) or Kanamycin (50 µg/mL) were warmed up to room temperature (RT). Under sterile conditions, 1mL of LB media was added to the mixture and placed into a shaking incubator at 220 rpm and 37 °C for 1 hour. Then, 50 µL of the transformation solution was added and spread on the agar plates. The plates were then placed in a stationary incubator at 37 °C overnight, allowing colonies to grow optimally.

c. Plasmid Amplification and DNA purification.

The following day after the bacterial transformation and upon the colonies' successful formation, the plasmid DNA is isolated from the bacteria. For small-scale

amplification (miniprep), a small isolated bacterial colony was picked from the agar plate and grown in a falcon tube containing 10mL of LB supplemented with the selection antibiotic. This falcon tube was then placed at 37 °C overnight in a shaking incubator, thus allowing optimal bacterial growth. Around 18 hours later, instructions from a commercially available kit (Macherey-Nalgen™, Germany) were followed to process the culture and extract the plasmid DNA. For the processing of a large-scale amplification of plasmid (maxiprep), an isolated colony was picked and placed into a falcon tube with 6mL LB media containing the appropriate antibiotic and grown in a shaking incubator at 200 rpm at 37 °C. When bacterial growth was visible in this LB media, it was transferred into a sterile Erlenmeyer containing 250 mL of LB media supplemented with the proper antibiotic. The Erlenmeyer was then placed into the shaking incubator at 200 rpm at 37 °C overnight.
The following day, an Invitrogen ™ PureLink HiPure Plasmid Maxiprep Kit was used to extract the amplified DNA.

Once the plasmid DNA was obtained using the kits, it was quantified on the BMG CLARIOstar plate reader and LVis Plate to obtain the purity (A_{260}/A_{280}) and concentration using UV/Vis absorbance.

d. PCR Amplification, PCR-Clean Up, Gibson Assembly & Bacterial Transformation

The Gibson Assembly method was used to create constructs with encoding fusion proteins or tags. In general, this molecular cloning method allows for the assembly of multiple linear DNA fragments, bypassing the usage of restriction enzymes.

First, each DNA fragment of the final construct was amplified via PCR to produce linear fragments using the primers designed on Benchling. This reaction's product

generated DNA fragments with at least 20 bp homology at the desired sites of the junction. To validate the amplification of the desired DNA fragments, agarose gel (1% w:v) electrophoresis was performed, and the parental template was digested with DpnI overnight at 37 °C.

Thereafter, a PCR clean-up kit (Macherey-Nagel-NucleoSpin® Gel and PCR clean-up Kit, Germany) was used to clean the DNA amplification sample. Briefly, the DNA fragments were bound to the provided silica membrane column, washed, dried, and eluted with the NE buffer. Then, the concentration of the final products was obtained using the CLARIOstar. Next, 50 ng of the backbone DNA vector fragment was combined with the insert at 1:2, 1:5 and 1:7 molar ratios in a final volume of 5 µL. To this mix, 15 µL of the Gibson Assembly master mix was added, containing T5 exonuclease, Phusion DNA polymerase and Taq DNA ligase. This complete mixture underwent a quick centrifuge spin and was then incubated in a preheated PCR Machine at 50 °C for 15 to 60 minutes. Thereafter, 2 to 10 µL of the assembly reaction product was used to perform bacterial transformation with *E.coli* DH5α competent cells following the protocol previously described in this section (**b**).

The next day, a portion of single colonies were picked to grow in LB culture; in parallel, the rest was added into a mixture containing DreamTaq PCR Master Mix and the primers that flank the insert region. The PCR was carried out, where the temperature was initially increased to 95 °C for 3 minutes before initiating 30 amplification cycles. Per cycle (lasting 3 min 30s), the temperature was maintained for denaturing of template DNA (98 °C, 10 s), then decreased for primer annealing (50- 75 °C, 20 s) and finally increased again for extension based (72 °C, 10 s). After completing all cycles, a final extension step was performed (72 °C, 10 min). The PCR products were then observed via agarose gel (1% w:v) electrophoresis. The colonies

that gave a band at the correct molecular weight were processed further for plasmid amplification and purification following section (**c**). To confirm that the correct clone was obtained, samples of the DNA were sent for Sanger sequencing using universal primers for pcDNA3.1 forward primer and BGH reverses primer by Eurofins Genomics.

e. Site-directed Mutagenesis.

The Site-directed Mutagenesis (SDM) technique is a PCR reaction where the primer pair have an overlapping region of 8 base pairs (bp) containing the mutation and a non-overlapping region complementary to the target sequence where the mutation is to be inserted. The primers were designed based on the protocol elaborated by (H. Liu & Naismith, 2008).

The PCR product is a linear double-stranded DNA. After DpnI digestion, 3 µl of the digest reaction was directly added to 50 µl of competent DH5α cells, incubated for 10 min on ice, heat-shocked at 42 °C for 1 min and then transferred to ice for 2 min. After adding 450 µl of LB, the cells were incubated on a shaker at 37 °C for 60 min. Duplicate aliquots of 250 µl of cell suspension were spread on LB plates containing Ampicillin (100 µg/ml). After incubating the plates overnight at 37 °C, we selected five colonies at random for each transformation and grew them overnight in 5 ml LB supplemented with Ampicillin medium at 37 °C. The plasmids were isolated using a miniprep kit. and each isolated plasmid, ~100 ng of the plasmid DNA, was sent for sequencing to confirm the mutation insertion.

2. Mammalian cell culture and maintenance

a. Culturing and maintenance Protocol for HEK293T cells

Human Embryonic Kidney (HEK293) cells acquired from ATCC® (CRL-1573™) were grown in Dulbecco's Modified Eagle's medium (DMEM)-high glucose (Sigma Aldrich) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (v:v) heat-inactivated Fetal Bovine Serum (PAN Biotech, Germany) at 37 °C in a 5% CO2 incubator.

HEK293 adherent cells were grown in T-75 cm^2 flasks. Upon reaching 80% confluency, cells were washed with DPBS without $Ca2^+$ and $Mg2^+$ ions, detached from the culture surface using 3 mL of a Trypsin-EDTA solution and incubated at 37 °C for 5 minutes. 10 mL DMEM-High Glucose media was used to neutralise the trypsin effect before room temperature centrifuging the cells at 1000 x g, for 5 min. The supernatant was discarded, and the cell pellet was resuspended in an adequate volume of DMEM-High Glucose media to give the desired cell density.

3. Transfection using Lipofectamine 3000

HEK293 cells were transiently transfected following the reverse transfection method using Lipofectamine™ 3000 (Thermo Fisher). Two transfection mixes (A & B) were prepared in Mix A; the plasmid DNA was diluted in an adequate volume of OptiMEM® media. To this, a 1:3 ratio of DNA(µg) to P3000™ (µl) was mixed. Thereafter, Lipofectamine™ was added to the OptiMEM® media, and each mix was incubated for 5 minutes. HEK293 cells that were previously seeded in 10 cm dishes or 6 well plates were washed with DPBS without Ca^{2+} and Mg^{2+}, and fresh DMEM-high Glucose media was added . Meanwhile, the diluted DNA mix was added to the Lipofectamine™ mix in a dropwise manner and incubated for 15 minutes at RT. The

transfection mix was added, and cells were incubated at 37 °C in a 5% CO2 incubator for 24 hours.

4. Real-Time Assays

a. cAMP Assay

The transfection mix was prepared such as, in Mix A, 3 µg of receptor and 1 µg of pGloSensor™-22F cAMP sensor plasmid (Promega); in some experiments, an additional 1 µg of pcDNA3.1 or accessory protein were added to OptiMEM® media. The appropriate amount of P3000™ (3:1 ratio) was added to this. In Mix B, the appropriate amount of Lipofectamine™ was added to the OptiMEM®.

Twenty-four hours after transfection via lipofectamine 3000, the cells were detached and reseeded in a 96-well white bottom plate at 60,000 cells per well for an extra 24 hours, then the cell culture media was removed. The cells were washed with the HBSS-based luminescent assays buffer (1X HBSS, 24 mM HEPES, 0.1% (w:v) BSA, 3.96 mM $NaHCO_3$, 1 mM $MgSO_4$, 1.3 mM $CaCl_2.2H_2O$). The plate was then equilibrated for 1 hour at 28 °C with 70-90 µl of cAMP Buffer supplemented with 0.45 mg/mL of Firefly D-Luciferin free acid (Nano Light Technology).

Where dynasore (HelloBio) is involved, after equilibration, 20 µl of dynasore (80 µM/well) is added and incubated for 15 mins at RT before initiating the basal readings. Where the use of an antagonist was incorporated into the assay, 10 µl of the antagonist was pre-incubated for 15 minutes before the basal readings were initiated.

A total of six basal reads were recorded before stimulating the cells with 10 µl of agonist. The bioluminescence was measured using the CLARIOstar® Plus Plate Reader (BMG LabTech, Germany). An average of these basal reads was used to

normalise the response of each well. Bioluminescence was measured for 36 cycles (1 min per cycle), with a 1 sec. integration time and no lens.

b. TRUPATH Assay

HEK293 cells were transiently transfected in a 10 cm dish following the reverse transfection method using Lipofectamine™ 3000 (Thermo Fisher). Two transfection mixes were prepared, the first containing 1 µg/plate of each of the G α, β, γ subunits, receptor plasmid either in with the empty vector pcDNA3.1 replacing the γ subunit which was added to 250 µl OptiMEM® media. To this, 12 µl/dish of P3000™ was added. Thereafter, 12 µl/dish of Lipofectamine™ was added to 250 µl OptiMEM® media, and each mix was incubated for 5 minutes. HEK293 cells were then washed without DPBS, without Ca^{2+} and Mg^{2+}, and 10 mL of fresh water was added . Meanwhile, the diluted DNA mix was added to the Lipofectamine™ mix in a dropwise manner and incubated for 15 minutes at RT. 500 µl of the transfection mix was added to each dish. The dish was incubated at 37 °C in a 5% CO2 incubator for 24 hours, then reseeded in white bottom 96-well plates at a density of 60000 cells per well.

Twenty-four hours later, the cell culture media was removed, and cells were washed with PBS and, after that, replaced with 80 µl of assay buffer (pH 7.4), followed by a 10 µl addition of freshly prepared 50 µM coelenterazine 400a (Nanolight Technologies) in the same buffer. After 5-10 min equilibration basal reads at RT in the CLARIOstar® Plus Plate Reader (BMG LabTech, Germany) with both the 515-30 and 410-80 emission filter for γ-GFP2 (Green Fluorescent Protein 2) and α-RLuc8 (Renilla Luciferase 8) coelenterazine 400a, respectively, at integration times of 1sec. per well are acquired. After equilibration, cells are treated with 10 µl of ligand and the assay completed within 15 cycles from basal.

BRET2 ratios were computed as the ratio of the GFP2 emission to Rluc8 emission. The BRET is calculated by dividing the acceptor emission value by the donor emission value for each sample to generate raw BRET ratio values. Then, we determined the mean BRET ratio for each set of samples and normalised the mean BRET values to no ligand control; the Net BRET ratio was calculated by subtracting the normalised mean BRET values for the donor-only control set. All concentration-response curves were fit to a four-parameter logistic equation in Prism (GraphPad Software). BRET2 concentration-response curves were analysed as either raw net BRET2 (fit Emax-fit Baseline) or by normalising to a reference receptor construct for each experiment.

c. β-arrestin recruitment Assay

To establish the optimal total DNA ratio of receptor: β-arrestin, HEK293 cells (60,000 cells/well) were seeded in white 96 well plates reverse co-transfected with the plasmids encoding the receptor (12.5 ng/well) and the β-arrestin1 or 2 fusions at increasing concentrations in the presence of empty vector (pcDNA3.1) to account for differences in the total amount of DNA/well and the ratio giving the better fold change to the absence of treatment was adopted. Based on this, 2.5 ng/well of SmBiT β-arrestin 1 or 2 were co-transfected with 100 ng/well of the receptor. After 24h, 25 µl/well of a 5X solution of the Nano-Glo® Live cell reagent was added, and luminescence readings were taken every minute at 37 °C until the signal was stable. Immediately after, 10 µl of agonists/vehicle were supplemented, and the luminescence was measured for 60 min (0.5s integration time and 1 min intervals for arrestins) at 37 °C using a CLARIOstar® Multimode Plate Reader (BMG Labtech, Germany). To account for differences in expression/cell density, the average of at least 3 stable pre-readings was used to normalise each well response.

d. Bystander β-arrestin recruitment Assay

The β-arrestin recruitment to the P.M was measured using a bystander NanoBiT approach with SmBiT-β-arrestin and the LgBiT-CAAX constructs. HEK 293 cells were seeded in white 96 well plates and reverse co-transfected with the plasmid encoding the receptor (50 ng/well), SmBiT-β-arrestin (2.5 ng/well), and LgBiT-CAAX (12.5 ng/well). 24h post-transfection, the cells were rinsed once with assay buffer, and the plates were pre-equilibrated for 1h at 37 °C with 80 µl of assay buffer. 10 µl/well of a 5X solution of the Nano-Glo® Live cell reagent was added, and luminescence readings were taken every minute at 37 °C until the signal was stable (3-5 min). Immediately after, 10 µl of agonist/vehicle were added and luminescence was further recorded for 30 min (no lens, 0.5 s integration time and 1 min intervals at 37 °C using a CLARIOstar® Plus Multimode Plate Reader (BMG Labtech, Germany). To account for differences in expression/cell density, the average of at least 3 stable pre-readings was used to normalise each well response.

e. Bystander BRET

HEK293 cells were transiently transfected with cDNA encoding wild-type or mutant D_1-Nluc (100 ng/well of a 6-well plate), as well as Venus/K-Ras (100 ng/well of a 6-well plate) or Venus/Rab proteins or empty vector (200 ng/well of a 6-well plate). 24 hours after transfection, cells were harvested and seeded into white bottom 96 well plates at 80 000 cells/well in DMEM containing 25mM HEPES, 0.3-mg/mL glutamine, 100-IU/ml penicillin, and 100-µg/mL streptomycin supplemented with 10% FBS. Forty-eight hours after transfection, the medium was removed, and cells were incubated in 80 µl of HBSS assay buffer for 1 hour at 37 °C in 5% CO_2. After basal measurements, 10 µl of furimazine was added after 10-15 min of equilibration,

the cells were treated with 10 µl HBSS buffer with or without agonist. Real-time BRET measurements were taken at 37°C using a CLARIOstar plate reader. Filtered light emissions were sequentially measured at 475-30 nm for Nluc and 515-30 nm for Venus. The "BRET ratio (ligand-vehicle)" was calculated by subtracting the ratio of 515-20 nm emission over 475–30 nm emission for a vehicle-treated cell sample from the same ratio for a sample treated with an agonist. BRET signals for assays where basal/constitutive localisation of Nluc-tagged wild-type D_1 or D_1 mutant proximal to Venus-tagged subcellular markers were calculated as described previously by subtracting the ratio of 515-30 nm emission over the 475-30 nm emission for a cell sample containing only the Nluc fusion protein from the same ratio of a second aliquot of cells containing both the Nluc and Venus fusion proteins.

5. Measuring ERK1/2 phosphorylation levels

To measure ERK1/2 (Thr202/Tyr204) phosphorylation in HEK293 cells, 50,000 cells per well were seeded in poly-D-lysine coated white clear 96 well plates and reverse transfected with 100 ng/well of receptor (D_1 WT and D_1 p.C347;351S mutant receptors). 24 hours after transfection, and the cell culture medium was removed, the cells were starved in FBS-free DMEM supplemented with 0.1% (w/v) BSA for 4 h at 37 °C in a 5% CO_2 humidified atmosphere. ERK1/2 phosphorylation levels were measured using the AlphaLISA SureFire Ultra™ ERK1/2 cellular assay kit following the manufacturer's instructions. In summary, after the starvation step, the cell media was removed and replaced with 50 µl of ERK media containing vehicle or, when necessary, the required antagonist and the plates were incubated for 20 min at 37 °C. After agonist stimulation (5 min), the media was quickly aspirated, replaced with 50 µl of the proprietary lysis buffer and the plates were agitated (350 rpm) for 30

min at RT. 10 µl of the Lysates were transferred to low-volume round bottom white 384 well/plates (ProxiPlate-384 Plus) and 5 µl of each donor/acceptor mix beads were added. Plates were sealed in foil, protected from light, and incubated for 24 hours at RT before the readings were acquired. The alpha screen signal was measured at 615 nm and analysed on a CLARIOstar Multimode Plate Reader (BMG Labtech, Germany) equipped with an Alpha Technology optical module.

6. Cell surface Staining for Flow Cytometry

HEK293 cells were transfected in 6 well poly-D lysine coated plates using lipofectamine 3000, after 24h cells were washed twice with cold PBS. Then the cells were detached with 10mM EDTA, 1% BSA in PBS and the cells were filtered using a 100 µm cell strainer. Afterwards, the required concentration of cells is transferred to labelled Eppendorf tubes and a washing step is performed by spinning at 1500 rpm for 5 min at 4 °C and the pellet is resuspended in 1 mL FACS buffer (1% BSA, 2mM EDTA in PBS). That was followed by an Incubation step on the ice for 30 minutes, followed by a centrifugation step at 1500 xg for 5 min at 4 °C. Then, the supernatant was removed and vortexed to resuspend the cells. After that, 100 µl of anti-Flag primary antibody (1:1000 dilution), or FACs buffer (for controls), is added to each tube and incubated on ice for 30 minutes. After that, 1 ml of cold FACS buffer is added to each tube and washed by centrifugation and the supernatant is removed. The cell pellet is resuspended in 100 µl of secondary antibody (1:10000 dilution) in FACs buffer or FACs buffer (for controls) and incubated on ice for 30 min in the dark.
Finally, 1 ml of cold FACS buffer was added to wash, after centrifugation the pellet was resuspended in FACS buffer and fixed with 1% paraformaldehyde for 10 minutes (100-200 µl) and then the cells underwent a final washing, and the pellet was

resuspended in 300-400 µl FACS in FACS tube and stored at 4 °C in the dark until acquisition.

7. Bret Saturation assay

In the BRET saturation experiments, cells were transfected with a constant amount of the BRET-donor D_1-Rluc8 tagged in the presence or absence of increasing amounts of the acceptors DHHCs-GFP10. Theoretically, for any specific interaction between the Receptor-donor and Receptor-acceptor fusions, the BRET ratio increases hyperbolically as a function of increasing GFP/Rluc value to reach an asymptote (saturation) when all donor molecules are associated with acceptors. By contrast, in the case of nonspecific interactions (bystander BRET), a linear plot is expected or eventually reaches a plateau for higher values of receptor density. The cells are pre-incubated in the absence of agonist drugs. BRET ratio measurements are acquired after adding coelenterazine-400a diluted in HBSS buffer to reach a final concentration of 5µM. BRET ratio readings are acquired using a CLARIOstar® Plus Plate Reader (BMG LabTech, Germany) that allows sequential integration of luminescence signals detected with two filter settings (515-30 and 410-80). The specific BRET ratio was calculated by subtracting from the mean BRET ratio value above the background BRET ratio, which corresponds to the signal obtained with cells expressing the BRET donor alone. BRET ratio values were plotted as a function of the GFP2/Rluc fusion protein ratio.

8. Acyl-Rac labelling

HEK 293 cells were seeded in 10 cm dishes and transfected 24h hours later with Flag-D_1 WT or mutant. 48 hours later, the cells were lysed in RIPA buffer (Tris

50 mM pH 7.5, NaCl 150 mM, Nonidet P-40 1%, deoxycholic acid 0.5%, SDS 0.1%, protease inhibitor cocktail) supplemented with 75 mM NEM (N-ethylmaleidimide) for 2 hours at 4 °C. The lysates were cleared by centrifugation at 13000 rpm for 15 min and incubated for 2 hours at room temperature. Two volumes of cold acetone were added to precipitate the proteins, and the samples were incubated overnight at -20 °C. The next day, the samples were centrifugated at 15000 rpm for 20 min then the cell pellets were washed once with cold acetone and centrifugated again for 10 min. The dried pellets were then resuspended in binding buffer (100 mM Hepes, 1 % SDS, 1 mM EDTA) containing 250 mM hydroxylamine or NaCl and incubated until the pellets were dissolved. Thiopropyl beads were then added to the samples and incubated for 2 hours at RT. After the incubation, the samples were washed 4 times with binding buffer and eluted with 3X Laemmli sample buffer containing 100 mM dithiothreitol. The samples were then resolved by SDS-PAGE electrophoresis followed by immunoblotting using an anti-Flag antibody.

9. bioinformatics (CSS-Palm)

CSS-Palm is a computer-based program for (Clustering and Scoring Strategy) palmitoylation site prediction for Palmitoylation sites prediction (F. Zhou et al., 2006). The experimentally verified palmitoylation sites were automatically clustered into three clusters by different thresholds of peptide similarity. When the program is given a putative palmitoylation site for prediction, the CSS-Palm will calculate a score between the sites with each cluster dependent on the BLOSUM62 matrix respectively. If the largest score was exceeded the cut-off value (2.6), the putative site would be predicted as a positive hit.

10. Sanger sequencing

Sanger sequencing is a method for determining the nucleotide sequence of DNA. The method was developed by Frederick Sanger and his colleagues in 1977. Sanger sequencing was performed in an automated fashion via a sequencing machine by Eurofins Genomics, Germany.

11. Live-cell Confocal imaging

Live-cell imaging was carried out using a Nikon spinning disk confocal microscope with a ×60, 1.4 numerical aperture, oil objective and a CO2 and 37 °C temperature-controlled incubator. A 488, 568 nm and 640 Voltran were used as light sources for imaging GFP, mRFP, and Snap-647 signals, respectively. Cells expressing both the Snap-tagged receptor (2 μg) and the indicated nanobody–GFP (200 ng) were plated onto glass coverslips. Receptors were surface labelled by the addition of Snap-Cell 647 SiR (1:1000, New England Biolabs) to the media for 20 min.

III. Data analysis and graphing

GraphPad Prism software (GraphPad Software, USA) was used for all cAMP data analysis. In the case of data obtained from BMG microplate readers (ClarioStar), the software provided by the manufacturers (MARS) was used for averaging, baseline corrections and area under the curve analysis. The baseline corrected data was obtained by averaging the last five points of the baseline before the effect to be measured took place. Each given point of datum was divided by this average baseline to give a fold-over-baseline value. Concentration-response curves were fitted using a four-parameter logistic nonlinear regression. As for the BRET acquired using the

TRUPATH open-source biosensor, GraphPad Prism software was used following the method of calculation and analysis detailed in the paper of Olsen et al. For several experiments performed, the R^2 values were calculated using GraphPad Prism software as a measure of goodness of fit of the data to the curve generated.

Chapter 3: investigating the Effects of loss of Palmitoylation on dopamine D_1 signalling

I. Introduction

G protein-coupled receptors serve as pivotal drug targets, their intricate involvement in human pathophysiology renders them subjects of extensive study (Hauser et al., 2017). The understanding of mechanisms regulating GPCR signalling is crucial in advancing therapeutic interventions. Palmitoylation is one of those regulatory mechanism and was shown to impact GPCRs signalling as reported in chapter 1.

The Dopamine D_1 undergoes palmitoylation exclusively at cysteines 347 and 351 on its C-tail (Jin et al., 1999). GPCR palmitoylation has been reported to have implications on these receptors' pharmacology, signalling and trafficking (Ernst et al., 2018; Qanbar & Bouvier, 2003). GPCRs interact with their heterotrimeric G proteins at the plasma membrane upon agonist stimulation and promote the dissociation of G-protein subunits. These G-proteins regulate different intracellular second messenger pathways depending on the type of Gα subunit involved. Since D_1 is coupled to $G\alpha_s$ it promotes the production of cAMP via the activation of adenylyl cyclase by stimulatory G proteins. cAMP activates protein kinase A that stimulates ERK1/2 activity (Fonseca et al., 2020), with both PKA and ERK leading to activation of a transcription factor called cAMP response element-binding protein (CREB), orchestrating the transcription of genes modulating cell behaviour (H. Zhang et al., 2020).

In addition, D_1 recruit β-arrestins (Kotowski et al., 2011), they play a crucial role in biological processes by recruiting effector proteins from diverse signalling pathways (e.g. ERK1/2, Raf-1, JNK3 and Akt) and important components of the endocytic machinery (DeWire et al., 2007; Jean-Charles et al., 2017).

While previous studies have touched on the palmitoylation of Dopamine D_1, they fell short of fully characterising its impact on receptor signalling and trafficking. These studies observed changes in cell surface internalisation without delving into intracellular localisation or functional outcomes post-receptor activation (Kong et al., 2011).

In this chapter, our focus is to comprehensively identify the impact of palmitoylation on the Dopamine D_1 receptor signalling. Our goal is to enhance the understanding of the role of palmitoylation of the C-tail in shaping receptor behaviour.

II. Outlines & Aims

To study the effects of loss of palmitoylation on D_1 pharmacology and protein-protein interactions, a palmitoylation-deficient mutant (D_1 p.C347S; C351S) was generated by mutating the cysteines in position 347 and 351 into serines using site-directed mutagenesis (SDM). D_1 Wild type and D_1 palmitoylation mutant sequences were validated by Sanger sequencing (**Fig.22**). To confirm the loss of palmitoylation in D_1 p.C347S;C351S an Acyl-RAC labelling was performed (**Fig.23**) (Tewari et al., 2020), this technique allows the detection of protein acylation.

Moreover, to investigate the effect of DHHCs and loss of palmitoylation on D_1 signalling a luminescent-based live-cell assay was used to look at cAMP production with a genetically engineered firefly luciferase that changes conformation when it binds to cAMP and generates light in the presence of its D-Luciferin substrate (**Fig.21**).

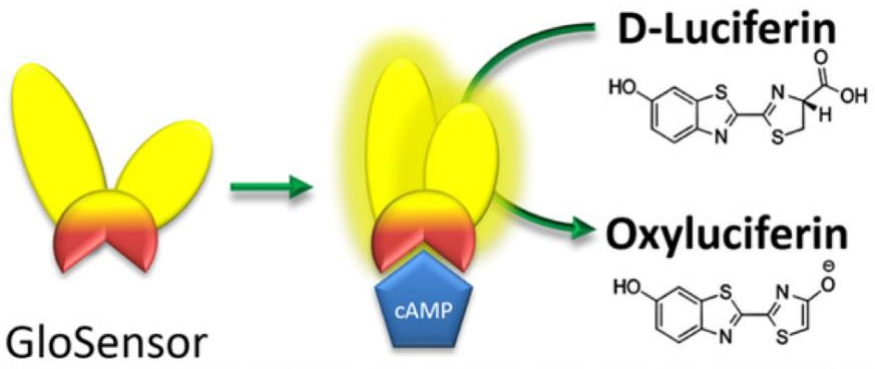


Figure 21-cAMP luciferase assay principle

Schematic representation of the biosensor (GloSensor) cAMP binding leads to a conformational shift in the biosensor that promotes an increase in luminescence activity in the presence of D-luciferin substrate.(DiRaddo et al., 2014)

Also, a measurement of β-arrestin related ERK1/2 production of D_1 was performed using AlphaLISA SureFire Ultra™ ERK1/2 cellular assay kit to investigate any changes in its production between D_1 WT and D_1 palmitoylation mutant. Finally, cAMP was profiled for D_1 WT and mutant in Hek293 β-arrestin 1&2 Knockout cells and the phenotype was compared between a restored β-arrestin 1&2 expression or an empty vector control.

III. Results

1. D_1 palmitoylation deficient mutant generation

Previous studies have shown that D_1 has two palmitoylation sites on the cysteines in positions 347 and 351 located on its C-terminal tail (H. Jin et al., 1999). This is also predicted by bioinformatics (CSS-Palm) and further confirmed by mutagenesis of the cystines into alanines at these sites leading to loss of the palmitoylation (H. Jin et al., 1999). Knowing that cysteine and serine are proteinogenic and the closest amino acids in structure, mutating cysteine into a serine will have

minimal effect on the structure and won't interfere with other local post-translational modifications. Using site-directed mutagenesis a D_1 palmitoylation deficient receptor was generated by mutating the two cysteines into serines. The sequence was confirmed by Sanger sequencing (**Fig.22**).

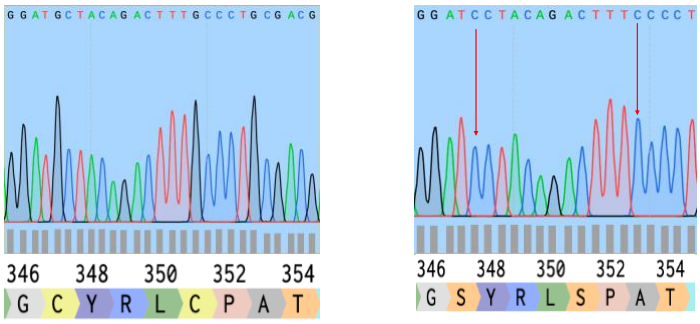


Figure 22- Sequence validation of the palmitoylation D1 mutant by Sanger sequencing

Electropherograms of DNA sequences of D_1 WT (right) and palmitoylation mutant (left). Arrows indicate mutated bases changing cysteine (TGC) to serine (TCC) at position 347 and 351.

2. D_1 p.C347;351S mutations lead to receptor loss of palmitoylation

After generating the palmitoylation mutant and validating the sequences by Sanger (**Fig.22**), loss of palmitoylation was validated by Acyl-RAC labelling. To this end, to check if the mutant has lost its ability to undergo palmitoylation. Equal amounts of D_1 WT or palmitoylation mutant were transfected in HEK 293. The lysates were then treated with NaCl (negative control) hydroxylamine-free buffer to make sure the protein could not bind the beads non-specifically (Forrester et al., 2011; Ulengin-Talkish et al., 2021). Whereas buffer with hydroxylamine breaks the bond between the palmitate group and the cysteine residues (Tewari et al., 2020). The freed cysteine residues could bind the thiopropyl beads if the protein was palmitoylated. The

samples were then eluted and resolved by SDS-PAGE electrophoresis followed by immunoblotting using an anti-Flag antibody against the Flag-D_1.

The results demonstrated that only D_1 WT was palmitoylated in HEK cells as it was pulled down with the thiopropyl beads, whereas the mutant could not be resolved for acylation (**Fig.23).**

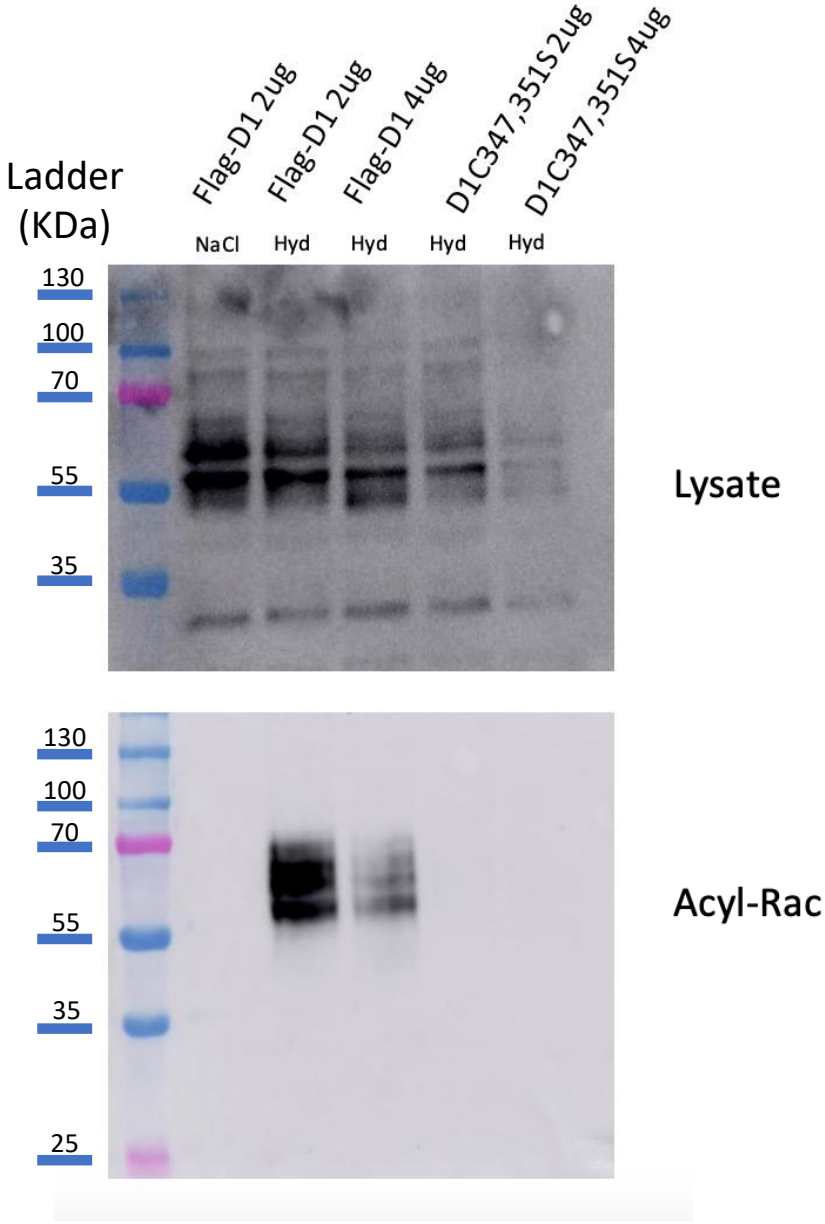


Figure 23-Validation of loss of palmitoylation in D_1 C347S; C351S mutant

Acyl-RAC detection of D_1 WT and D_1 p.C347S; C351S mutant. Equal amounts of total protein lysates from HEK293 were run on SDS-PAGE in duplicate and were analysed by Western blotting against Flag-D_1. Equal amounts of total protein lysates were processed for acyl-RAC Hyd: Hydroxylamine.

3. The p.C347;351S on D_1 reduces cAMP production

To further evaluate the effects of mutating the cysteines into serines and confirm that the receptor still presents a cAMP production profile similar to previously published papers where the cysteines palmitoylated residues were mutated into

alanines (H. Jin et al., 1997, 1999; Kong et al., 2011), D_1 cAMP production was assayed in live-cells where the receptor was co-transfected with a genetically engineered firefly luciferase that changes conformation when it binds to cAMP and generates light in the presence D-Luciferin. To this end, HEK293 cells were transfected with equal amount of D_1 WT or palmitoylation mutant with a cAMP Glo sensor and treated with a concentration gradient of either SKF 81297 (D_1 selective agonist) (Vermeulen et al., 1994), dopamine or A-68930 hydrochloride (D1-Like selective agonist) (Johnson et al., 1992; Langen & Dost, 2011). The D_1 palmitoylation mutant showed a reduced ability to produce cAMP compared to D_1 WT, its cAMP production was 51%, 42% and 41% lower than D_1 WT with SKF 81297, A-68930 and dopamine respectively (**Fig.24; Table 11**).

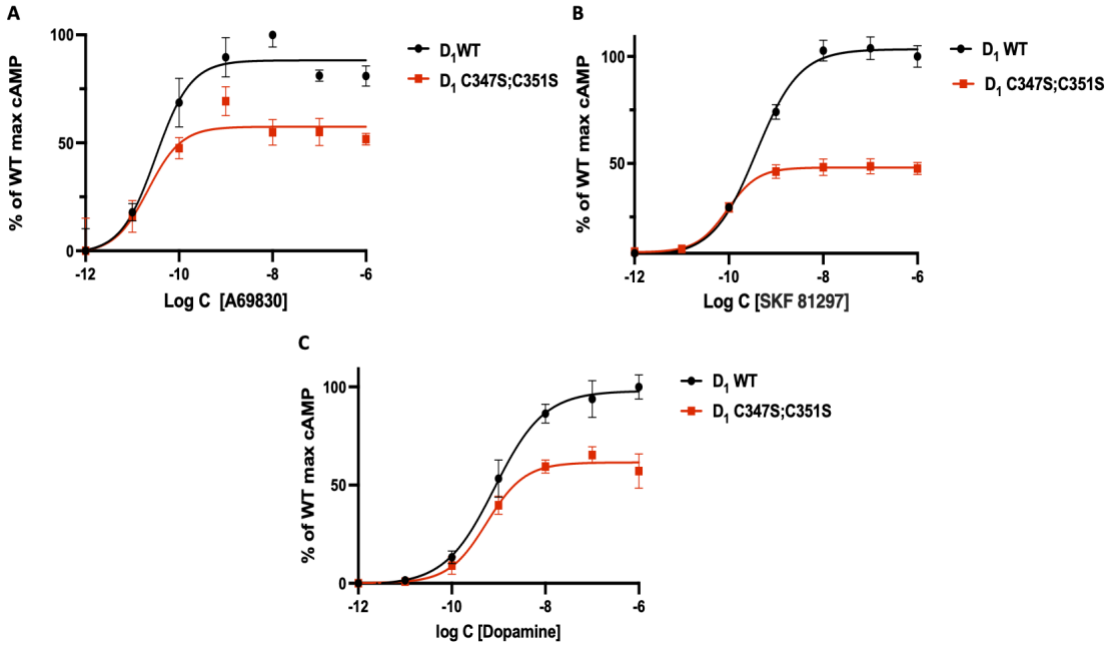


Figure 24- Characterisation of D_1 cAMP production

*D_1 WT and D_1 palmitoylation deficient mutant cAMP production assay performed in HEK 293 cells. Concentration response of cAMP production where cells were treated, respectively, with vehicle or a gradient of the D_1 agonist A-69830 (**A**), SKF 81297(**B**), and Dopamine(**C**). cAMP production was plotted as a percentage of D_1 WT maximum production of cAMP (n of at least 3 independent repeats performed in triplicate).*

Table 11- Comparing D_1 WT pEC_{50}&E_{max} with various agonists.

	A-69830	SKF 81297	Dopamine
D_1 WT pEC_{50}	10.5± 0.33	10.13± 0.08	9.08± 0.11
D_1 $C_{347S;C351S}$ pEC_{50}	10.66± 0.12	10.41± 0.09	9.25± 0.14
D_1 WT E_{max}	96.98± 3.11	103.4± 3.22	98.86± 2.03
D_1 $C_{347S;C351S}$ E_{max}	55.45± 8.11***	49.62± 1.2***	57.34± 1.04***

Values are representative of n of at least 3 independent repeats performed in triplicate. Concentration responses shown in ***Fig.24*** *were analysed by nonlinear regression using Prism, and the pEC_{50}, and E_{max} values were derived. Statistical significances of the differences were determined using unpaired Student's* t *test.*

4. Effect of DHHCs overexpression on D_1 signalling

Starting from the hypothesis that if a DHHC interacts with D_1 it was assumed that this would lead to a change in cAMP production in response to agonist stimulation for D_1 WT but not for the palmitoylation mutant. To test this hypothesis, various DHHCs were overexpressed with D_1 WT or mutant with the cAMP GloSensor (**Fig.25**).

The results showed that DHHC 7 and 21 could not generate a significant difference in cAMP production between D_1 WT and D_1 palmitoylation mutant. The other DHHCs reduced cAMP production for both constructs, with DHHC 17 driving the most significant cAMP reduction between D_1 WT and the palmitoylation null mutant and DHHC 5 inducing a higher cAMP for the mutant than the WT. Surprisingly, overexpression of DHHCs did not statistically t impact receptor affinity to SKF81297 (**Table 12**).

These findings align with publications arguing and demonstrating that DHHCs are enzymes that palmitoylate a pleiotropy of proteins. Therefore, DHHCs can impact D_1

by modulating its palmitoylated accessory proteins, such as G proteins and ion channel transporters (S. Chen et al., 2018; Jennings & Linder, 2010, 2012; Ohno et al., 2006).

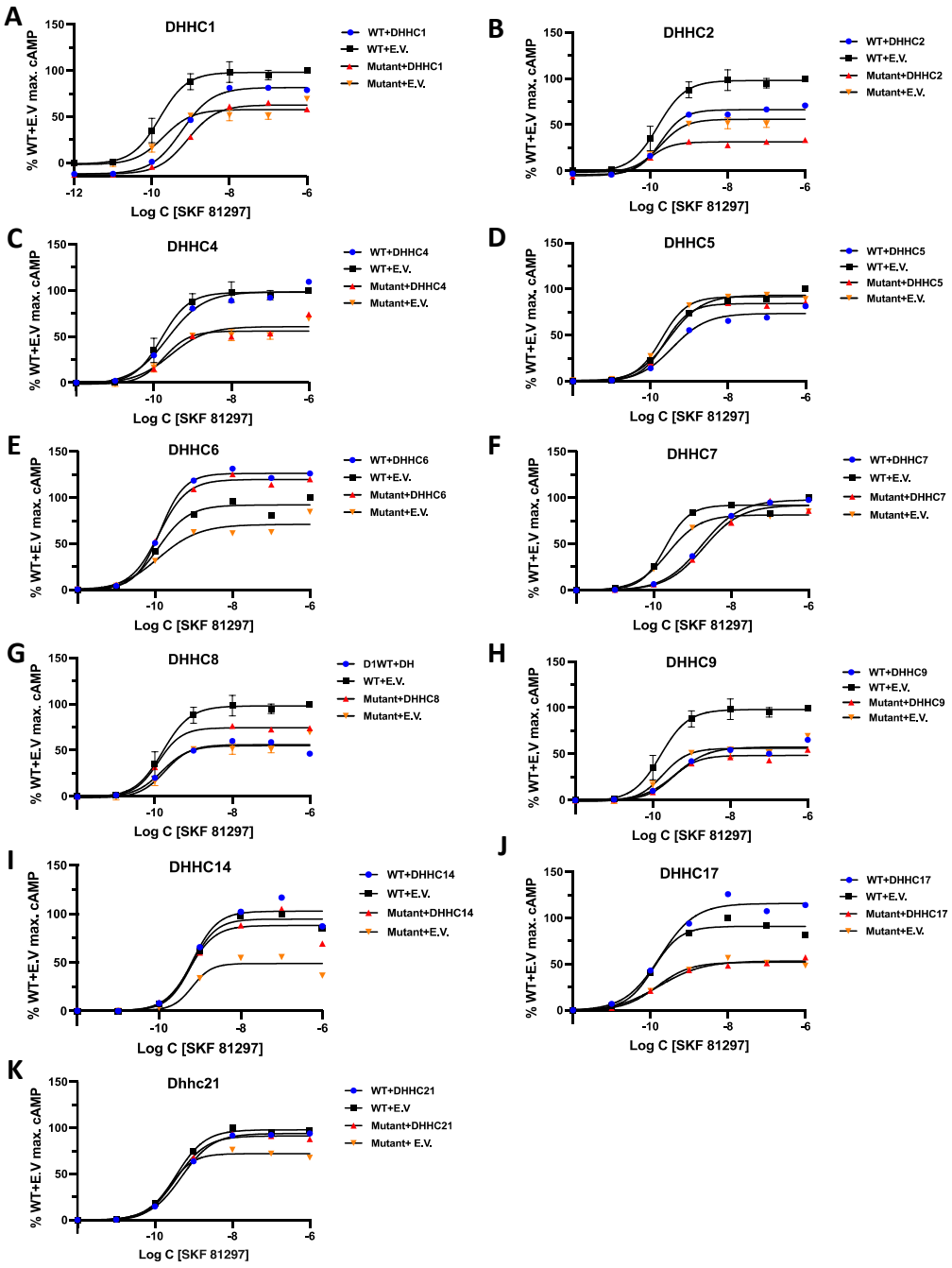


Figure 25- Effect of DHHCs overexpression on D_1 cAMP production

D_1 WT and D_1 palmitoylation mutant cAMP production in DHHCs overexpression conditions. The assay was performed in HEK 293 cells with D_1 WT or palmitoylation mutant in various DHHC overexpression conditions and D_1 WT and palmitoylation mutant transfected with an empty vector as a control. The concentration response of

cAMP production was measured by treating with vehicle or a gradient of concentration of the D_1 agonist SKF 81297 panel A to K represent independent experiments of D_1 cAMP production with the available DHHCs (1;2;4;5;6;7;8;9;14;17&21). cAMP production was plotted as a percentage of D_1 WT+EV E_{max} (n of 3 independent experiments).

Table 12- Comparing D_1 WT pEC50&Emax with various DHHCs.

	E.V	**DHHC1**	**DHHC2**	**DHHC4**
D_1 WT pEC_{50}	9.8± 0.1	9.22± 0.12	9.73± 0.1	9.64± 0.14
D_1 $C_{347S;C351S}$ pEC_{50}	9.74± 0.12	9.08± 0.08	10.06± 0.14	9.54± 0.8
D_1 WT E_{max}	98.06± 4.43	81.63± 1.93	66.32± 2.62	98.59± 5.21
D_1 $C_{347S;C351S}$ E_{max}	53.59± 6.42***	62.8± 2.23***	31.42± 1.63***	60.1± 3.41***

	DHHC5	**DHHC6**	**DHHC7**	**DHHC8**
D_1 WT pEC_{50}	9.44± 0.13	9.87± 0.05	8.76± 0.02	9.82± 0.16
D_1 $C_{347S;C351S}$ pEC_{50}	9.68± 0.09	9.9± 0.06	8.71± 0.12	9.91± 0.05
D_1 WT E_{max}	74.01± 4.08	126.2± 2.25	97.68± 0.45	50.55± 3.12
D_1 $C_{347S;C351S}$ E_{max}	83.32± 1.1**	119.6± 2.51*	97.92± 3.65	75.36± 1.23***

	DHHC9	**DHHC14**	**DHHC17**	**DHHC21**
D_1 WT pEC_{50}	9.4± 0.14	9.18± 0.2	9.75± 0.17	9.32± 0.19
	9.51± 0.16	9.25± 0.26	9.8± 0.15	9.48± 0.22
D_1 WT E_{max}	57.4± 3.54	148.2± 9.76	115.91± 1.33	102.3± 2.85
D_1 $C_{347S;C351S}$ E_{max}	48.01± 2.42**	133.58± 5.26*	55.33± 2.3***	99.4± 3.88

*Concentration responses shown in **Fig.25** were analysed by nonlinear regression using Prism, and the pEC_{50}, and E_{max} values were derived. Statistical significances of*

the differences were determined using unpaired Student's t test (Values are representative of n of at least 3 independent repeats performed in triplicate).

5. Loss of palmitoylation reduces D_1 ERK 1/2 production

After agonist activation of a GPCR, Gs cAMP production stimulation and β-arrestins recruitment one of the downstream effects of these cascades is the production of ERK1/2. To measure ERK 1/2 production, we used the AlphaLISA SureFire Ultra™ ERK1/2 cellular assay kit. ERK1/2 was measured 10 min after SKF 81297 D_1 selective agonist or dopamine treatment as recommended after optimisation of treatment time and cell number (Garbison et al., 2015) (**Fig.26**). The results showed that regardless of the D_1 activating agonist, the palmitoylation mutant receptor had a 33% reduction in ERK1/2 production compared to D_1 WT with dopamine and SKF 81297 (**Table 13**).

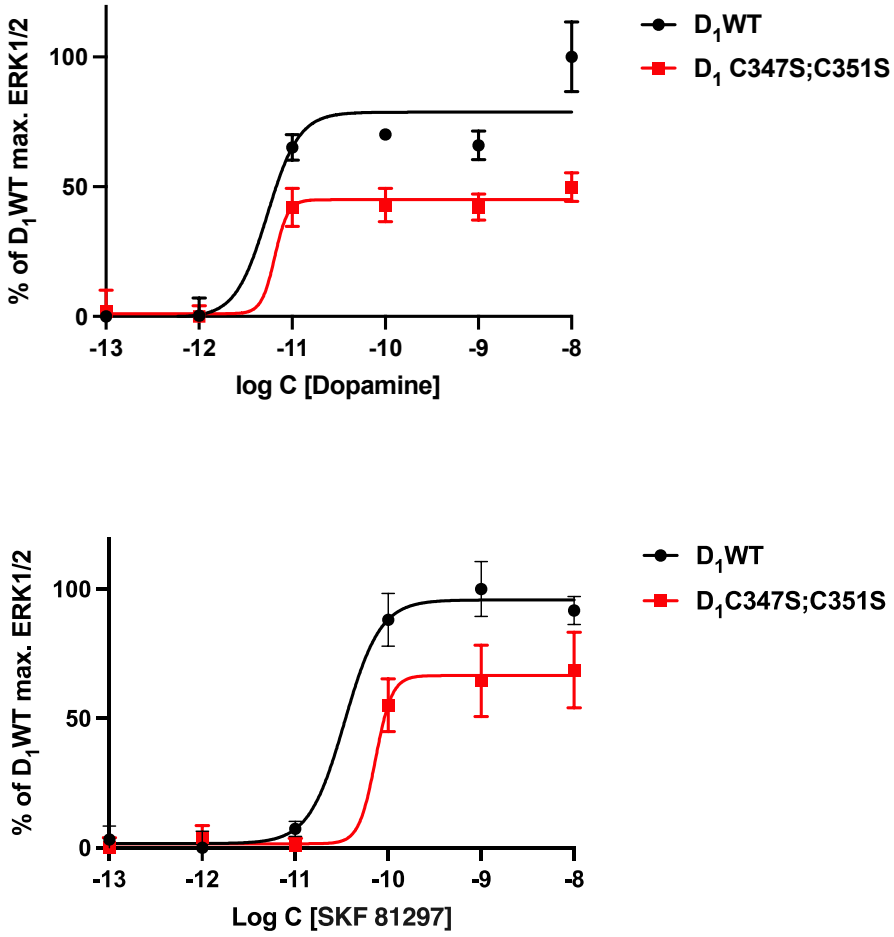


Figure 26- D_1 Wild type and D_1 palmitoylation deficient mutant

Agonist-induced ERK1/2 phosphorylation in response to D_1 agonist dopamine (top) SKF 81297 (bottom). Values represent the mean E_{max} ± SEM percentage normalised to WT receptor maximal ERK1/2 production (n=3).

Table 13- Statistical evaluation of ERK1/2 phosphorylation profile of D_1 WT and the palmitoylation mutant

	Dopamine	**SKF81297**
D_1 WT E_{max}	78.71± 12.54	105.48± 7.21
D_1 $C_{347S;C351S}$ E_{max}	45.01± 2.42**	72.08± 8.43***

*Concentration responses shown in **Fig.26** were analysed by nonlinear regression using Prism, and the E_{max} values were derived. Statistical significances of the differences were determined using unpaired Student's* t *test (Values are representative of n of at least 3 independent repeats performed in triplicate).*

6. Investigating the impact of β-arrestin 1&2 on D_1 cAMP production

The data showed that the D_1 palmitoylation mutant has a reduced ERK1/2 phosphorylation compared to WT (**Fig.27)**, as reported in HEK 293 cells expressing the D_1, β-arrestin recruitment contributes to the activation of ERK1/2 (Kaya et al., 2020). To understand the impact of β-arrestin 1&2 absence on D_1 signalling, HEK 293 β-arrestin 1&2 knockout cells provided by Dr Asaka Inoue are extensively used to understand loss on arrestins impact on various receptors (Tréfier et al., 2018; Wan et al., 2018) were chosen to evaluate the impact of β-arrestin 1&2 on D_1 WT and palmitoylation mutant cAMP profile. For that purpose, HEK293 β-arrestin 1&2 Knockout cells were transfected with D_1 WT or palmitoylation mutant with the Glo sensor and cAMP production was assayed with a gradient of SKF 81297 result showed

that in the absence of β-arrestin 1&2 the mutant loses the cAMP production difference previously observed between D_1 WT and the palmitoylation mutant, in fact the difference in cAMP production is nullified with no statistically significant impact on pEC_{50} (**Fig.27& table 14**). This suggested that arrestins play a major role in driving the cAMP production differential between D_1 WT and the palmitoylation mutant. To validate this hypothesis, a phenotype rescue experiment was performed where D_1 WT or the mutant with the Glo sensor were transfected with β-arrestin 1&2 or an empty vector plasmid and assayed for cAMP the results demonstrated that restoring β-arrestin 1&2 expression rescued the cAMP production phenotype of the wild type receptor previously observed in HEK293 normal cells (**Fig.27 B, C& table 14**).

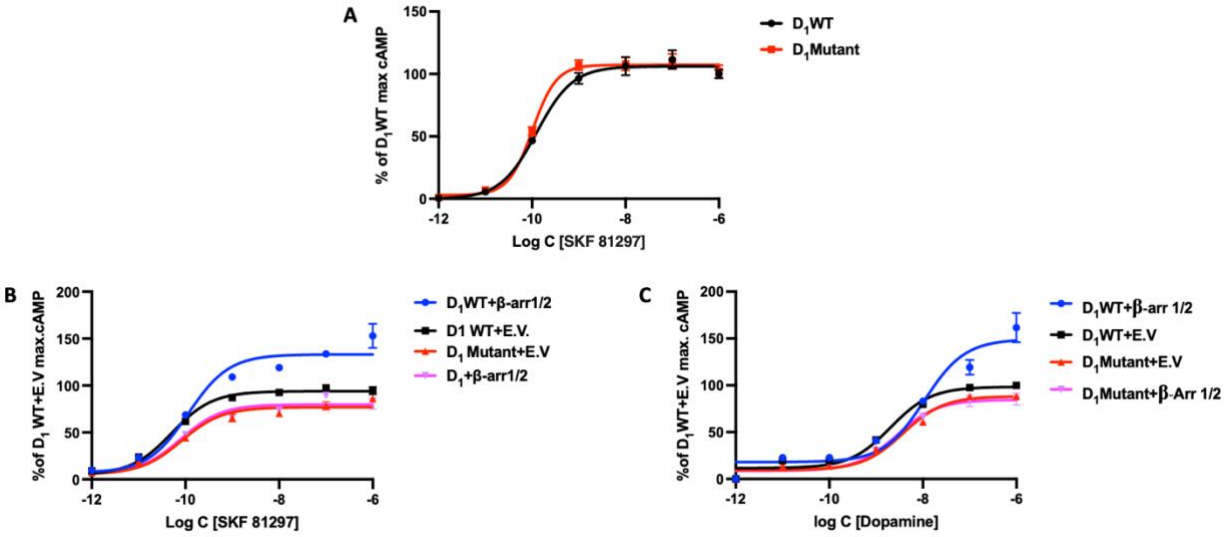


Figure 27- Functional characterisation of D_1 in HEK 293 KO cells for β-arrestin 1&2

D_1 WT and D_1 palmitoylation deficient mutant cAMP production assay performed in HEK 293 KO cells for β-arrestins 1&2 ***(A)*** *concentration response of cAMP production a gradient of the D_1 agonist SKF 81297. (**B**)&(**C**)HEK 293 KO cells for β-arrestins 1&2 were transfected with D_1 WT or D_1 palmitoylation mutant with β-arrestins 1&2or an empty vector control. Values are mean ± S.D. of n = 3 independent experiments of D_1 WT (A) or D_1 WT+EV maximum cAMP production percentage normalised to vehicle-treated cells and basal reading.*

Table 14- Statistical evaluation of cAMP production profile of D_1 WT and the palmitoylation mutant in HEK 293 β-arrestin1&2 KO cells

Dopamine	**pEC_{50}**	**E_{max}**
D_1 WT+ β-arrestin 1&2	7.97 ±0.93	**149.2 ±11.3****
D_1 Mutant+ β-arrestin 1&2	8.56 ±0.55	84.59 ±10.24
D_1 WT+ E.V	8.70 ±0.56	98.4 ±6.5
D_1 Mutant+ E.V	8.42 ±0.44	88.3 ±9.2
SKF 81297		
D_1 WT+ β-arrestin 1&2	9.94 ±0.56	**133.2 ±7.95****
D_1 Mutant+ β-arrestin 1&2	10.1 ±0.65	79.83 ±10.38
D_1 WT+ E.V	10.29 ±0.26	89.07 ±5
D_1 Mutant+ E.V	10.09 ±0.57	94 ±5.24
D_1 WT	9.90 ±0.1	106.1 ±4.32
D_1 Mutant	9.99 ±0.1	107.3 ±4.25

*Concentration responses shown in **Fig.27** were analysed by nonlinear regression using Prism, and the pEC_{50}, and E_{max} values were derived. Statistical significances of the differences with WT+E.V control or WT were determined using unpaired Student's* t *test (Values are representative of n of at least 3 independent repeats performed in triplicate).*

IV. Discussion & conclusion

The data demonstrated a 50% decrease in cAMP production for D_1 palmitoylation mutant compared to D_1 WT, in line with Jin et al. findings (Jin et al., 1999). Furthermore, the D_1 palmitoylation mutant decreased cAMP production was coupled to a reduced capacity to promote ERK1/2 phosphorylation. Interestingly, in HEK 293 β-arrestin 1&2 KO cells, the mutant's decreased cAMP production compared to WT was nullified. However, restoring β-arrestin 1&2 expression rescued D_1 WT phenotype. The data seem to indicate that arrestin is required for D_1 cAMP

response; this is supported by new studies on the biochemical basis of GPCR G protein activation that are looking at the subcellular organisation of GPCR signalling (Calebiro & Koszegi, 2019; Lobingier & von Zastrow, 2019; Sutkeviciute & Vilardaga, 2020).

GPCRs are not confined to the plasma membrane, they transit in the endocytic pathway (Hanyaloglu & von Zastrow, 2008). GPCR cellular cAMP signalling can be determined by many factors, including the ligand's binding affinity for receptors (Feinstein et al., 2013; Ferrandon et al., 2009) and specific features of the receptor's trafficking itinerary (Sposini et al., 2017) and β-arrestins binding at the GPCR carboxy terminus can co-exist with G protein binding in endosomes, which sustains G protein signalling inside the cell (Thomsen et al., 2016). One important factor is the interaction between GPCRs and β-arrestin, they were discovered as scaffolding proteins which are recruited to and functionally desensitise activated receptors at the plasma membrane (Gurevich & Gurevich, 2019). Additionally, they serve as essential endocytic adaptor protein for many GPCRs, promoting receptor endocytosis via clathrin-coated pits and driving receptor delivery to endosomes (Moo et al., 2021). However, endocytosis was long believed only to impact the longer-term homeostatic regulation of GPCRs and not affect the response to acute agonist application. This view has changed due to the accumulation of evidence that various GPCRs can engage G proteins after endocytosis, as well as from the plasma membrane, and can leverage the endocytic network to promote or sustain cellular signalling (Calebiro & Koszegi, 2019; Lobingier & von Zastrow, 2019; Sutkeviciute & Vilardaga, 2020). For GPCRs that signal by coupling to stimulatory heterotrimeric G_s, a number of them have now been shown to engage G_s on the endosome as well as the plasma membrane, enabling receptors to initiate signalling from each location (Calebiro et al., 2009; Calebiro &

Koszegi, 2019; Feinstein et al., 2013; Irannejad et al., 2013; Sutkeviciute & Vilardaga, 2020). This highlights how spatiotemporal aspects of GPCR activation can profoundly influence cAMP production. However, how such signalling diversity is programmed remains poorly understood. Moreover, recent work using HEK293 cells in which β-arrestin1 and β-arrestin 2 are stably knocked out to delineate D_1 pharmacology and signalling mechanisms, Dose responses with the D_1 agonist SKF-81297 in β-arrestin1/2 KO impaired D_1 desensitisation (Jain et al., 2020).

In addition, when looking at the effect of DHHC enzymes overexpression on D_1 cAMP signalling, the co-expression with both D_1 WT and the mutant served to underscore the intricacies of post-translational modification regulation. In retrospect, a Forskolin control for all cAMP production experiments would have been of use to make sure there is no system production saturation. Also performing the ERK1/2 phosphorylation assay in the HEK 293 β-arrestin 1&2 KO cells with a phenotype rescue and delineating the impact of β-arrestin 1 from 2 on D_1 WT.

However, studying palmitoylation faces challenges due to a lack of defined and characterised pharmacological inhibitors. Moreover, the mutation of palmitoylated cysteines may have unintentional consequences on other cysteine modifications, such as redox modifications, S-glutathionylation and S-nitrosylation, as it is typically solvent exposed cysteines that participate in palmitoylation and are thus open to additional post-translational modifications (Main et al., 2021). DHHCs can show some form of redundancy, limiting the information gained from individual knockout or over-expression models on their function and any subsequent change in substrate behaviour. Moreover, pharmacologically inhibiting selected DHHCs for shorter periods would be very instructive. Nevertheless, DHHCs-specific inhibitors targeting the active site have yet to be fruitful. This is due to the lack of structural information

on these proteins for example, DHHC inhibitor 2-bromopalmitate (2-BP). 2-BP, which is thought to irreversibly alter the DHHC active site cysteine through nucleophilic displacement and alkylation, has been widely used to determine the effect of reducing palmitoylation on proteins of interest (Jennings et al., 2009). However, there are several caveats to its use, including poor potency and bioavailability and extensive off-target effects, suggesting up to 99% of its targets are not DHHCs (Chase & Tubbs, 1972; Jennings et al., 2009). Mass spectrometry supports this, suggesting it does not favour DHHCs over other proteins (Davda et al., 2013; Draper & Smith, 2009; B. Zheng et al., 2013). Another example of a palmitoylation inhibitor is the antibiotic tunicamycin, which has been shown to inhibit the palmitoylation of calcium channels (Hurley et al., 2000) and presynaptic plasticity protein GAP-43.

This chapter's findings put forward the question of arrestin 1&2 interactions with the palmitoylation mutant and their impact on ERK1/2 phosphorylation, as previous studies reported that knocking out β-arrestin 1&2 impacted ERK1/2 phosphorylation (Urs et al., 2011). Moreover, the data presented in this chapter indicate that loss of palmitoylation of D_1 might be having the same effect that previous studies already characterised for knocking out β-arrestin 1&2 on D_1 as it reduced its ERK1/2 phosphorylation (Bono et al., 2023) and impaired its desensitisation.

GPCRs can be regulated by: desensitisation, in which the receptor loses its ability to signal in the presence of stimuli; internalisation, which lowers the number of receptors on the cell surface; and degradation, which lowers the total level of receptors inside the cell (Krupnick & Benovic, 1998), exploring the effects of loss of palmitoylation on D_1 G protein coupling and β-arrestin 1&2 recruitment becomes vital. In parallel, we need to identify the DHHCs responsible for the D_1 palmitoylation, since pharmacological approaches are not the best suited; thus, we will use techniques aimed

at the study of protein-protein interactions such as Co-IP (Co-immunoprecipitation) and Bioluminescence resonance energy transfer as they are valuable approaches to overcome the lack of pharmacological tools.

Chapter 4: investigating D_1 loss of palmitoylation effects on G protein activation, DHHCs and arrestin interactions

I. Introduction

GPCR studies have historically heavily relied on radioligand-binding assays to study the pharmacology of the receptors (Beerkens et al., 2022; Zhang & Xie, 2012). The use of biochemical methods, like SDS-PAGE, cysteine cross-linking and co-immunoprecipitation, were the first employed assays to study GPCRs in tissues and cell models (Avissar et al., 1983; Bai et al., 1998; Fraser & Venter, 1982; Limbird et al., 1975; Rogers, 1984). However, these methods relied on membrane solubilisation using detergents under denaturating conditions such as boiling or relying on poor-quality antibodies and tagged constructs, thus prone to artefactual results (Guo et al., 2017). Even though these biochemical tools contributed to uncovering many GPCRs related mechanisms (Maziarz et al., 2020; Mo & Fu, 2016; Wan et al., 2018), they lacked precision and presented the inconvenience of not being adapted to high throughput screening. The development of biophysical approaches such as protein complementation and BRET have provided useful tools to understand GPCR pharmacology and related mechanisms (signalling, kinetics, protein-protein interactions…) in cells, tissues, and whole organisms.

Previous studies in GPCRs indicated that the proximal part of the C-terminus between TM7 and the palmitoylation site forms the short helix 8 parallel to the plane of the membrane. Since helix 8, along with the ICLs, is part of the cytoplasmic "face" of GPCRs that is recognised by intracellular signal transducers. Not surprisingly, helix 8 was shown to play a role in arrestin-1 binding (Kirchberg et al., 2011). Interestingly, biophysical studies using fluorescently labelled vasopressin V2 receptor (Rahmeh et al., 2012) and β2AR (Liu et al., 2012) found that G protein-biased agonists perturb TM6 and arrestin-biased agonists perturb TM7 and helix 8, whereas unbiased agonists

induce both perturbations. Furthermore, the D_1 receptor mutated in helix 8 displays enhanced G protein signalling but reduced arrestin-mediated desensitisation (Yang et al., 2019). These data suggest that helix 8 and TM7 play a major role in arrestin binding. Indeed, helix 8 of rhodopsin was found to contact the finger loop of arrestin-1 in the complex (Y. Kang et al., 2015). Similarly, T491 and T360 on the C-terminus of M2R and V2Rpp, respectively, establish interactions with R25 (N-domain) and K294 (gate loop) of arrestins (Staus et al., 2020).

Experimental data identifying the receptor residues participating in arrestin binding are incomplete in the case of the dopamine D_1 receptor (Kaya et al., 2020) and virtually absent for hundreds of other GPCRs. The fact that palmitoylation can impact both TM7 and helix 8, it became evident that the impact of D_1 loss of palmitoylation on β-arrestins recruitment and G protein activation needed to be investigated.

II. Outlines & Aims

In the previous chapter, investigating the effects of D_1 loss of palmitoylation on its cAMP production and downstream ERK1/2 phosphorylation led to question and hypothesise whether loss of palmitoylation has an impact on D_1 protein interactions such as β-arrestin 1&2 and G protein recruitment. In addition, uncovering the DHHCs involved in D_1 palmitoylation would be a breakthrough as they are yet to be identified. To this end, BRET and Luciferase protein complementation assays (**Fig.28**) were used to characterise the effects of loss of palmitoylation on D_1 G protein activation by TRUPATH BRET2 (**Fig.29**), β-arrestin 1&2 recruitment and identify the DHHCs responsible of palmitoylating D_1.

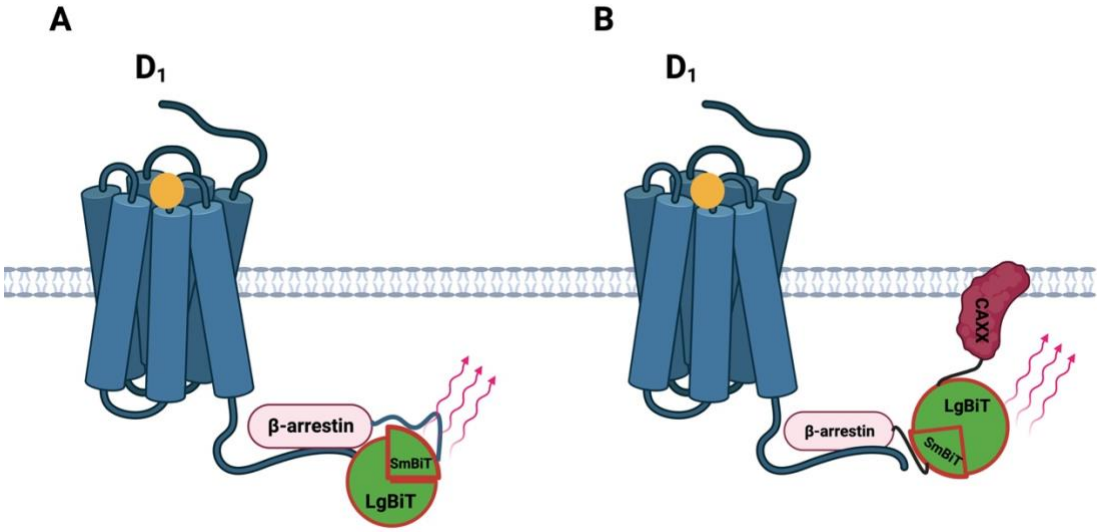


Figure 28- Schematic of the NanoBiT complementation assay for measuring D_1 β-arrestin 1&2 recruitment.

Engineered split-luciferase system to detect Protein-protein interaction between D_1-LgBiT and SmBiT-β-arrestin 1&2 ***(A)*** *or indirectly between D_1 and SmBiT -β-arrestin 1&2 Via their interaction in a Bystander fashion with a Plasma membrane marker the LgBiT-CAXX probe* ***(B).*** *Genetically fusing the proteins of interest to the two split components of NanoLuc, termed LgBiT and SmBiT. These chimeric proteins are then expressed in cells and interactions between them are detected by the addition of a cell-permeable furimazine substrate that is converted to light by the reconstituted NanoLuc. As the NanoBiT components have negligible affinity for one another (~190 μM), their association and the subsequent reconstitution of functional NanoLuc are dependent on the PPI being studied.*

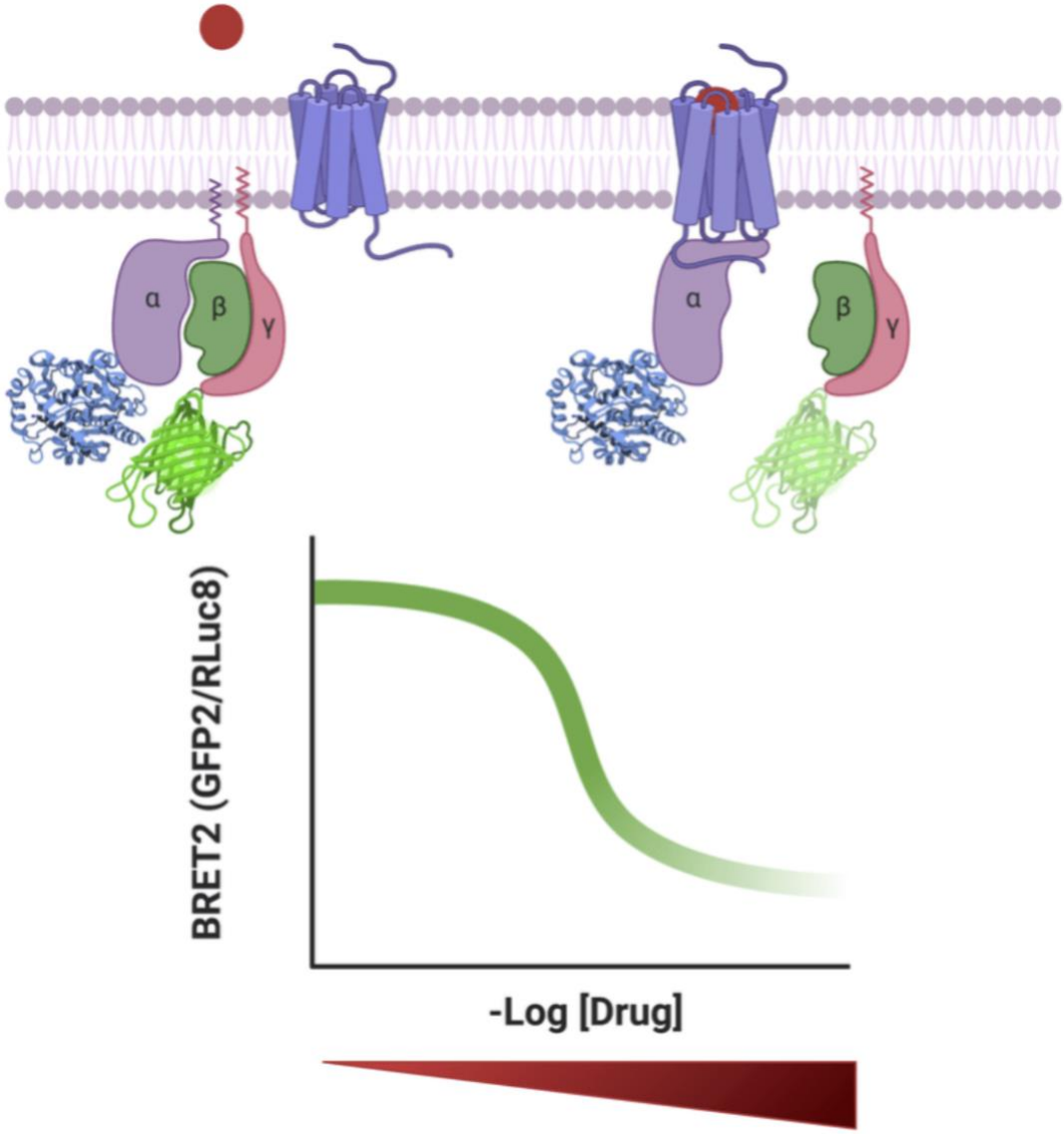


Figure 29- TRUPATH measures heterotrimeric G protein dissociation by bioluminescence resonance energy transfer 2, or BRET2.

The TRUPATH BRET2 biosensor system was developed by constructing luciferase donor Rluc8-Gα chimaeras, acceptor GFP2-Gγ chimaeras, and untagged Gβ constructs. For the Gγ constructs, the GFP2 tag is fused to the N-terminus of the protein. For the Gα constructs, the localisation of the Rluc8 was optimised by protein engineering and experimental refinement to determine the optimal localisation of the Rluc8 insert within the Gα. With the binding of the drug, the alpha and beta subunits dissociate resulting in decreased BRET2 signal. With increased concentration of the drug, the BRET2 signal decreases.

III. Results

1. Loss of palmitoylation impairs D_1 β-arrestin 1&2 recruitment

After D_1 agonist activation and G protein complex dissociation is the recruitment of β-arrestin 1&2, with β-arrestin 2 being mainly responsible for D_1 mediated ERK1/2 phosphorylation and desensitising the receptor (Kaya et al., 2020; Y. Yang et al., 2022).

D_1-LgBiT constructs were generated by Gibson assembly and, as previously found they had a similar cAMP profile to D_1 WT and Palmitoylation mutant (**Fig.30A**). The split Nanoluc system was used to investigate D_1-LgBiT constructs recruitment of β-arrestin 1&2 tagged SmBiT. If arrestins are recruited to D_1, the functional Nanoluc will be reconstituted, and in the presence of Furimazine, its substrate, light will be emitted. With β-arrestin 1&2, the mutant could not exhibit any statistically significant recruitment, and no concentration response could be derived. As for D_1 WT, it was able to recruit both β-arrestin 1&2 (**Fig.30B**). One primary concern was that the LgBiT tag on D_1 influenced β-arrestin 1&2 recruitment in comparison to D_1 untagged C-terminally as the pEC50 for the WT was 9.34 and 10.92 for β-arrestin 1 and 2 respectively in comparison to a 7.8 in literature for β-arrestin 2 (Conroy et al., 2015). Therefore, to rule out a potential effect of the LgBiT tag of the D_1 on its arrestin , we used another approach to monitor arrestin recruitment using a plasma membrane-specific Probe LgBiT-CAXX (Janetzko et al., 2021). When arrestins are recruited to the untagged receptor, they will come in proximity of the LgBiT-CAXX, and the functional Nanoluc will be reconstituted and can emit light. The results demonstrated that loss of palmitoylation still reduces significantly β-arrestin 1&2 recruitment to D_1 with a reduction of 70 and 64% for β-arrestin 1&2 respectively, and the pEC50 for

D_1 WT were comparable to what is reported in the literature for β-arrestin 2 recruitment stimulation by Conroy& Sibley (Conroy et al., 2015).

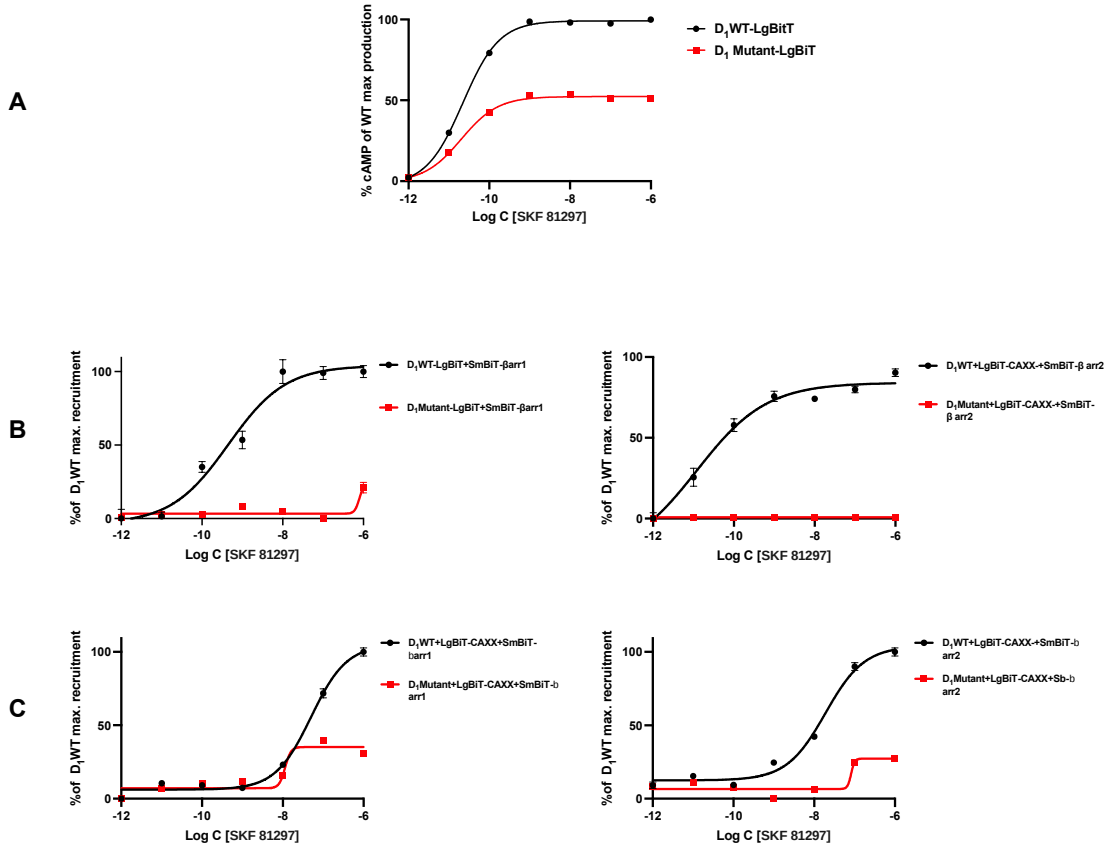


Figure 30- Effects of loss of palmitoylation on D_1 β-arrestin1&2 recruitment

*Agonist-mediated ß-arrestin 1&2 recruitment plotted as dose-dependent recruitment of β-arrestin 1&2 to D_1 WT and D_1 palmitoylation deficient mutant. (**A**) D_1 WT and D_1 palmitoylation deficient mutant tagged with LgBiT cAMP production assay performed in HEK 293 cells. Concentration response of cAMP production, cells were treated respectively, with vehicle or a gradient of the D_1 agonist SKF 81297. (**B**) luminescence in response to serial dilution of D_1 agonist SKF-81297 over vehicle in HEK293 cells transfected with either 12.5 µg D_1-LgBiT WT (black) or palmitoylation mutant (red) and with either 2.5 µg of SmBiT-βarrestin1 or βarrestin2-SmBiT. (**C**) The concentration response of luminescence was measured in response to a serial dilution of D_1 agonist SKF-81297 or vehicle in HEK293 cells transfected with either 50 µg D_1 WT (black) or palmitoylation mutant (red) and with either 2.5 µg of SmBiT-βarrestin1 or βarrestin2-SmBiT and 12.5 µg of LgBiT-CAXX probe. Data are mean ± SEM percentage of activation normalised to agonist-induce maximal response of four individual experiments each performed in triplicate. Dose-response curves were analysed by nonlinear regression using Prism.*

Table 15- Statistical evaluation of D_1 β-arrestin1&2 recruitment

A	pEC_{50}	E_{max}
D_1 WT-LgBiT	10.66 ±0.14	99.19 ±3.2
D_1 Mutant-LgBiT	10.7 ±0.33	**52.38 ±2.07*****

B		
D_1 WT-LgBiT (β-arrestin1)	9.34 ±0.5	104.1 ±18.1
D_1 Mutant-LgBiT (β-arrestin1)	-	-
D_1 WT-LgBiT (β-arrestin2)	10.92 ± 1	93.18 ± 14.1
D_1 Mutant-LgBiT (β-arrestin2)	-	-

C		
D_1 WT (β-arrestin1)	7.3 ±0.38	98.78 ±12.69
D_1 Mutant (β-arrestin1)	7.94 ± 0.26	**28.09 ±6.33****
D_1 WT (β-arrestin2)	7.72 ±0.43	91.49 ±5.94
D_1 Mutant (β-arrestin2)	7.08 ±0.54	**27.3 ±12.11****

*The concentration responses shown in **Fig.30** were analysed by nonlinear regression using Prism, and the pEC_{50}, and E_{max} values were derived. Statistical significances to WT with the same condition of transfection were determined using an unpaired Student's* t *test.*

2. Loss of palmitoylation reduces D_1 trimeric G protein activation in HEK293 cells but not HEK 293 β-arrestin 1&2 KO cells

The changes in signalling at the cAMP and ERK1/2 level suggest a difference in G protein-coupling. D_1 has been described previously to couple to Gαs G proteins (Moritz et al., 2023). Gαs coupling leads to increased cAMP production. The effects of loss of palmitoylation on D_1 palmitoylation mutant reduced cAMP accumulation, and ERK1/2 phosphorylation could therefore be through a decrease in Gαs coupling. Using the TRUPATH biosensor platform, the effect of D_1 WT or the palmitoylation mutant on agonist-mediated $G\alpha_{sL}$ (GαsLong) dissociation was investigated. HEK293 cells were transfected with GαsL-Rlu8, Gβ1, and Gγ1- GFP2, and stimulated with SKF 81297 in the concentration range 1 μM to 1 pM SKF 81297 was used as the stimulating agonists due to its high potency in cAMP accumulation.

The results showed a reduced decrease of BRET percentage for the D_1 palmitoylation mutant compared to D_1 WT of 30% in HEK 293 cells after stimulation with a gradient of concentration of SKF81297 (**Fig31A, Table 16)**. In contrast, no statistically significant difference was observed in in HEK 293 β-arrestin 1&2 KO cells (**Fig31B, Table 16)**.

The data suggests that the reduced cAMP production observed for the D_1 palmitoylation (**Fig.24**) mutant is potentially due to a reduced ability to drive G protein activation after agonist stimulation. How this might occur is unclear, but it is tempting to speculate that the change in flexibility of helix 8 due to not being "pinned" closer to the plasma membrane might influence the receptor's ability to drive G-protein activation. In studies of the Beta-Adrenergic receptors, the c-tail has been implicated in influencing G-protein association, supporting a role for the c-tail in G protein association/activation. Interestingly, this difference in G protein dissociation was not observed in cells lacking β-arrestin 1&2 (**Fig.31B, Table 17**). These results imply that D_1 needs to recruit β-arrestins to signal properly and activate its associated G protein.

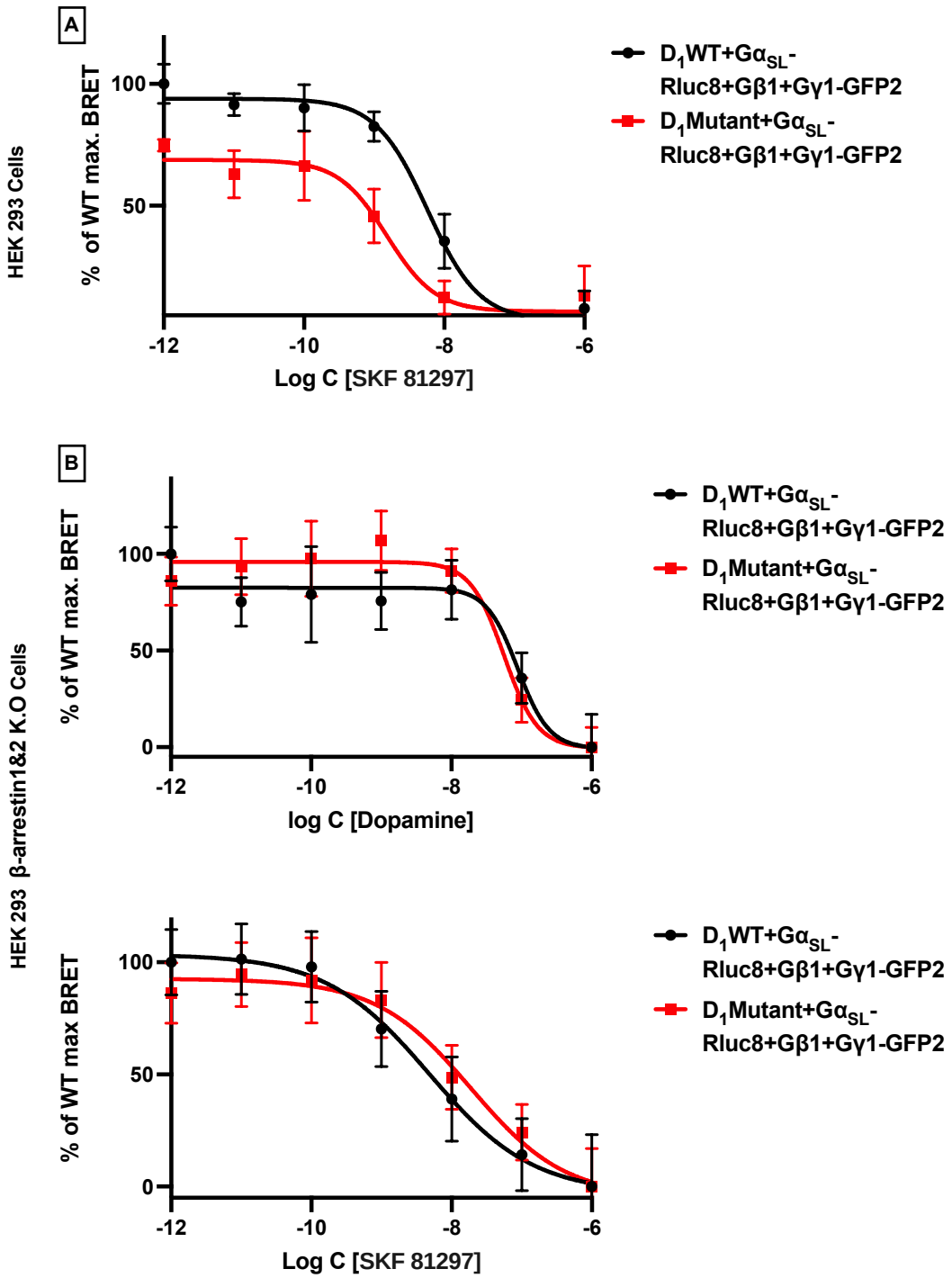


Figure 31- Characterising the effects of D_1 loss of palmitoylation on its Gαs *subunits dissociation from its trimeric G protein complex.*

*WT D_1 and palmitoylation deficient mutant D_1 BRET TRUPATH assay in HEK 293 cells(**A**) and HEK293* β-arrestin 1&2 K. *Cells (**B**) were transfected with (1:1:1:1) ratio of receptor: GαSL-Rluc8: Gβ1: Gγ1-GFP2. Rluc8 being the BRET donor and GFP2 acting as the BRET acceptor were treated, respectively, with vehicle treatment and a gradient of the D_1 agonist SKF 81297 or dopamine. Net BRET was plotted as a percentage of D_1 WT maximal BRET signal (n = 4 independent experiments).*

Table 16- Statistical evaluation of the effects of D_1 loss of palmitoylation on its Gαs subunits dissociation from its trimeric G protein complex

Cell type	Agonist	Receptor	pEC50	E_{max}
HEK 293	**SKF 81297**	**D_1 WT**	8.27 ±0.35	95.91 ±7.9
		D_1 Mutant	8.9 ± 0.73	66.78 ±8.01*
***HEK293* β-arrestin 1&2 KO**	**SKF 81297**	**D_1 WT**	8.31 ±0.42	103.6 ±13.2
		D_1 Mutant	7.89 ±0.57	92.6 ±11.1
	Dopamine	**D_1 WT**	7.56 ±0.73	83.45 ± 12.2
		D_1 Mutant	7.25 ±0.65	92.8 ±15.3

*The percentages of BRET shown in **Fig.31** were analysed by nonlinear regression using Prism, and the pEC_{50}, and E_{max} values were derived. Statistical significances to WT with the same condition of transfection were determined using an unpaired Student's* t *test.*

3. Investigating D_1 Protein-Protein interaction with DHHCs

To identify potential DHHCs that could be involved in the palmitoylation of D_1, we screened a panel of DHHC enzymes for their ability to interact with D_1 by co-immunoprecipitation. HEK 293 cells were transfected with Flag-D_1 and available HA-DHHC family members. To determine the transfection efficiency for the DHHC, an equal amount of total cell lysate was loaded into each well and blotted for -HA. DHHC expression was not uniform across the family members (Pre-IP) (**Fig.32**). A Co-IP was performed to elute the Flag-D_1 with their interacting protein partners. The samples were then resolved by SDS-PAGE electrophoresis followed by immunoblotting using an anti-Flag antibody (**Fig. 32**). The western blot suggested that D_1 WT can bind to several DHHCs, at least in an overexpressed setting.

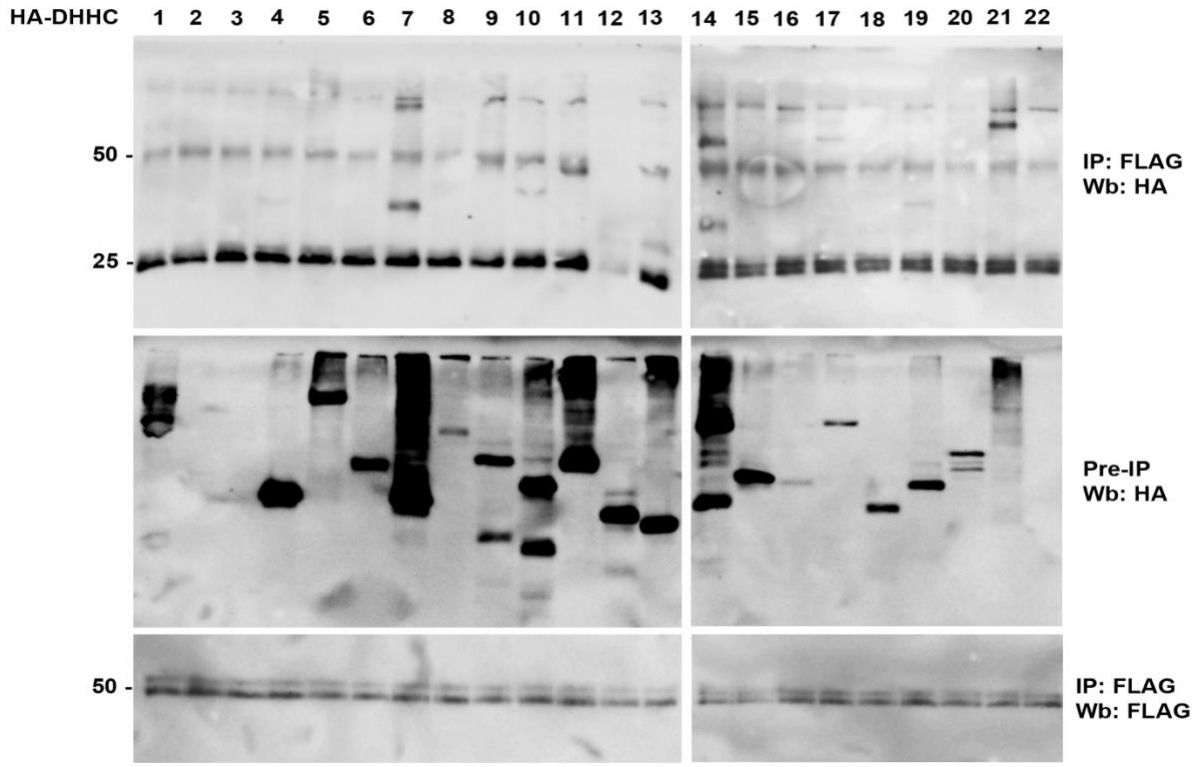


Figure 32- Co-IP of D_1 WT with various DHHCs. (Etienne S.)

Co-immunoprecipitation (Co-IP) assays showing interactions of FLAG-D1 WT with 22 HA-DHHCs found in humans (DHHC1–22). The cells were lysed then subjected to immunoprecipitation with anti-Flag antibodies and then blotted with anti-HA antibodies to look for which DHHCs can co-precipitate. As a control for transfection and expression, we also re-blotted for D_1 WT with anti-Flag (bottom panel). The middle panel shows that each DHHC is expressed. Co-precipitating DHHCs can be observed in the upper panel and include 1, 4, 7, 11, 14, 17, 19 and 21.

Although the co-IP screen implicated several DHHCs in their ability to interact with D_1, we wanted to validate these interactions using an alternative approach. To accomplish this, we used BRET assays using D_1 C-terminally tagged with Rluc8 and DHHC tagged with GFP10 constructs provided by Dr Stephane Lefrancois. In this assay, if D_1 is found at an interacting distance with the DHHC, we expect energy transfer to occur in the presence of Coelenterazine 400a, the substrate of Rluc8. Then we proceeded to try and validate our potential interactions by BRET (**Fig.33**). Our data supported a potential interaction between DHHC9 and the D_1 WT compared to

the D_1 palmitoylation mutant that did not show interaction with any DHHC (**Fig.33, Table 17**).

A saturation BRET was performed To define if the interaction between D_1 WT and DHHC 9 is specific, where the amount of BRET donor D_1-Rluc8 construct was kept constant, and the amount of BRET acceptor DHHCs-GFP10 was titrated. Suppose the BRET signal comes from random collisions. In that case, the signal should increase linearly (Besson et al., 2022), such was the case for DHHC7 (**Fig.34B**). On the other hand if a protein pair are interacting, the net BRET increases as a hyperbolic function reaching saturation as the limited amount of donor protein will not be able to bind with all the excess amount of acceptor protein (Sauvageau & Lefrancois, 2019)which was the case for D_1 WT and DHHC 9 (**Fig.34A**). Taken together, our co-IP and BRET experimental results both suggest that DHHC 9 can interact with D_1 WT.

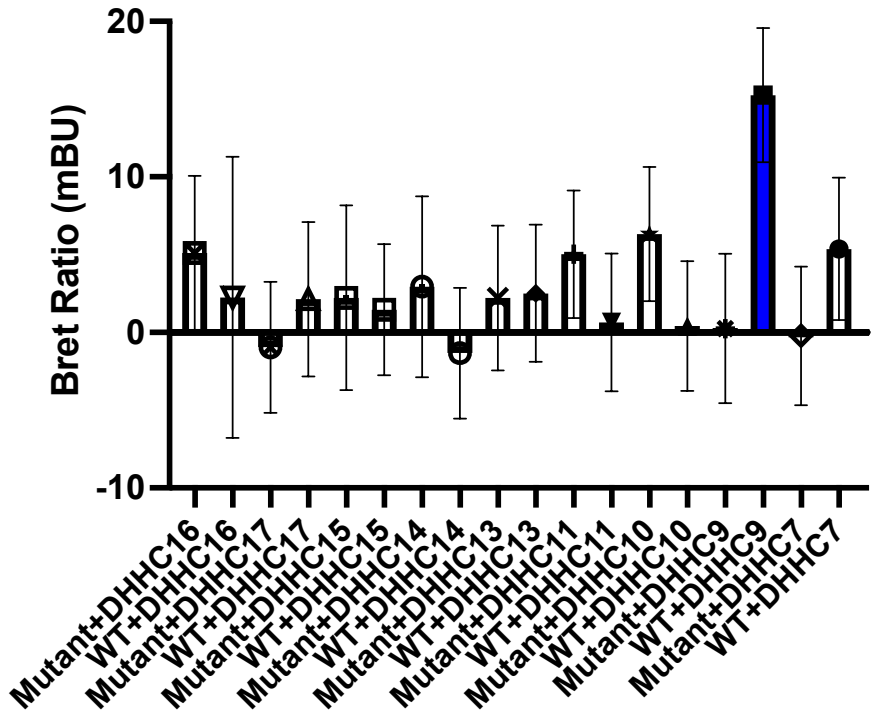


Figure 33- Screening for interaction Between D_1 and available DHHCs-GFP10

WT D_1 and palmitoylation deficient mutant BRET with DHHCs-GFP10, assay in HEK 293 cells. Cells were transfected with a (1:1) ratio of receptor: D_1-Rluc8: DHHC's-GFP10 or empty vector. Rluc8 is the BRET donor and GFP10 acts as the BRET acceptor. Net BRET was obtained by normalising the raw BRET values to the BRET of donor only (n = 3 independent experiments).

Table 17- Statistical evaluation of the BRET between D_1 and the DHHCs-GFP10 of figure 33.

	D_1 WT-Rluc8	**D_1 Mutant-Rluc8**
DHHC7-GFP10	2.36±04.57	-0.2±4.45
DHHC9-GFP10	**16.3±4.3***	**0.21±3.6**
DHHC10-GFP10	0.4±4.2	0.4 ±4.1
DHHC11-GFP10	0.64±4.43	0.64 ±4.3
DHHC13-GFP10	2.51±4.41	2.5±4.41
DHHC14-GFP10	-1.3±4.2	-1.3±4.21
DHHC15-GFP10	1.45±4.3	2.22±5.93

DHHC16-GFP10	2.5±9	5.11±4.96
DHHC17-GFP10	2.2±4.97	-0.95±4.21

BRET value represented in Fig.33 in mBU (milliBRET units) statistical significances to WT were determined using unpaired Student's t *test.*

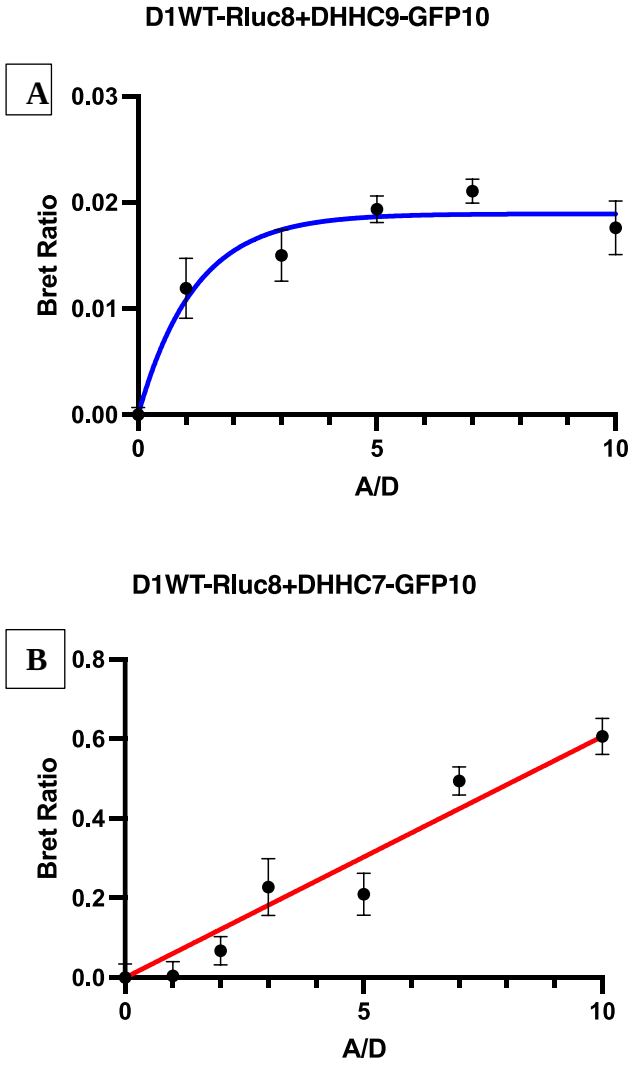


Figure 34- Detection of spontaneous interaction between D_1 WT and DHHC 9&7 by BRET titration experiments.

*The existence of an interaction between D_1 WT and DHHC9 validation using quantitative BRET saturation curves assay in HEK 293 cells co-transfected with a constant amount of D_1 WT-Rluc8 plasmid and an increasing amount of the DHHC 9 (**A**) or 7(**B**)-GFP10 plasmid. BRET signals were plotted as a function of the expression ratio of receptorDHHC-GFP10 over D_1-Rluc8 (n = 3). The amount of receptor effectively expressed in transfected cells was monitored for each individual experiment by correlating both total luminescence and total fluorescence. While*

D_1WT-Rluc8/DHHC 7-GFP10 yields a straight line, D_1WT-Rluc8/DHHC 9-GFP10 yields a clear hyperbola.

IV. Discussion & Conclusion

This chapter explored the impact of palmitoylation on the ability of D_1 to signal. Previous studies using a palmitoylation deficient receptor had already indicated reduced cAMP production using single endpoint assays (H. Jin et al., 1999). Here, we revisited this using a real-time assay reaffirmed the diminished cAMP production in the D_1 palmitoylation mutant, consistent with existing literature. Further examination of the receptor ability to activate G-protein revealed a significantly reduced ability of the mutant receptor to activate G-protein. Suggesting a potential link between palmitoylation and alterations in membrane interaction or conformation of the cytosolic loop housing the palmitoylated cysteine. For instance, the C-terminal tails of AMPAR or NMDAR (ionotropic glutamate receptors) are palmitoylated, enabling attachment of the receptor tail to the plasma membrane, therefore enabling their interaction with downstream signalling protein partners that are important for the signalling activity of these receptors (Hayashi, 2021).

Palmitoylation, is executed by a family of acyl transferases, plays a crucial role in receptor function. Screening several members of this enzyme family identified a potential interaction with multiple DHHCs. Subsequent BRET interaction screening narrowed down the potential involvement to DHHC 9, localised in the Golgi apparatus (Mansilla et al., 2007; Swarthout et al., 2005). This finding positions DHHC 9 as a plausible candidate implicated in D_1 palmitoylation.

Also the data are promising to support this, the Co-Ip can benefit from appropriate controls, such as a D_1 control with a given amount of purified recombinant protein,

this would give a single defined clean band which could be used as a reference and included aliquots of all the supernatants, washes and flow through to make sure the immunoprecipitation is specific not elution dependant, and that the targeted D_1 not being lost in any other fractions, one last useful control would be to load a lane where the beads have been denatured to release the antibodies, to detect bands that might be coming from the light and heavy chains of the primary antibody this will ensure that the antibody was efficiently crosslinked to the beads. The TRUPATH experiment, investigating G protein interaction in HEK293 β-arrestin 1&2 KO cells, could benefit from a phenotype rescue control. Reinstating β-arrestin 1&2 expression could elucidate whether the observed phenotype in HEK293 is reinstated, providing valuable insights into the role of β-arrestin in the observed cAMP result. Finally, the BRET in the saturation assay using a fixed ratio of donor and variable acceptor amount can significantly be affected, in particular for high A/D plasmid ratios, such a problem defeats one of the main interests of the fix donor method, developed to determine the homomeric stoichiometry of proteins based on a single variable (James et al., 2006) as a decrease of expression of the Rluc-8 at the highest concentration of acceptor can occur and affect the energy transfer events. Therefore, using a variable expression of both donor and acceptor, BRET saturation assays provide a better range for acceptor detection and improve the robustness of the assay. Alternatively, validating D_1 loss of palmitoylation in HEK293 DHHC 9 KO cells could provide additional insights.

The data strongly indicate that the lack of palmitoylation influences receptor function. However, D_1 agonist-induced palmitoylation needs to be evaluated to understand if D_1 interaction with DHHC9 or other DHHCs is modulated by receptor activation, as it was reported in the literature that some GPCRs such as the serotonin receptor 5-HT_7

and adrenergic receptor β2AR (Kvachnina et al., 2009; O'Dowd et al., 1989) can undergo agonist-mediated palmitoylation.

The requirement for β-arrestin 1&2 in the cAMP result is interesting and suggests a few different possibilities. The first is the possibility of the formation of some super-complex involving receptors, arrestins, and G-proteins (Thomsen et al., 2016). Alternatively, this could be due to a trafficking event that requires β-arrestins. The upcoming chapter aims to delve into the trafficking disparities between WT and mutant D_1, examining the potential role of palmitoylation in receptor sub-cellular localisation.

Chapter 5: investigating D_1 loss of palmitoylation effects on its expression and trafficking

I. Introduction

More than 200 different post-translational modifications have been reported (Zmuda & Chamberlain, 2020). Palmitoylation and S-acylation refer to the same PTM because most protein acyl transferases that attach fatty acids to the SH group of cysteine residues prefer to use palmitoyl-CoA as a substrate, and palmitate is one of the most abundant fatty acids in cells (Lemonidis et al., 2017). Palmitoylation is reversible, with depalmitoylation being performed by acyl-protein thioesterases (Linder & Deschenes, 2007; Y. Peng et al., 2018). Therefore, This reversibility allows the organism to regulate its protein function and/or localisation via palmitoylation (Ko & Dixon, 2018; Zmuda & Chamberlain, 2020). DHHCs catalysing S-palmitoylation are polytopic membrane proteins, and most of them are found in the ER or the Golgi membranes. Depending on cell type, 2-3 of them localise to the plasma membrane (Chopard et al., 2018; Ko & Dixon, 2018).

Palmitoylation of a membrane protein can affect its localisation and, for instance, from the Golgi apparatus to the plasma membrane as for c-Met (D. T. Coleman et al., 2016), or from the plasma membrane to the nucleus or the mitochondria like for EGFR ((Bollu et al., 2014). Kong et al. demonstrated that palmitoylation of the D_1 receptor is critically involved in agonist-dependent receptor internalisation (Kong et al., 2011). Moreover, our results indicated that loss of palmitoylation impaired β-arrestin 1&2 recruitment to D_1. β-arrestins are master regulators of cellular signalling that operate by desensitising ligand-activated GPCRs at the plasma membrane and promoting their subsequent endocytosis. Receptor endocytosis was demonstrated to contribute to distinct cAMP signalling profiles for different GPCRs. Regulated trafficking of GPCRs has clear consequences for their signalling. The discovery of endosomal G

protein signalling indicates that receptor endocytosis and localisation to endosomal compartments contribute to “spatial bias” in GPCR signalling (Calebiro et al., 2009; Ferrandon et al., 2009). For example, inhibiting endocytosis partially decreases cAMP production by the activated $G\alpha_s$-coupled dopamine receptor D_1 and the β2-adrenergic receptor (Irannejad et al., 2013; Kotowski et al., 2011). Moreover, there is evidence of GPCR activation and signalling in the Golgi. Conformational biosensors, as well as mini-G protein biosensors which mimic Gα subunit interactions with active GPCR conformations (Nehmé et al., 2017), revealed activation of GPCRs Gαs proteins in the Golgi (Irannejad et al., 2017; Nash et al., 2019).

Given the pivotal roles of β-arrestin recruitment and palmitoylation in GPCR internalisation and trafficking, this chapter undertakes an in-depth investigation into the effects of palmitoylation on Dopamine D_1 receptor localisation and trafficking. The aim is to unravel the dynamic interplay between these molecular processes and their implications on GPCR functionality.

II. Outlines & Aims

Previously, in chapters 3 and 4, we reported that loss of D_1 palmitoylation reduced its cAMP production and downstream ERK1/2 phosphorylation. Moreover, this reduced signalling was coupled to an altered β-arrestin 1&2 and G protein recruitment. In addition, uncovering that DHHC9, which is located in the trans Golgi apparatus, was involved in D_1 palmitoylation.

Trafficking of receptors from or to the plasma membrane and their shuttling to specialised intracellular compartments are pivotal processes to maintain cellular homeostasis. The signal transduction of G protein-coupled receptors is closely regulated by endocytosis, targeting of receptors to endosomes and their sorting to

lysosomes or recycling to the plasma membrane (Di Fiore & von Zastrow, 2014; Posner & Laporte, 2010). To this end, bystander BRET, dynasore as a pharmacological agent that inhibits dynamin GTPase activity blocking dynamin-dependent endocytosis in cells (Macia et al., 2006), and Western blotting were used to look at the effect of agonist treatment on D_1 WT at various time points. In addition, the FACS technique was used to understand the effects of loss of palmitoylation on D_1 plasma membrane expression and trafficking to various subcellular compartments and its signalling effects (**Fig.35**).

Finally, conformational biosensors based on nanobodies have recently emerged as a powerful method that complements traditional signalling assays to study spatially restricted signalling. These nanobodies are a single protein domain derived from the antigen-binding region of heavy-chain only antibodies produced in camelid species (Manglik et al., 2017). Several generated nanobodies specifically bind the active conformation of a specific GPCR or family of GPCRs (Manglik et al., 2017) or recognise a nucleotide-free Gαs conformation as a readout of GDP exchange by the Gαs subunit of the activated G protein (Irannejad et al., 2013). Therefore, two nanobodies were selected as biosensors to visualise D_1 in its active conformation (Nb6B9) or the D_1-G protein complex (Nb37).

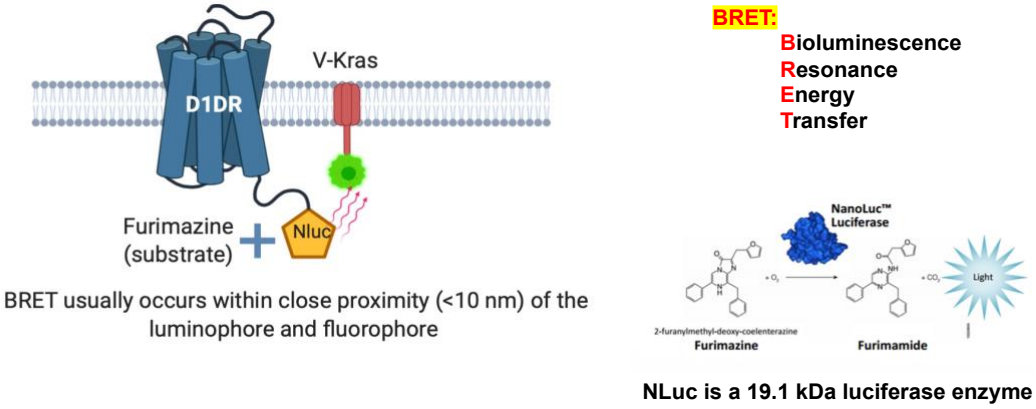


Figure 35- Bystander BRET principle

D_1-Nluc act as the BRET donor for a compartment marker tagged with Venus (Kras for example). When D_1-Nluc is within 10 nm of the acceptor in presence of Furimazine the substrate transfer of energy will occur.

III. Results

1. Dynasore reduces D_1 cAMP production

Dynamin has a function in membrane tubulation and fission and structuring vesiculo-tubular structures (Kirchhausen et al., 2008). It is vital for clathrin-dependent endocytosis from the plasma membrane, for the fission of caveolae in the plasma membrane to form free transport vesicles, and for vesicle formation at the trans-Golgi network (Cao et al., 2000; Corda et al., 2002; Nichols, 2003; Takei et al., 2005). Treating cells with dynasore inhibits clathrin-mediated endocytosis.

To assess the effects of D_1 loss of palmitoylation on its cAMP production in the absence of endocytosis, D_1 WT and the palmitoylation mutant were pre-treated with 80 µM of dynasore or vehicle and cAMP production was measured after addition of a gradient of concertation of SFK81297.

The results demonstrated that dynasore treatment drastically reduced cAMP production of D_1 WT and palmitoylation mutant compared to vehicle treatment by 73 and 35 % respectively (**Fig.36, Table 18**). This result highlighted that clathrin-mediated endocytosis is central to D_1 signalling regardless of its palmitoylation state. Taking into consideration that D_1 can signal intracellularly from the Golgi apparatus (Puri et al., 2022) and endosomes (G. E. Peng et al., 2021), exploring the effects of loss of palmitoylation on D_1 intracellular trafficking will be the focus of the future approaches.

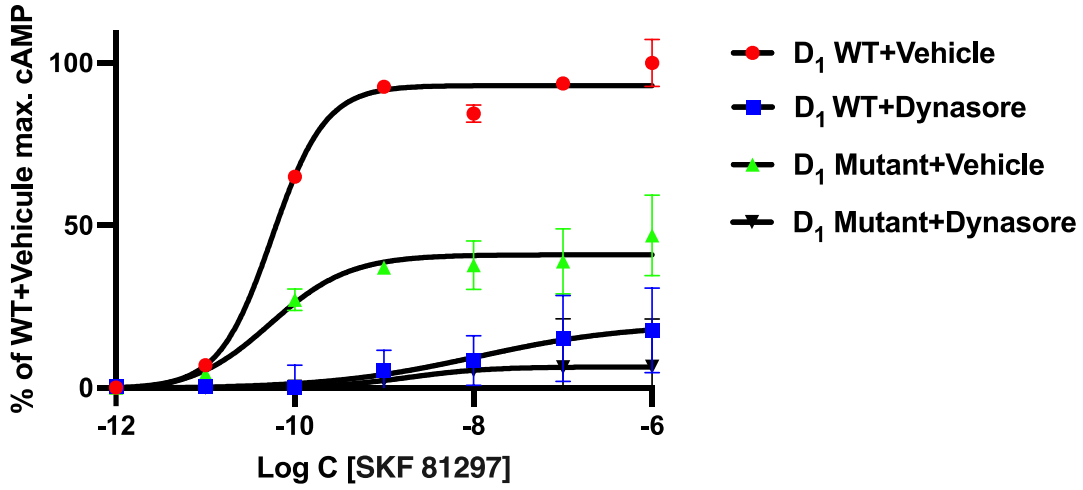


Figure 36- Dynasore effects on D_1 cAMP production

D_1 WT and D_1 palmitoylation deficient mutant cAMP production assay performed in HEK 293 cells. Concentration response of cAMP production where cells were treated, respectively, with vehicle and a gradient of the D_1 agonist SKF 81297 or 80 µM Dynasore and a gradient of SKF 81297. Statistical analysis of the cAMP production normalised to the D_1 WT+ vehicle production of cAMP.

Table 18- Statistical evaluation of the effects of dynasore on D_1 cAMP production

	pEC_{50}	**E_{max}**
D_1 WT+vehicle	10.25±0.34	93±3.48
D_1 Mutant+vehicle	10.27±0.46	40.93±14.36*
D_1 WT+Dynasore	7.94±4.2	19.57±10.8**
D_1 Mutant+Dynasore	8.72±5.1	6.43±4.79***

*Concentration responses shown in **Fig.36** were analysed by nonlinear regression using Prism, and the pEC_{50}, E_{max} values were derived. Statistical significance to WT+vehicle was determined using unpaired Student's* t *test.*

4. Investigating the trafficking properties of D_1 WT and D_1 palmitoylation mutant using bystander BRET

To examine the distribution and trafficking of D_1 WT and D_1 palmitoylation mutant, a bystander BRET method was followed (Lan et al., 2011, 2012). Taking advantage of several Venus-tagged subcellular localisation markers Rab4, Rab5, Rab6, Rab7, Rab8, Rab9 and Rab11a in addition to K-Ras (D. D. Jensen et al., 2013; Lan et al., 2011; Szakadáti et al., 2015; Tiulpakov et al., 2016).

To investigate the effect of palmitoylation on receptor trafficking, D_1 WT and palmitoylation mutant were C-ter tagged with Nluc using the Gibson assembly cloning method. The insertion of the Nluc tag did not affect the D_1 cAMP production profile as the mutant cAMP production was reduced by 32% compared to WT (**Fig.37 & Table 19**). Moreover, the pEC_{50} of both Nluc tagged constructs was not affected and was in line with the results obtained with the untagged version of the receptor (**Fig.24 & Table 12)**.

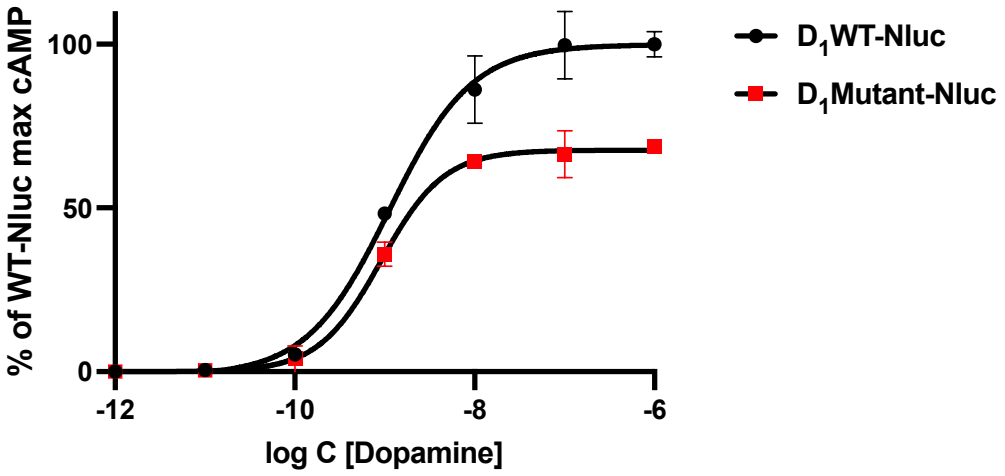


Figure 37- D1*-Nluc constructs validation.*

D1 WT and D1 palmitoylation deficient mutant tagged with Nluc on the C-ter, cAMP production assay performed in HEK 293 cells. Concentration response of cAMP production where cells were treated, respectively, with vehicle and a gradient of the D1 agonist Dopamine. Values are mean ± S.D of n = 3 independent experiments.

Table 19- Statistical evaluation of D1-Nluc constructs cAMP production.

	pEC50	**Emax**
D1 WT-Nluc	9.01±0.18	99.84±9.16
D1 Mutant-Nluc	9.04±0.22	67.64±5.4*

*Concentration-response curves (**Fig.37**) were analysed by nonlinear regression using Prism (GraphPad Software), and the EC50 values were derived from the curves. Statistical significances of the differences were determined using unpaired Student's* t *test.* $p < 0.05$ *was considered statistically significant.*

To study D1 trafficking, selective markers of various compartments of interest were needed. To this end, small GTPases that must localise at the plasma membrane for biological activity (Szakadáti et al., 2015) and Rab-GTPase (Rab) proteins associated with intracellular membrane trafficking that have been identified to localise to specific domains of the endocytic compartments (Pfeffer, 2013) (**Fig.38**) were tagged with Venus and paired with D1-Nluc for bystander BRET. To this end, Rab5 was used as a marker for early endosomes, Rab4 was used to monitor fast recycling to the plasma membrane on the other hand Venu-Rab11a to monitor the slow recycling

process (Coppens & Romano, 2020; Hutagalung & Novick, 2011; Pavlos & Friedman, 2017; Shearer & Petersen, 2019; Sposini et al., 2017a; Tiulpakov et al., 2016). Rab9 monitors D_1 sorting toward the endo-lysosomal compartments (Kucera et al., 2016). Rab7 was used to look at trafficking in the late endocytic and autophagic pathways (Guerra & Bucci, 2016; Kucera et al., 2016). Finally, Rab6 and Rab8 were used to look at the trans-Golgi and Cis-Golgi trafficking respectively (Antony et al., 1992; Dickson et al., 2020; Henry & Sheff, 2008).

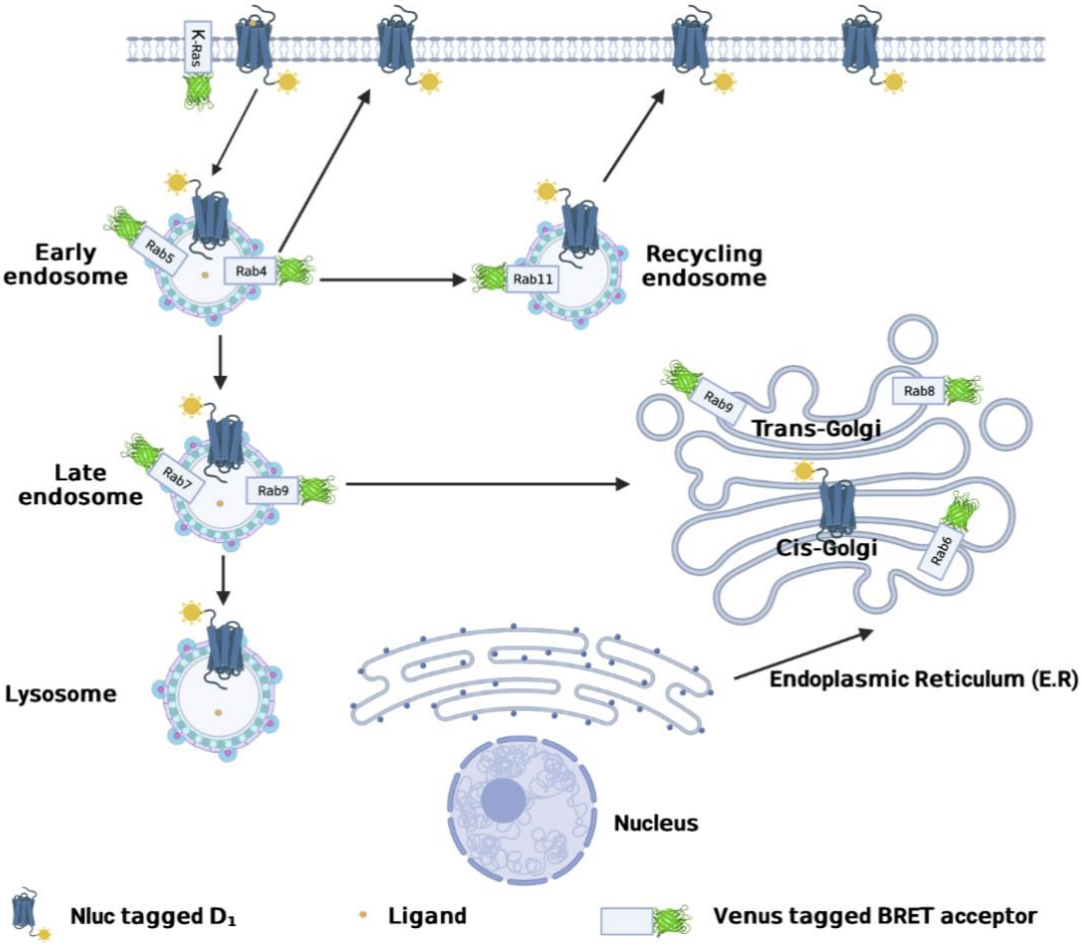


Figure 38- A simplified schematic representation of subcellular markers and their localisation for receptor trafficking BRET experiment.

A simplified schematic representation of subcellular marker localisation and receptor trafficking. Ligand-induced trafficking, as well as constitutive localisation, was monitored using Nluc-tagged wild-type or palmitoylation mutant D_1 by measuring proximity via BRET with the plasma membrane marker Venus/K-Ras, or the subcellular compartment markers Rabs: Venus/Rab5 for early endosomes; Venus/Rab4 for early endosome recycling; Venus/Rab11 for recycling endosomes;

Venus/Rab7 for late endosomes/lysosomes; Venus/Rab9 for late endosome trafficking to the trans-Golgi network; Venus/Rab6 for Golgi apparatus and trans-Golgi network; or Venus/Rab8 for trans-Golgi network to plasma membrane.

Therefore, D_1-Nluc constructs (WT and p.C347S; C351S) were co-transfected with the trafficking markers or an empty vector (donor-only control) in HEK 293 cells, and BRET was generated after treatment with 1µM of SKF 81297 or vehicle.

The results showed that upon agonist treatment, D_1 WT receptors BRET increases plasma membrane whereas D_1 palmitoylation mutant levels are not affected by the treatment (**Fig.39 &table 20**),this is shown in the bystander BRET with the K-Ras construct. Shortly after the D_1 selective agonist SKF81297 treatment, the BRET signal value of for D_1 WT peaks at 53 milliBRET Units (mBU), whereas the mutant is unaffected. A significant difference was found with Rab 8, after treating with the agonist, the D_1 mutant receptor BRET peaks at 12 mBU while the D_1 WT Bret peaks at 1 mBU after going below basal for the first 5 min, suggesting that when treated D_1 WT is trafficked out of the trans-Golgi whereas the palmitoylation mutant receptor is trapped in the Golgi compartment and cannot exist at the same rate. Moreover, no statistical difference was observed between D_1 WT and the palmitoylation mutant with Rab's 4, 5, 7, 9 and 11. Moreover, in HEK293 β-arrestin 1&2 Knockout cells, no D_1 agonist mediated trafficking to the plasma membrane was observed for D_1 WT and the palmitoylation mutant (**Fig.40**).

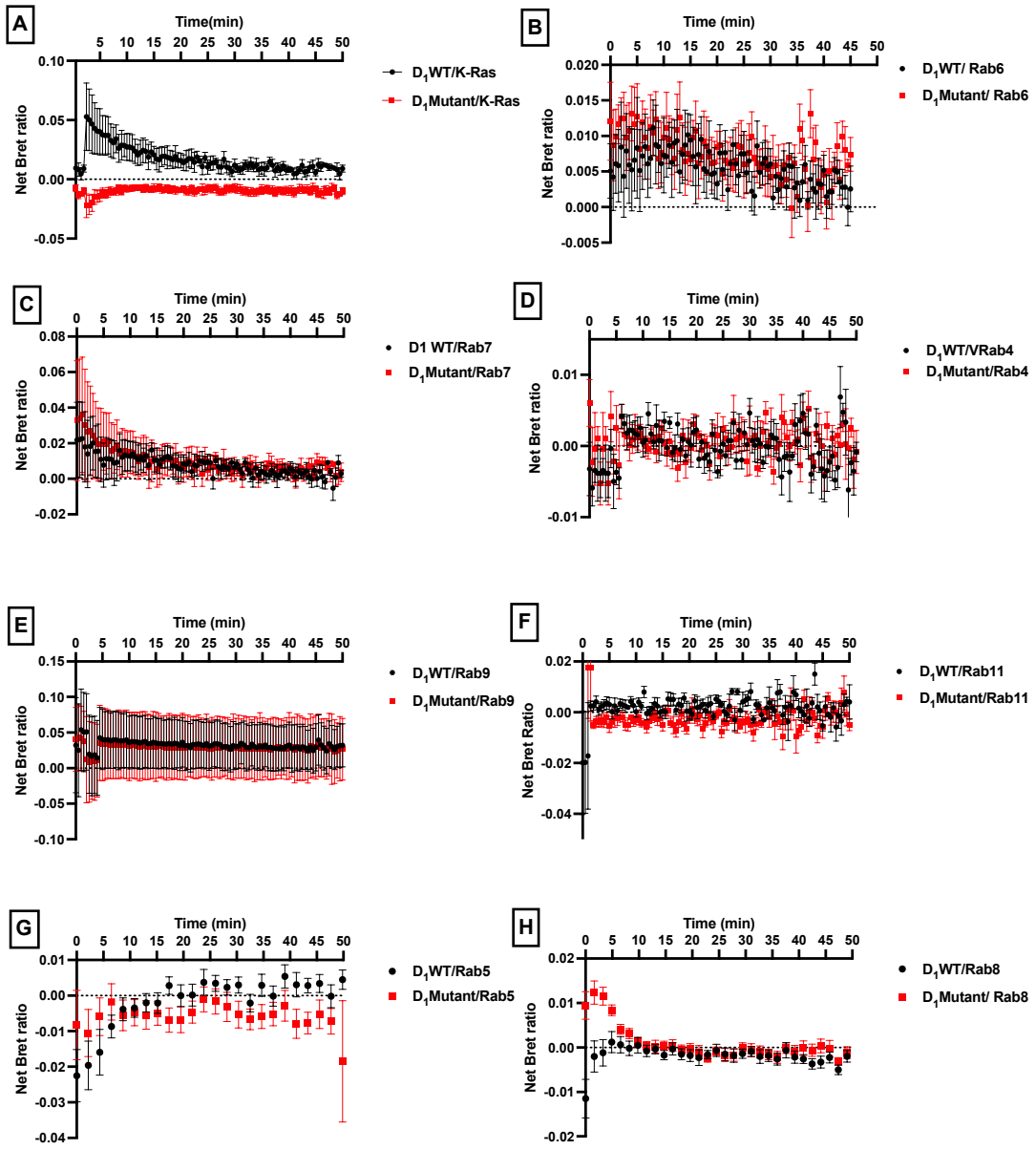


Figure 39- Bystander BRET of D_1 WT and palmitoylation mutant with various cellular compartments markers.

HEK 293 cells were transiently transfected with wild-type D1 (Black), D_1 p.C347S; C351S palmitoylation mutant (Red), tagged with Nluc and various cellular markers K-Ras and Rab's 4;5;6;7;8;9;11 tagged with Venus (panel A to H respectively). BRET ratio was calculated as described in Materials and Methods by normalising the SKF91297 agonist treated conditions to the untreated conditions. SKF 81297 (1µM) was added at t = 0 after the establishment of the baseline. Points represent the mean ± SEM of 3 independent experiments.

Table 20- Statistical evaluation of maximum BRET over basal determined from Figure 39.

	D_1 WT	D_1 Mutant
K-Ras	0.053±0.015**	-0.008±0.002
Rab4	0.005±0.002	0.004±0.002
Rab5	0.004 ±0.004	-0.001±0.004
Rab6	0.011±0.004	0.013±0.003
Rab7	0.016±0.009	0.019±0.021
Rab8	0.001±0.002**	0.012±0.002
Rab9	0.04±0.04	0.036±0.05
Rab11	0.008±0.002	0.001±0.002

*BRET signals represented in (**Fig.39**) were analysed and the maximum values for D_1 WT and D_1 palmitoylation mutant were compared for the various Bystander BRET markers used. Statistical significances were determined using unpaired Student's* t *test.* $p < 0.05$ *was considered statistically significant.*

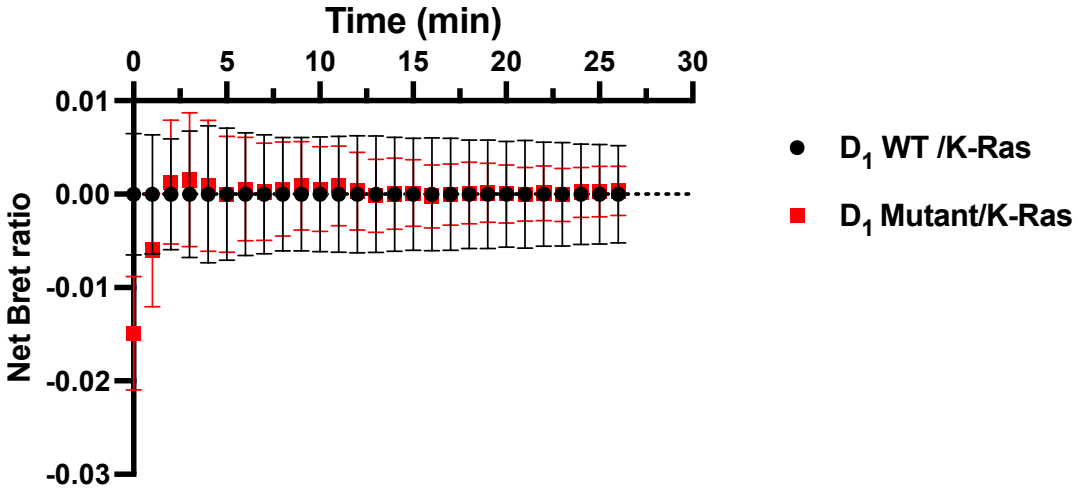


Figure 40-Bystander BRET of D_1 WT and palmitoylation mutant with plasma membrane marker Venus/K-Ras in HEK293 β-arrestin 1&2 Knockout cells.

HEK293 β-arrestin 1&2 Knockout cells were transiently transfected with wild-type D_1 (Black), D_1 p.C347S; C351S palmitoylation mutant (Red), tagged with Nluc and cellular markers K-Ras tagged with Venus. BRET ratio was calculated as described in Materials and Methods by normalising the SKF91297 agonist treated conditions to the untreated conditions. SKF 81297 (1μM) was added at t = 0 after the establishment of the baseline. Points represent the mean ± SEM of 3 independent experiments.

5. Detection of active D_1 WT and palmitoylation mutant at subcellular membranes using Nanobody-based conformational-sensitive biosensors

It has been previously shown that a single-domain camelid antibody, nanobody 80 (Nb80) (Rasmussen et al., 2011), can be repurposed as a conformational biosensor to detect activated β2AR and β1AR in living cells (Irannejad et al., 2017). Through directed evolution on Nb80, a high-affinity nanobody (Nb6B9) was generated that stabilises the active conformation of epinephrine-bound β2AR (Ring et al., 2013). Given that β2AR/Nb6B9 binding sites are highly conserved among other aminergic receptors such as β1AR and D_1 (Rasmussen et al., 2011), this nanobody could also be used as a conformational-sensitive biosensor to detect activated D_1 WT and palmitoylation mutant in real time and living cells (**Fig.41**).

Preliminary experiments performed in collaboration with Dr. Roshanak Irannejad at the University of California San Francisco, demonstrated that Hela cells expressing Snap-tagged D_1 and Nb6B9 (**Fig.41A**) and NB37 **(Fig.41B)** fused to GFP was diffuse throughout the cytoplasm (**Fig.41A**). Upon stimulation of these cells with 10 μM DA, Nb6B9-GFP was rapidly recruited first to the plasma membrane and shortly after to the Golgi apparatus (**Fig.41A**, 5 min, 10min). These data suggest that the D_1 WT and palmitoylation mutant Golgi and P.M receptors are activated in response to extracellular DA addition. We suspect that shortly after treatment the activated D_1 WT in the Golgi diffuses outside whereas the D_1 palmitoylation mutant localisation is unaffected as it can be seen in Figure 41.A that at 10 min after treatment D_1 WT colocalisation is reduced with the Golgi marker GalT-mRFP but the Palmitoylation mutant colocalisation is unchanged. Moreover, Nb6B9 dynamics look relatively comparable between the D_1 WT and the palmitoylation mutant, suggesting that the

mutations do not affect the receptor changing into its active conformation upon binding the ligand (DA) (**Fig.42**).

To investigate whether palmitoylation affected activated D_1 coupling to G proteins to elicit a G-protein-mediated response at the P.M, another nanobody-based biosensor was used, Nb37-GFP that was previously employed to detect transiently active β1AR/Gs and β2AR/Gs complexes (Irannejad et al., 2017). Nb37-GFP was recruited to the plasma membrane upon stimulation with DA, suggesting that the D_1 WT and palmitoylation pool couples to G protein and activates it (**Fig.41B**). However, the kinetics suggested that the palmitoylation mutants result in earlier Nb37 recruitment suggesting that D_1-G protein coupling/activation occurs earlier for palmitoylation mutants compared to D_1 WT (**Fig.42**).

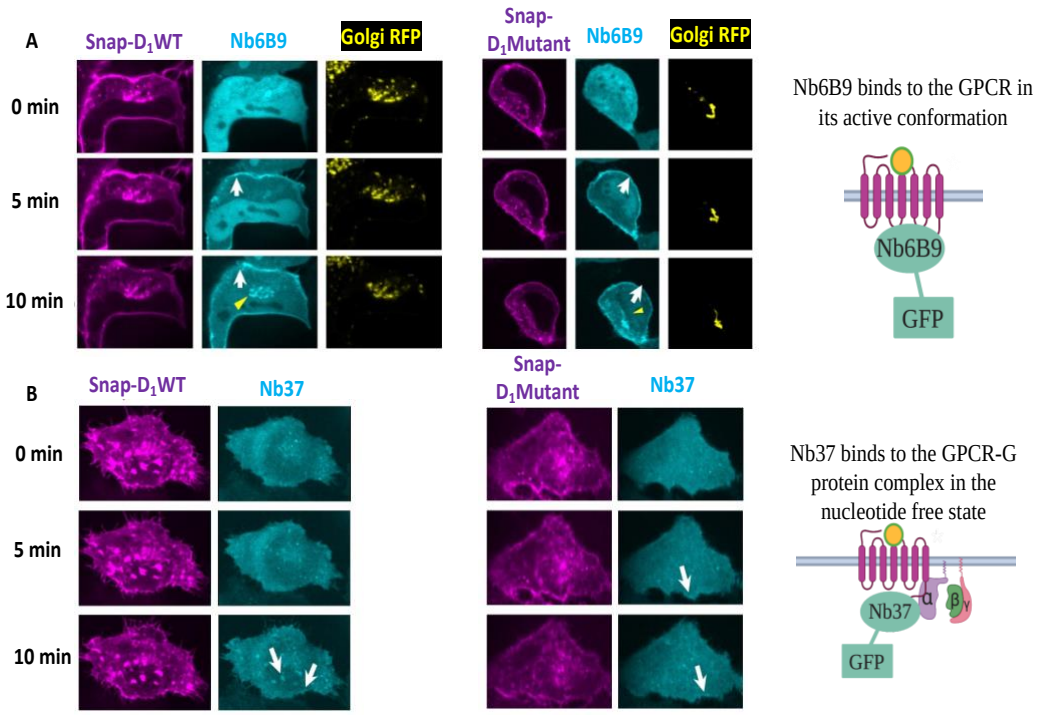


Figure 41- Conformational biosensors detects activated D_1 at the plasma membrane and the Golgi upon dopamine stimulation.

*(**A**)Nb6B9 binds to the receptor exclusively in its active conformation. Nb6B9 is fused to GFP and used it as a conformational biosensor for D1DR. Confocal images of representative Snap-D_1 WT and palmitoylation mutant expressing HeLa with Nb6B9-*

*GFP and GalT-mRFP expression before and after 10 µM DA addition. Stimulation with 10 µM DA results in the recruitment of Nb6B9 to active D_1 at the plasma membrane and the Golgi in HeLa cells. (**B**)Representative Hela cells expressing Snap-D_1 WT and palmitoylation mutant and Nb37-GFP before and after 10 µM DA addition. DA stimulates G protein activation at the Golgi in D1DR-expressing Hela cells.*
White arrow: P.M activation
Yellow arrowhead: Golgi activation

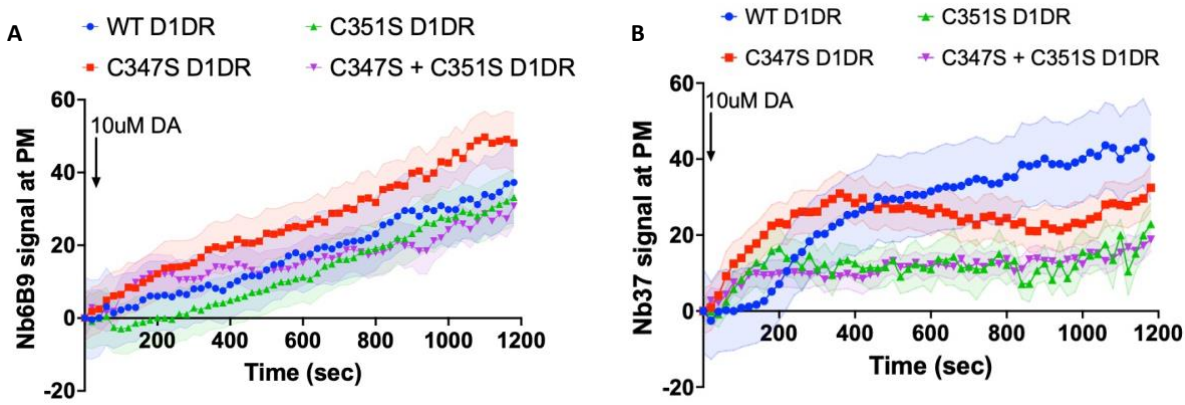


Figure 42- Quantification of Nb6B9-GFP and Nb37-GFP recruitment at the Plasma membrane.

Quantification of Nb6B9-GFP (A) and Nb37-GFP (B) recruitment at the plasma membrane upon 10 µM DA stimulation in Hela cells; normalised fluorescence intensity of Nb6B9-GFP relative to Snap D_1 at the P.M (n = 8 and 10 For D_1 WT and D1 palmitoylation mutant respectively, 1 biological replicates).

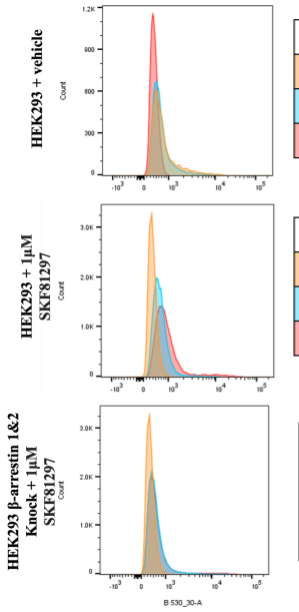
6. Investigating the effect of palmitoylation on plasma membrane D_1 in HEK293 β-arrestin 1&2 Knockout cells and HEK293 cells.

As previously discussed in chapter 4 loss of D_1 palmitoylation impaired its β-arrestin 1&2 recruitment. Moreover, loss of palmitoylation affected the P.M trafficking of D_1 in response to agonist treatment. To quantify D_1 on the plasma membrane.

HEK293 & HEK293 β-arrestin 1&2 Knockout cells were transfected with equal amounts of D_1 WT or palmitoylation mutant. Cells were sorted using the Flow cytometry (FACS) sorting to assay D_1 protein expression on the surface of the cells. Flow cytometry uses a laser-based technology to count, sort, and profile cells in a heterogeneous fluid mixture. With Flag-D_1 being fluorescently labelled with a secondary antibody against Flag that is coupled to a fluorochrome, the fluorescence intensity represents the amount of D_1 on the cell surface (**Fig.43**).

The FACS result (**Fig.43**) showed a similar number of cells positive for D_1 WT and D_1 palmitoylation mutant. Mean Fluorescence Intensity (MFI) was not different between the D_1 WT sample and the palmitoylation mutant in HEK293 cells in the absence of agonist treatment but when treated for 10 min with SKF 81297 D_1 WT was more found at the P.M than the palmitoylation mutant. In Contrast, in HEK293 β-arrestin 1&2 Knockout cells SKF81297 treatment was unable to generate a higher fluorescent signal for D_1 WT when compared to the palmitoylation mutant as both fluoresced at similar levels.

This preliminary data suggests no significant difference in the basal surface expression of D_1 WT and palmitoylation mutant in HEK293 cells. However, after agonist stimulation, D_1 WT can traffic better to the P.M. In contrast the palmitoylation mutant P.M levels aren't affected. Moreover, in the absence of β-arrestin 1&2 D_1 WT and palmitoylation mutant are found at similar levels afters agonist treatment suggesting a dual role of palmitoylation and β-arrestin 1&2 in D_1 trafficking on the P.M in response to agonist activation.



Sample	Cell Count	Mean fluorescence intensity
D_1 WT	15451	834
D_1 palm. Mutant	15482	748
Unstained sample	15860	435

Sample	Cell Count	Mean fluorescence intensity
Unstained sample	46808	400
D_1 palm. Mutant	46466	701
D_1 WT	46137	1002

Sample	Cell Count	Mean fluorescence intensity
Unstained sample	46808	400
D_1 palm. Mutant	45495	637
D_1 WT	45301	680

Figure 43- FACS sorting of D_1 WT and palmitoylation mutant in HEK293 cells and HEK293 β-arrestin 1&2 Knockout cells after 10 min SKF81297 selective D_1 agonist treatment.

Flag-D_1 WT, palmitoylation mutant or vehicle empty vector were equally transfected in HEK 293 cells. The Flag tag was detected using an anti-mouse Flag tag antibody that was coupled later to an anti-mouse Alexa Fluor® 488.(N=1)

IV. Discussion & Conclusion

Data previously presented in chapters 3&4 demonstrated a decrease in cAMP production for D_1 palmitoylation mutant in comparison to D_1 WT, D_1 palmitoylation mutant decreased cAMP production is correlated with a less efficient trimeric G protein dissociation and an impaired β-arrestin1&2 recruitment. In HEK 293 β-arrestins 1&2 KO the difference between D_1 WT and mutant cAMP production was lost suggesting that D_1 palmitoylation is central to β-arrestin1&2 recruitment and impacts receptor downstream signalling. Plasma membrane and Golgi trafficking data suggest that the D_1 WT intracellular pool can exist the Golgi and get shuttled to the plasma membrane after agonist treatment whereas the palmitoylation mutant remains

trapped. These findings support the previous data that demonstrated that DHHC 9 is the enzyme suspected of palmitoylating D_1 located in the Golgi and is responsible for modulating D_1 trafficking to the plasma membrane. Similar to D_1, DHHC 9's role extends beyond D_1 palmitoylation, impacting the localisation and function of various peripheral membrane proteins. For instance, N/H-Ras localisation and signalling from the P.M and Golgi critically depend on the dynamics of palmitate turnover. These two small GTPases are palmitoylated by DHHC9 in the Golgi. This lipidation mediates their localisation to the plasma membrane (Vartak et al., 2014). In addition, the glucose transporter GLUT1 S-palmitoylation is required for maintaining GLUT1 P.M localisation and DHHC9 is the palmitoyl transferase responsible for this critical post-translational modification (Zhang et al., 2021).

To further support our findinds, the FACS and Nanobody-based biosensor data require meticulous repetition for robust statistical analysis. Moreover, The FACS experiments are missing a vehicle treatment control for the HK293 β-arrestin1&2 KO cells. The data would benefit from a phenotype rescue by transfecting β-arrestin1&2 to see if the restoration of β-arrestin1&2 expression leads to observing a phenotype similar to that seen in HEK293 cells. Also, the nanobody-based conformational-sensitive biosensors experiment needs to be repeated in HEK293 cells with SKF81297 to rule out any impact of differential agonist effect or cell type on the results observed. Moreover, the bystander BRET experiments could benefit from more optimisation by including a Vasopressin receptor used in the original paper where these constructs were used as a positive control (Tiulpakov et al., 2016). In addition, switching to well-mode kinetics and automatic injections with a fixed gain instead of manual injection would produce a better temporal resolution with more consistency limiting human error and intervention. Lastly, the inclusion of a control that allow to determine that the BRET

signal differences are not the result of differences in the conformations between D_1 WT and the palmitoylation mutant knowing that protein palmitoylation can regulate and impact its P.M distributions, density, conformation, orientation (Jeyifous et al., 2016; Naumenko & Ponimaskin, 2018).

Finally, dynasore has undesirable non-specific and specific binding properties in common with many other small-molecule inhibitors. For example, it binds to serum proteins, causing it to lose dynamin inhibitory activity (Kirchhausen et al., 2008), limiting its use for many experimental designs. Dynasore also exhibits cytotoxicity and stoichiometric binding to the trace level of detergents commonly used in biological assays (McCluskey et al., 2013). Therefore, the dynasore experiment should incorporate a cell viability control to discern whether the observed reduction in cAMP is attributable to dynasore inhibiting dynamin activity, preventing endocytosis, rather than inducing cell death. Alternatively, using siRNAs against clathrin could be explored as an alternative to dynasore (Mayle et al., 2012).

This chapter underscores the multifaceted impact of palmitoylation on D_1 function, signalling, and trafficking. Rigorous experimental refinement and additional controls will fortify the validity and interpretability of the presented data, paving the way for a more comprehensive understanding of the intricate interplay between palmitoylation, receptor dynamics, and downstream signalling events.

Chapter 6: General discussion and Conclusion

I. Discussion

Dopamine is a neurotransmitter of the central nervous system that acts through the activation of a huge variety of different receptors to modulate various aspects of human and animal behaviour. Signalling properties of the D_1 receptors are under tight control of multiple factors regulating their functional activity, affecting behaviour. One of these factors is receptor palmitoylation, a post-translational receptor modification. Palmitoylation is a dynamic modification, and repeated cycles of palmitoylation/depalmitoylation are known to modulate different protein functions (Zhang & Hang, 2017). More than 70% of all known GPCRs contain potential palmitoylation site(s) downstream of their seventh transmembrane domain, suggesting that palmitoylation can represent a general feature of neurotransmitter receptors (Probst et al., 1992). Disruption of palmitoylation could significantly affect a variety of neurotransmitter receptor properties, including conformation, trafficking, and localisation on the plasma membrane and downstream signalling.

1. D_1 palmitoylation is central for its G protein activation and β-arrestin 1&2 interactions

Understanding the basis governing D_1 palmitoylation effects on its protein-protein interactions required the development of suitable tools to delineate the influence of each of the individual component contributions in the overall system responses. Our first objective was to implement an approach flexible enough to readily answer how D_1 interacts with DHHCs and how palmitoylation affects D_1's direct recruitment of downstream effectors (G proteins and Arrestin). Thus, we chose to use a BRET approach and a NanoBiT complementation assay, which, as discussed in chapter 4, provided assays suitable for studying GPCR dynamics (Dixon et al., 2016).

For the first time, DHHC9 is reported to palmitoylate D_1. Moreover, the palmitoylation state of D_1 affects the trimeric G protein dissociation and β-arrestin 1&2 recruitment. The specificity of D_1 WT interaction with certain DHHC was raised after the CO-IP experiment and Acyl-Rac validation of the palmitoylation state of D_1 WT and the p.C347S;C351S mutant. Then, the BRET interaction study of D_1 WT with the available panel of DHHCs identified DHHC9, which is located in the Golgi apparatus as the enzyme behind D_1 palmitoylation.

In addition, non-visual arrestins (β-arrestin-1 and β-arrestin-2), which are adaptor proteins that function to regulate G protein-coupled receptor signalling and trafficking (Burtey et al., 2007), were shown to be poorly recruited to D_1 palmitoylation mutant in comparison to D_1 WT. These results were in line with the work of Kong et al. that demonstrated that the internalisation of the D_1 receptor palmitoylation mutant was attenuated when treated with inhibitors of clathrin-mediated endocytosis. However, these treatments did not wholly abolish endocytosis, and in the presence of the cholesterol depleter, methyl-β-cyclodexytrin inhibitors of clathrin-mediated endocytosis, the palmitoylation mutant receptor internalisation was still more resistant to cholesterol depletion than wild type D_1 (Kong et al., 2011). These results are in line with the findings presented in this work as it shows that the D_1 palmitoylation mutant recruitment of β-arrestin-1 and β-arrestin-2 is impaired compared to the WT receptor and since receptor clathrin-mediated endocytosis requires the coordinated interaction of β-arrestins with clathrin (Tian et al., 2014) it would be expected for its internalisation to be less sensitive to clathrin mediated endocytosis inhibitors.

In parallel, a BRET-based assay using the TRUPATH probes (Olsen et al., 2020) to monitor the trimeric G protein dissociation for $G\alpha_s$. This assay demonstrated that D_1

WT dissociates the trimeric G protein more than the palmitoylation mutant receptor, thus explaining the reduced cAMP production observed for the mutant receptor.

Since palmitoylation influences all aspects of GPCR signalling, the palmitoylation state of certain receptors can preferentially direct signalling through particular G proteins and hence give different responses to the same ligand. Up to two palmitate groups can be found on D_1 and different palmitoylation profiles can result in various conformations of the carboxy-terminal tail, which may select for certain G protein interactions (Chini & Parenti, 2009). Therefore, investigating the whole panel of G protein activity of D_1 single-site palmitoylation mutants can be an addition to the present work. Moreover, Palmitoylation can influence the phosphorylation state of the receptor, modulating desensitisation (Naumenko & Ponimaskin, 2018). Therefore, uncovering how palmitoylation affects the phosphorylation profile of D_1 is interesting to explore using Phosphoproteomic techniques such as mass spectrometry, and Western blotting using commercially available Anti-D_1 phospho antibodies.

2. D_1 palmitoylation is essential for agonist-induced trafficking and proper Golgi transport

To understand the impact of palmitoylation on D_1 trafficking, bystander BRET and nanobody-based conformational-sensitive biosensors were used. This approach highlighted the differential trafficking of D_1 depending on its palmitoylation state, showing that agonist treatment directed intracellular D_1 WT to the plasma membrane; when intracellular trafficking was checked as suspected, the Golgi trafficking after agonist treatment was impaired for the palmitoylation mutant receptor. When D_1 internalisation was tested in HEK 293 β-arrestin 1&2 KO cells, we found that in the absence of arrestins, the agonist-induced trafficking of D_1 WT to the P.M was terminated. To better understand D_1 trafficking, it would be beneficial to test D_1 WT

trafficking and the palmitoylation mutant intracellular recycling and degradation path in the β-arrestin 1&2 KO HEK 293 and try to rescue the phenotype with arrestin transfection to understand which of 1 or 2 is responsible for the agonist-induced effect on D_1 WT. One of the limiting factors in studying D_1 internalisation and trafficking was the overlap in Rab's presence in multiple sub-compartments, such as Rab4's presence in early endosomes and recycling vesicles and Rab7's presence in late endosomes and lysosomes. Therefore, having more specific markers and relying on antibodies and confocal microscopy might provide more flexibility to assay and discriminate subpopulations of vesicles and trafficking compartments at the expense of losing temporal resolution.

II. Future directions

The work presented in this thesis highlights the effect of D_1 loss of palmitoylation on its G protein activation and β-arrestin 1&2 recruitment and how that impacts its trafficking and localisation. The biochemical basis of GPCRs activation has been studied to an atomic detail level (Weis & Kobilka, 2018). However, we are only now beginning to understand the subcellular organisation of GPCR signalling (Calebiro & Koszegi, 2019; Lobingier & von Zastrow, 2019). Many GPCRs are not restricted to the plasma membrane and transit the endocytic pathway (Hanyaloglu & von Zastrow, 2008). However, endocytosis was long believed only to impact the longer-term homeostatic regulation of GPCRs and not their acute signalling. Over the past years, this view has changed due to new evidence that GPCRs can engage G proteins after endocytosis and leverage the endocytic network to promote and attenuate cellular responses (Sutkeviciute & Vilardaga, 2020). Support for this emerging view is particularly well-developed for signalling mediated by GPCRs that

couple to stimulatory heterotrimeric G proteins (Gs) and activate adenylyl cyclases to produce cAMP. Several such GPCRs have now been shown to engage Gs on the endosome and the plasma membrane and stimulate sequential phases of cAMP production from both locations (Godbole et al., 2017). It is increasingly clear that GPCRs vary considerably in the magnitude and duration of cAMP production that they stimulate from endosomal membranes compared to the plasma membrane (Cahill et al., 2017; Thomsen et al., 2016; Tsvetanova & von Zastrow, 2014). Such differences have been clearly shown to impact downstream cellular responses mediated by GPCRs, both at the cell and tissue levels (Calebiro et al., 2009; Godbole et al., 2017), but little is known about how such signalling diversity is programmed.

The current view holds that endosomal signalling by GPCRs is strictly dependent on β-arrestin and that receptor-specific signalling is programmed according to the overall stability of the GPCR /β-arrestin complex (Godbole et al., 2017; Sutkeviciute & Vilardaga, 2020; Thomsen et al., 2016). Some GPCRs require binding to β-arrestin for internalisation and then remain bound, using β-arrestin as a molecular scaffold to stabilise receptors together with other signalling proteins on the endosome limiting membrane (Nguyen et al., 2019; Oakley et al., 2000), this resulted in a sustained cAMP elevation (Cahill et al., 2017; Feinstein et al., 2013; Nguyen et al., 2019). Other GPCRs similarly require β-arrestin to internalise but then dissociate from β-arrestin during or shortly after endocytosis (Oakley et al., 2000). This behaviour is still β-arrestin-dependent due to β-arrestin being needed to internalise, but receptors dissociated from β-arrestin transit endosomes rapidly and produce a transient cAMP response (Cahill et al., 2017).

On the other hand, some GPCRs do not require β-arrestin for endocytosis (Moo et al., 2021). Therefore, an additional mode of GPCR signalling from endosomes might exist that does not strictly require β-arrestin (Blythe & von Zastrow, 2023).

Since D_1 loss of palmitoylation impaired its β-arrestin recruitments, reduced its cAMP production, and altered its localisation. It became evident that it is of interest to map D_1 cAMP microdomains signalling to understand if the drop in cAMP production for the palmitoylation mutant is resulting in altered P.M or/and intracellular G_s signalling. This could be achieved using conformational fluorescent GPCR biosensors based on a variety of strategies, such as fluorescent resonance energy transfer (Kim et al., 2022). More recently, a strategy of biosensors utilising nanobodies has been developed to detect the active GPCR conformation (De Groof et al., 2019; Puri et al., 2022).

Furthermore, D_1 arrestin interaction on the endosomes needs to be evaluated to understand how the loss of palmitoylation is impacting its β-arrestin interaction on the endosomes. This could be achieved using an endosomal bystander LgBiT, utilising the FYVE domain of endofin (Namkung et al., 2016a), in combination with the SmBiT-βarrs. This assay robustly detected endosomal translocation of all GPCRs known to colocalise with βarrs at the endosome (Janetzko et al., 2022). One interesting question would be to understand the phosphorylation profile of D_1 WT vs the palmitoylation mutant to understand the reasons behind β-arrestin not being recruited to the receptor. Since agonist activation of D_1 rapidly leads to receptor desensitisation and a return to basal levels of signalling. This desensitisation process is intimately linked with receptor phosphorylation. The D_1 receptor is highly phosphorylated, with 32 intracellular serine and threonine residues, and is known to be phosphorylated by several kinases including protein kinase A, protein kinase C, and G protein-coupled receptor kinases (Moritz et al., 2023). Previous studies indicate that the D_1 is

phosphorylated on its third intracellular loop (ICL3) and C-terminus in a hierarchical fashion, in that phosphorylation must first occur on the C-terminus before the ICL3 can be phosphorylated (Moritz et al., 2023). These results indicate that a large fraction of DA-induced D_1 phosphorylation occurs on residues T360 and S362 in the proximal C-terminus and that these residues are also responsible for most DA-induced β-arrestin recruitment to the D_1. Thus, Using the same approach, we can identify the effect of loss of palmitoylation on D_1 phosphorylation and understand if β-arrestin impaired recruitment in the palmitoylation mutant is resulting from a difference in its phosphorylation or the hypothesis that it is caused by a conformational change in the C-tail and ICL3 blocking access of Kinases or/and β-arrestin. Finally, D_1 differential localisation and trafficking of D_1, depending on its palmitoylation profile can be monitored using microscopy imaging.

III. Concluding remarks

The modern challenges in the GPCR field involve translating the continuing boom in structural and signalling information to decipher the molecular processes underpinning these receptors' dynamic and protein-protein interactions to incorporate them into drug discovery. Nevertheless, among the pending questions, we need more fundamental information about GPCRs PTMs and their role in tuning receptor activity. Accordingly, even if our knowledge of GPCR signalling has developed extensively, little is known about what mechanisms drive these structures' spatial and temporal functions. Therefore, to take advantage of the new information arising from palmitoylation and its impact on receptor signalling and trafficking, two of the main objectives of this thesis were to investigate the mechanistic basis for palmitoylation

effect on D_1 to potentially explore its in vivo relevance in the context of disease in future studies.

Past behavioural and physiological studies have provided some insight into D_1-mediated β-arrestin functions. Urs et al. showed that both β-arrestin2 and D_1 knockout mice had lower morphine-induced locomotor activity than control mice (Urs et al., 2011). They concluded that the D_1-dependent, β-arrestin-related ERK signalling cascade was required for acute morphine-induced locomotor activity. These data support the importance of β-arrestin signalling in locomotor functions. A phase I clinical trial evaluated a novel D_1 agonist with partial cAMP activity but no activation of β-arrestin, which suggested its superiority to levodopa in treating late-stage Parkinson's patients. These data suggest that understanding mechanisms that can govern D_1 receptor/β-arrestin interactions and signalling is of therapeutic importance. Such studies, however, are in their infancy, and future research is necessary as the data suggest that functional selectivity at D_1-mediated β-arrestin may be a route to precision medicine. For some conditions, such as young adult rats in the T-maze task (Yang et al., 2021), bias toward D_1-mediated β-arrestin appears essential.

In contrast, bias against D_1-mediated β-arrestin may be more beneficial for other conditions, at least in non-human primates (Wang et al., 2019). Several ongoing clinic trials are using novel D_1 drugs that are biased against β-arrestin (Huang et al., 2020; Yang et al., 2022). All the compounds currently in clinical trials, however, are partial cAMP agonists. Determining if the lack of β-arrestin activity helps or hinders therapeutic responses will be very useful. Precise functional targeting may be crucial for maximising the effectiveness of D_1 agonists.

The data presented in this thesis added and reinforced the current knowledge on the importance of palmitoylation of neurotransmitter receptors, in this case, D_1 and its

crucial role in the regulation of receptor functions such as trafficking signalling and β-arrestin interactions, and consequently, in the control of different kinds of physiological and potential pathological behaviours. We are only beginning to uncover how these diverse extracellular signals integrate to establish unique patterns of cAMP-signalling within a given cell type. Although the medicines we use to modulate the cAMP pathway are helping patients, they are also associated with unwanted adverse effects. We need a detailed understanding of the system's complexity to increase the specific efficacy of drugs targeting these signalling pathways. To achieve this, we must strive to map out all cellular cAMP signalosomes. The unique combination of phosphoproteomics, interactome analysis, and imaging may support the discovery of new pharmacological targets by increasing the chance of identifying biologically meaningful interactions. Functional phosphoproteomic analysis downstream of D_1 may help define novel signalosomes by identifying new signalling targets. Interactomes of new or known signalosome components may support the identification of suitable targeting domains for resonance energy transfer sensors that can then be used to characterise cAMP signalling at that site in space and time. With a detailed model of the spatiotemporal distribution of cyclic nucleotides in D_1 WT versus D_1 palmitoylation mutant cells, we may be able to design specific targeted interventions, such as signalosome disruptor peptides or small molecules, to correct pockets of aberrant cyclic nucleotide signalling for precision medicine that can be used to on a broader scale for address similar signalling phenotypes occurring in disease models.

Although this thesis has focused on the palmitoylation of the D_1 receptor, many other GPCRs are known to be palmitoylated. For example: The serotonin receptor 5-HT_{1A}, where the lack of palmitoylation impairs the interaction with its $G\alpha_i$ subunits,

thus losing the ability to inhibit cAMP production (Papoucheva et al., 2004). Also, the β_2 Adrenergic receptor In the absence of palmitoylation, agonist activation promotes reduced cAMP production and a different phosphorylation profile of the receptor (Liu et al., 2012; Loisel et al., 1996; Moffett et al., 2001). In addition, upon ligand activation, palmitoylated C-C chemokine receptor type 5 (CCR5) is phosphorylated on four serine residues in position 336, 337, 342, and 349, but these phosphorylations could not be found in unpalmitoylated CCR5 (Kraft et al., 2001). Moreover, inhibition of palmitoylation affected CCR5 signalling (Blanpain et al., 2001; Kraft et al., 2001). CCR5 palmitoylation is essential for its incorporation into plasma membrane raft domains, raft localisation, caveolae-dependent internalisation, transport to the plasma membrane, and its protein stability (Boncompain et al., 2019; Jansen & Beaumelle, 2022; Venkatesan et al., 2002).

All the above examples highlight the importance of palmitoylation and show that it is responsible for modulating receptor trafficking, phosphorylation, and signalling and works in tandem with other regulatory mechanisms. Therefore, the information and approaches used here can now be applied to these other receptors to understand how general these findings are.

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References

Adams, M. N., Christensen, M. E., He, Y., Waterhouse, N. J., & Hooper, J. D. (2011). The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. *PloS One*, *6*(11), e28018. https://doi.org/10.1371/journal.pone.0028018

Ahearn, I. M., Haigis, K., Bar-Sagi, D., & Philips, M. R. (2011). Regulating the regulator: post-translational modification of RAS. *Nature Reviews. Molecular Cell Biology*, *13*(1), 39–51. https://doi.org/10.1038/nrm3255

Ahn, K. H., Nishiyama, A., Mierke, D. F., & Kendall, D. A. (2010). Hydrophobic residues in helix 8 of cannabinoid receptor 1 are critical for structural and functional properties. *Biochemistry*, *49*(3), 502–511. https://doi.org/10.1021/bi901619r

Aitken, A., Cohen, P., Santikarn, S., Williams, D. H., Calder, A. G., Smith, A., & Klee, C. B. (1982). Identification of the NH2-terminal blocking group of calcineurin B as myristic acid. *FEBS Letters*, *150*(2), 314–318. https://doi.org/10.1016/0014-5793(82)80759-x

Albert, P. R., Sajedi, N., Lemonde, S., & Ghahremani, M. H. (1999). Constitutive Gi2-dependent Activation of Adenylyl Cyclase Type II by the 5-HT1A Receptor: INHIBITION BY ANXIOLYTIC PARTIAL AGONISTS*. *Journal of Biological Chemistry*, *274*(50), 35469–35474. https://doi.org/https://doi.org/10.1074/jbc.274.50.35469

Alexander, G. E. (2004). Biology of Parkinson's disease: pathogenesis and pathophysiology of a multisystem neurodegenerative disorder. *Dialogues in Clinical Neuroscience*, *6*(3), 259–280. https://doi.org/10.31887/DCNS.2004.6.3/galexander

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Sharman, J. L., Southan, C., & Davies, J. A. (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: G protein-coupled receptors. *British Journal of Pharmacology*, *176 Suppl*(Suppl 1), S21–S141. https://doi.org/10.1111/bph.14748

An, J. H., Oh, B.-K., & Choi, J. W. (2013). Detection of tyrosine hydroxylase in dopaminergic neuron cell using gold nanoparticles-based barcode DNA. *Journal of Biomedical Nanotechnology*, *9*(4), 639–643. https://doi.org/10.1166/jbn.2013.1525

Andres, D. A., Seabra, M. C., Brown, M. S., Armstrong, S. A., Smeland, T. E., Cremers, F. P., & Goldstein, J. L. (1993). cDNA cloning of component A of Rab geranylgeranyl transferase and demonstration of its role as a Rab escort protein. *Cell*, *73*(6), 1091–1099. https://doi.org/10.1016/0092-8674(93)90639-8

Antony, C., Cibert, C., Géraud, G., Santa Maria, A., Maro, B., Mayau, V., & Goud, B. (1992). The small GTP-binding protein rab6p is distributed from medial Golgi to the trans-Golgi network as determined by a confocal microscopic approach. *Journal of Cell Science*, *103 (Pt 3*, 785–796. https://doi.org/10.1242/jcs.103.3.785

Aubert, I., Ghorayeb, I., Normand, E., & Bloch, B. (2000). Phenotypical characterization of the neurons expressing the D1 and D2 dopamine receptors in the monkey striatum. *Journal of*

Comparative Neurology, *418*(1), 22–32. https://doi.org/10.1002/(SICI)1096-9861(20000228)418:1<22::AID-CNE2>3.0.CO;2-Q

Audagnotto, M., & Dal Peraro, M. (2017). Protein post-translational modifications: In silico prediction tools and molecular modeling. *Computational and Structural Biotechnology Journal*, *15*, 307–319. https://doi.org/10.1016/j.csbj.2017.03.004

Avissar, S., Amitai, G., & Sokolovsky, M. (1983). Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: subunit assembly may explain high- and low-affinity agonist states. *Proceedings of the National Academy of Sciences of the United States of America*, *80*(1), 156–159.

Ayoubi, T. A., & Van De Ven, W. J. (1996). Regulation of gene expression by alternative promoters. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *10*(4), 453–460. http://www.ncbi.nlm.nih.gov/pubmed/8647344

Baccouch, R., Rascol, E., Stoklosa, K., & Alves, I. D. (2022). The role of the lipid environment in the activity of G protein coupled receptors. *Biophysical Chemistry*, *285*, 106794. https://doi.org/10.1016/j.bpc.2022.106794

Bai, M., Trivedi, S., & Brown, E. M. (1998). Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *The Journal of Biological Chemistry*, *273*(36), 23605–23610.

Baillie, G. S., Sood, A., McPhee, I., Gall, I., Perry, S. J., Lefkowitz, R. J., & Houslay, M. D. (2003). β-arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates β-adrenoceptor switching from Gs to Gi. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(3), 940–945. https://doi.org/10.1073/pnas.262787199

Barbieri, M. A., Hoffenberg, S., Roberts, R., Mukhopadhyay, A., Pomrehn, A., Dickey, B. F., & Stahl, P. D. (1998). Evidence for a Symmetrical Requirement for Rab5-GTP in in Vitro Endosome-Endosome Fusion*. *Journal of Biological Chemistry*, *273*(40), 25850–25855. https://doi.org/https://doi.org/10.1074/jbc.273.40.25850

Barkan, K., Lagarias, P., Stampelou, M., Stamatis, D., Hoare, S., Safitri, D., Klotz, K.-N., Vrontaki, E., Kolocouris, A., & Ladds, G. (2020). Pharmacological characterisation of novel adenosine A(3) receptor antagonists. *Scientific Reports*, *10*(1), 20781. https://doi.org/10.1038/s41598-020-74521-y

Barnett-Norris, J., Lynch, D., & Reggio, P. H. (2005). Lipids, lipid rafts and caveolae: their importance for GPCR signaling and their centrality to the endocannabinoid system. *Life Sciences*, *77*(14), 1625–1639. https://doi.org/10.1016/j.lfs.2005.05.040

Basith, S., Cui, M., Macalino, S. J. Y., Park, J., Clavio, N. A. B., Kang, S., & Choi, S. (2018). Exploring G protein-coupled receptors (GPCRs) ligand space via cheminformatics approaches: Impact on rational drug design. *Frontiers in Pharmacology*, *9*(MAR), 128. https://doi.org/10.3389/fphar.2018.00128

Beaulieu, J.-M. M., & Gainetdinov, R. R. (2011). The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacological Reviews*, *63*(1), 182 LP – 217. https://doi.org/10.1124/pr.110.002642

Beerkens, B. L. H., Koç, Ç., Liu, R., Florea, B. I., Le Dévédec, S. E., Heitman, L. H., IJzerman, A. P., & van der Es, D. (2022). A Chemical Biological Approach to Study G Protein-Coupled Receptors: Labeling the Adenosine A1 Receptor Using an Electrophilic Covalent Probe. *ACS Chemical Biology*, *17*(11), 3131–3139. https://doi.org/10.1021/acschembio.2c00589

Ben-Jonathan, N., & Hnasko, R. (2001). Dopamine as a Prolactin (PRL) Inhibitor. *Endocrine Reviews*, *22*(6), 724–763. https://doi.org/10.1210/edrv.22.6.0451

Bence, K., Ma, W., Kozasa, T., & Huang, X. Y. (1997). Direct stimulation of Bruton's tyrosine kinase by G(q)-protein alpha-subunit. *Nature*, *389*(6648), 296–299. https://doi.org/10.1038/38520

Benghezal, M., Lipke, P. N., & Conzelmann, A. (1995). Identification of six complementation classes involved in the biosynthesis of glycosylphosphatidylinositol anchors in Saccharomyces cerevisiae. *The Journal of Cell Biology*, *130*(6), 1333–1344. https://doi.org/10.1083/jcb.130.6.1333

Berg, T. J., Gastonguay, A. J., Lorimer, E. L., Kuhnmuench, J. R., Li, R., Fields, A. P., & Williams, C. L. (2010). Splice variants of SmgGDS control small GTPase prenylation and membrane localization. *The Journal of Biological Chemistry*, *285*(46), 35255–35266. https://doi.org/10.1074/jbc.M110.129916

Berman, D. M., & Gilman, A. G. (1998). Mammalian RGS proteins: Barbarians at the gate. *Journal of Biological Chemistry*, *273*(3), 1269–1272. https://doi.org/10.1074/jbc.273.3.1269

Besson, B., Eun, H., Kim, S., Windisch, M. P., Bourhy, H., & Grailhe, R. (2022). Optimization of BRET saturation assays for robust and sensitive cytosolic protein–protein interaction studies. *Scientific Reports*, *12*(1), 9987. https://doi.org/10.1038/s41598-022-12851-9

Bibb, J. A., Snyder, G. L., Nishi, A., Yan, Z., Meijer, L., Flenberg, A. A., Tsai, L. H., Kwon, Y. T., Girault, J. A., Czernik, A. J., Huganir, R. L., Hemmings, H. C., Nairn, A. C., & Greengard, P. (1999). Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature*, *402*(6762), 669–671. https://doi.org/10.1038/45251

Bizzozero, O. A., & Lees, M. B. (1986). Fatty acid acylation of rat brain myelin proteolipid protein in vitro: identification of the lipid donor. *Journal of Neurochemistry*, *46*(2), 630–636. https://doi.org/10.1111/j.1471-4159.1986.tb13013.x

Blanpain, C., Wittamer, V., Vanderwinden, J. M., Boom, A., Renneboog, B., Lee, B., Le Poul, E., El Asmar, L., Govaerts, C., Vassart, G., Doms, R. W., & Parmentier, M. (2001). Palmitoylation of CCR5 Is Critical for Receptor Trafficking and Efficient Activation of Intracellular Signaling Pathways. *Journal of Biological Chemistry*, *276*(26), 23795–23804. https://doi.org/10.1074/jbc.M100583200

Blaskovic, S., Blanc, M., & Van Der Goot, F. G. (2013). What does S-palmitoylation do to membrane proteins? *FEBS Journal*, *280*(12), 2766–2774. https://doi.org/10.1111/febs.12263

Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., & Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics*, *4*(6), 1633–1649. https://doi.org/10.1002/pmic.200300771

Blumer, J. B., & Lanier, S. M. (2014). Activators of G Protein Signaling Exhibit Broad Functionality

and Define a Distinct Core Signaling Triad. *Molecular Pharmacology*, *85*(3), 388 LP – 396. https://doi.org/10.1124/mol.113.090068

Blythe, E. E., & von Zastrow, M. (2023). β-Arrestin-independent endosomal cAMP signaling by a polypeptide hormone GPCR. *Nature Chemical Biology*. https://doi.org/10.1038/s41589-023-01412-4

Bockaert, J., & Philippe Pin, J. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *The EMBO Journal*, *18*(7), 1723–1729. https://doi.org/https://doi.org/10.1093/emboj/18.7.1723

Bollu, L. R., Ren, J., Blessing, A. M., Katreddy, R. R., Gao, G., Xu, L., Wang, J., Su, F., & Weihua, Z. (2014). Involvement of de novo synthesized palmitate and mitochondrial EGFR in EGF induced mitochondrial fusion of cancer cells. *Cell Cycle (Georgetown, Tex.)*, *13*(15), 2415–2430. https://doi.org/10.4161/cc.29338

Boncompain, G., Herit, F., Tessier, S., Lescure, A., Nery, E. Del, Gestraud, P., Staropoli, I., Fukata, Y., Fukata, M., Brelot, A., Niedergang, F., & Perez, F. (2019). Targeting CCR5 trafficking to inhibit HIV-1 infection. *Science Advances*, *5*(10), 1–14. https://doi.org/10.1126/sciadv.aax0821

Bono, F., Tomasoni, Z., Mutti, V., Sbrini, G., Kumar, R., Longhena, F., Fiorentini, C., & Missale, C. (2023). G Protein-Dependent Activation of the PKA-Erk1/2 Pathway by the Striatal Dopamine D1/D3 Receptor Heteromer Involves Beta-Arrestin and the Tyrosine Phosphatase Shp-2. In *Biomolecules* (Vol. 13, Issue 3). https://doi.org/10.3390/biom13030473

Borovac, J. A. (2016). Side effects of a dopamine agonist therapy for Parkinson's disease: a mini-review of clinical pharmacology. *The Yale Journal of Biology and Medicine*, *89*(1), 37–47.

Boutin, J. A., Ferry, G., Ernould, A. P., Maes, P., Remond, G., & Vincent, M. (1993). Myristoyl-CoA:protein N-myristoyltransferase activity in cancer cells. Purification and characterization of a cytosolic isoform from the murine leukemia cell line L1210. *European Journal of Biochemistry*, *214*(3), 853–867. https://doi.org/10.1111/j.1432-1033.1993.tb17989.x

Brami-Cherrier, K., Valjent, E., Garcia, M., Pagès, C., Hipskind, R. A., & Caboche, J. (2002). Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: A new route to cAMP response element-binding protein phosphorylation. *Journal of Neuroscience*, *22*(20), 8911–8921. https://doi.org/10.1523/jneurosci.22-20-08911.2002

Brisch, R., Saniotis, A., Wolf, R., Bielau, H., Bernstein, H.-G., Steiner, J., Bogerts, B., Braun, K., Jankowski, Z., Kumaratilake, J., Henneberg, M., & Gos, T. (2014). The role of dopamine in schizophrenia from a neurobiological and evolutionary perspective: old fashioned, but still in vogue. *Frontiers in Psychiatry*, *5*, 47. https://doi.org/10.3389/fpsyt.2014.00047

Brooks, D. J. (2000). Dopamine agonists: their role in the treatment of Parkinson's disease. In *Journal of neurology, neurosurgery, and psychiatry* (Vol. 68, Issue 6, pp. 685–689). https://doi.org/10.1136/jnnp.68.6.685

Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B., & Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*, *70*(5), 715–728. https://doi.org/https://doi.org/10.1016/0092-8674(92)90306-W

Buhl, A. M., Johnson, N. L., Dhanasekaran, N., & Johnson, G. L. (1995). G alpha 12 and G alpha 13

stimulate Rho-dependent stress fiber formation and focal adhesion assembly. *The Journal of Biological Chemistry*, *270*(42), 24631–24634. https://doi.org/10.1074/jbc.270.42.24631

Burtey, A., Schmid, E. M., Ford, M. G. J., Rappoport, J. Z., Scott, M. G. H., Marullo, S., Simon, S. M., McMahon, H. T., & Benmerah, A. (2007). The conserved isoleucine-valine-phenylalanine motif couples activation state and endocytic functions of beta-arrestins. *Traffic (Copenhagen, Denmark)*, *8*(7), 914–931. https://doi.org/10.1111/j.1600-0854.2007.00578.x

Busquets-Hernández, C., & Triola, G. (2021). Palmitoylation as a Key Regulator of Ras Localization and Function. *Frontiers in Molecular Biosciences*, *8*, 659861. https://doi.org/10.3389/fmolb.2021.659861

Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G., & Sefton, B. M. (1987). Myristoylated alpha subunits of guanine nucleotide-binding regulatory proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(21), 7493–7497. https://doi.org/10.1073/pnas.84.21.7493

Buss, J. E., & Sefton, B. M. (1985). Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. *Journal of Virology*, *53*(1), 7—12. https://doi.org/10.1128/jvi.53.1.7-12.1985

Cadet, J. L., Jayanthi, S., McCoy, M. T., Beauvais, G., & Cai, N. S. (2010). Dopamine D1 receptors, regulation of gene expression in the brain, and neurodegeneration. *CNS & Neurological Disorders Drug Targets*, *9*(5), 526–538. https://doi.org/10.2174/187152710793361496

Cahill, T. J., Thomsen, A. R. B., Tarrasch, J. T., Plouffe, B., Nguyen, A. H., Yang, F., Huang, L.-Y., Kahsai, A. W., Bassoni, D. L., Gavino, B. J., Lamerdin, J. E., Triest, S., Shukla, A. K., Berger, B., Little, J., Antar, A., Blanc, A., Qu, C.-X., Chen, X., … Lefkowitz, R. J. (2017). Distinct conformations of GPCR–β-arrestin complexes mediate desensitization, signaling, and endocytosis. *Proceedings of the National Academy of Sciences*, *114*(10), 2562–2567. https://doi.org/10.1073/pnas.1701529114

Calebiro, D., & Koszegi, Z. (2019). The subcellular dynamics of GPCR signaling. *Molecular and Cellular Endocrinology*, *483*, 24–30. https://doi.org/10.1016/j.mce.2018.12.020

Calebiro, D., Nikolaev, V. O., Gagliani, M. C., De Filippis, T., Dees, C., Tacchetti, C., Persani, L., & Lohse, M. J. (2009). Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biology*, *7*(8), e1000172. https://doi.org/10.1371/journal.pbio.1000172

Callier, S., Snapyan, M., Le Crom, S., Prou, D., Vincent, J. D., & Vernier, P. (2003). Evolution and cell biology of dopamine receptors in vertebrates. *Biology of the Cell*, *95*(7), 489–502. https://doi.org/10.1016/S0248-4900(03)00089-3

Canto, I., & Trejo, J. (2013). Palmitoylation of protease-activated receptor-1 regulates adaptor protein complex-2 and -3 interaction with tyrosine-based motifs and endocytic sorting. *The Journal of Biological Chemistry*, *288*(22), 15900–15912. https://doi.org/10.1074/jbc.M113.469866

Cantrell, A. R., Smith, R. D., Goldin, A. L., Scheuer, T., & Catterall, W. A. (1997). Dopaminergic modulation of sodium current in hippocampal neurons via cAMP-dependent phosphorylation of specific sites in the sodium channel α subunit. *Journal of Neuroscience*, *17*(19), 7330–7338. https://doi.org/10.1523/jneurosci.17-19-07330.1997

Cao, H., Thompson, H. M., Krueger, E. W., & McNiven, M. A. (2000). Disruption of Golgi structure and function in mammalian cells expressing a mutant dynamin. *Journal of Cell Science*, *113 (Pt 1*, 1993–2002. https://doi.org/10.1242/jcs.113.11.1993

Caragea, C., Sinapov, J., Silvescu, A., Dobbs, D., & Honavar, V. (2007). Glycosylation site prediction using ensembles of Support Vector Machine classifiers. *BMC Bioinformatics*, *8*, 1–13.

Caras, I. W., Weddell, G. N., Davitz, M. A., Nussenzweig, V., & Martin, D. W. J. (1987). Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science (New York, N.Y.)*, *238*(4831), 1280–1283. https://doi.org/10.1126/science.2446389

Carman, C. V, & Benovic, J. L. (1998). G-protein-coupled receptors: turn-ons and turn-offs. *Current Opinion in Neurobiology*, *8*(3), 335–344. https://doi.org/10.1016/s0959-4388(98)80058-5

Carmona-Rosas, G., Alcántara-Hernández, R., & Hernández-Espinosa, D. A. (2019). Chapter 12 - The role of β-arrestins in G protein-coupled receptor heterologous desensitization: A brief story. In A. K. B. T.-M. in C. B. Shukla (Ed.), *G Protein-Coupled Receptors, Part B* (Vol. 149, pp. 195–204). Academic Press. https://doi.org/https://doi.org/10.1016/bs.mcb.2018.08.004

Caron, J. M. (1997). Posttranslational modification of tubulin by palmitoylation: I. In vivo and cell-free studies. *Molecular Biology of the Cell*, *8*(4), 621–636. https://doi.org/10.1091/mbc.8.4.621

Carpenter, B., Nehmé, R., Warne, T., Leslie, A. G. W., & Tate, C. G. (2016). Structure of the adenosine A(2A) receptor bound to an engineered G protein. *Nature*, *536*(7614), 104–107. https://doi.org/10.1038/nature18966

Carr, S. A., Biemann, K., Shoji, S., Parmelee, D. C., & Titani, K. (1982). n-Tetradecanoyl is the NH2-terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. *Proceedings of the National Academy of Sciences of the United States of America*, *79*(20), 6128–6131. https://doi.org/10.1073/pnas.79.20.6128

Casey, P. J., Solski, P. A., Der, C. J., & Buss, J. E. (1989). p21ras is modified by a farnesyl isoprenoid. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(21), 8323–8327. https://doi.org/10.1073/pnas.86.21.8323

Cepeda, C., Colwell, C. S., Itri, J. N., Chandler, S. H., & Levine, M. S. (1998). Dopaminergic modulation of NMDA-induced whole cell currents in neostriatal neurons in slices: Contribution of calcium conductances. *Journal of Neurophysiology*, *79*(1), 82–94. https://doi.org/10.1152/jn.1998.79.1.82

Chalhoub, G., & McCormick, P. J. (2022). Palmitoylation and G-protein coupled receptors. *Progress in Molecular Biology and Translational Science*, *193*(1), 195–211. https://doi.org/10.1016/bs.pmbts.2022.09.002

Chamberlain, L. H., & Shipston, M. J. (2015). The physiology of protein s-acylation. *Physiological Reviews*, *95*(2), 341–376. https://doi.org/10.1152/physrev.00032.2014

Channer, B., Matt, S. M., Nickoloff-Bybel, E. A., Pappa, V., Agarwal, Y., Wickman, J., & Gaskill, P. J. (2023). Dopamine, Immunity, and Disease. *Pharmacological Reviews*, *75*(1), 62–158. https://doi.org/10.1124/pharmrev.122.000618

Chao, S. Z., Lu, W., Lee, H. K., Huganir, R. L., & Wolf, M. E. (2002). D1 dopamine receptor

stimulation increases GluR1 phosphorylation in postnatal nucleus accumbens cultures. *Journal of Neurochemistry*, *81*(5), 984–992. https://doi.org/10.1046/j.1471-4159.2002.00877.x

Charron, G., Zhang, M. M., Yount, J. S., Wilson, J., Raghavan, A. S., Shamir, E., & Hang, H. C. (2009). Robust fluorescent detection of protein fatty-acylation with chemical reporters. *Journal of the American Chemical Society*, *131*(13), 4967–4975. https://doi.org/10.1021/ja810122f

Charych, E. I., Jiang, L. X., Lo, F., Sullivan, K., & Brandon, N. J. (2010). Interplay of palmitoylation and phosphorylation in the trafficking and localization of phosphodiesterase 10A: Implications for the treatment of schizophrenia. *Journal of Neuroscience*, *30*(27), 9027–9037. https://doi.org/10.1523/JNEUROSCI.1635-10.2010

Chase, J. F., & Tubbs, P. K. (1972). Specific inhibition of mitochondrial fatty acid oxidation by 2-bromopalmitate and its coenzyme A and carnitine esters. *The Biochemical Journal*, *129*(1), 55–65. https://doi.org/10.1042/bj1290055

Chatterjee, S., & Mayor, S. (2001). The GPI-anchor and protein sorting. *Cellular and Molecular Life Sciences : CMLS*, *58*(14), 1969–1987. https://doi.org/10.1007/PL00000831

Chen, R., Anderson, V., Hiroi, Y., & Medof, M. E. (2003). Proprotein interaction with the GPI transamidase. *Journal of Cellular Biochemistry*, *88*(5), 1025–1037. https://doi.org/10.1002/jcb.10439

Chen, S., Zhu, B., Yin, C., Liu, W., Han, C., Chen, B., Liu, T., Li, X., Chen, X., Li, C., Hu, L., Zhou, J., Xu, Z.-X., Gao, X., Wu, X., Goding, C. R., & Cui, R. (2017). Palmitoylation-dependent activation of MC1R prevents melanomagenesis. *Nature*, *549*(7672), 399–403. https://doi.org/10.1038/nature23887

Chen, S., Zhu, B., Yin, C., Liu, W., Han, C., Xu, X., Gao, X., Wu, X., Goding, C. R., & Cui, R. (2018). *HHS Public Access*. *549*(7672), 399–403. https://doi.org/10.1038/nature23887.Palmitoylation-dependent

Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G. F., Thian, F. S., Kobilka, T. S., Choi, H.-J., Kuhn, P., Weis, W. I., Kobilka, B. K., & Stevens, R. C. (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science (New York, N.Y.)*, *318*(5854), 1258–1265. https://doi.org/10.1126/science.1150577

Chergui, K., & Lacey, M. G. (1999). Modulation by dopamine D1-like receptors of synaptic transmission and NMDA receptors in rat nucleus accumbens is attenuated by the protein kinase C inhibitor Ro 32-0432. *Neuropharmacology*, *38*(2), 223–231. https://doi.org/10.1016/S0028-3908(98)00187-7

Chini, B., & Parenti, M. (2009). G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *Journal of Molecular Endocrinology*, *42*(5), 371–379. https://doi.org/10.1677/JME-08-0114

Cho, D. I., Zheng, M., & Kim, K.-M. (2010). Current perspectives on the selective regulation of dopamine D2 and D3 receptors. *Archives of Pharmacal Research*, *33*(10), 1521–1538. https://doi.org/10.1007/s12272-010-1005-8

Chopard, C., Tong, P. B. V., Tóth, P., Schatz, M., Yezid, H., Debaisieux, S., Mettling, C., Gross, A., Pugnière, M., Tu, A., Strub, J. M., Mesnard, J. M., Vitale, N., & Beaumelle, B. (2018).

Cyclophilin A enables specific HIV-1 Tat palmitoylation and accumulation in uninfected cells. *Nature Communications*, *9*(1), 1–15. https://doi.org/10.1038/s41467-018-04674-y

Chun, L., Zhang, W., & Liu, J. (2012). Structure and ligand recognition of class C GPCRs. *Acta Pharmacologica Sinica*, *33*(3), 312–323. https://doi.org/10.1038/aps.2011.186

Cismowski, M. J., Ma, C., Ribas, C., Xie, X., Spruyt, M., Lizano, J. S., Lanier, S. M., & Duzic, E. (2000). Activation of Heterotrimeric G-protein Signaling by a Ras-related Protein: IMPLICATIONS FOR SIGNAL INTEGRATION*. *Journal of Biological Chemistry*, *275*(31), 23421–23424. https://doi.org/https://doi.org/10.1074/jbc.C000322200

Cole, R. L., Konradi, C., Douglass, J., & Hyman, S. E. (1995). Neuronal adaptation to amphetamine and dopamine: Molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron*, *14*(4), 813–823. https://doi.org/10.1016/0896-6273(95)90225-2

Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., & Sprang, S. R. (1994). Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. *Science (New York, N.Y.)*, *265*(5177), 1405–1412. https://doi.org/10.1126/science.8073283

Coleman, D. T., Gray, A. L., Kridel, S. J., & Cardelli, J. A. (2016). Palmitoylation regulates the intracellular trafficking and stability of c-Met. *Oncotarget*, *7*(22), 32664–32677. https://doi.org/10.18632/oncotarget.8706

Collins, F. S., Lander, E. S., Rogers, J., & Waterson, R. H. (2004). Finishing the euchromatic sequence of the human genome. *Nature*, *431*(7011), 931–945. https://doi.org/10.1038/nature03001

Colombo, S., Longhi, R., Alcaro, S., Ortuso, F., Sprocati, T., Flora, A., & Borgese, N. (2005). N-myristoylation determines dual targeting of mammalian NADH-cytochrome b5 reductase to ER and mitochondrial outer membranes by a mechanism of kinetic partitioning. *The Journal of Cell Biology*, *168*(5), 735–745. https://doi.org/10.1083/jcb.200407082

Conroy, J. L., Free, R. B., & Sibley, D. R. (2015). Identification of G protein-biased agonists that fail to recruit β-arrestin or promote internalization of the D1 dopamine receptor. *ACS Chemical Neuroscience*, *6*(4), 681–692. https://doi.org/10.1021/acschemneuro.5b00020

Coppens, I., & Romano, J. D. (2020). Sitting in the driver's seat: Manipulation of mammalian cell Rab GTPase functions by apicomplexan parasites. *Biology of the Cell*, *112*(7), 187–195. https://doi.org/10.1111/boc.201900107

Corda, D., Hidalgo Carcedo, C., Bonazzi, M., Luini, A., & Spanò, S. (2002). Molecular aspects of membrane fission in the secretory pathway. *Cellular and Molecular Life Sciences : CMLS*, *59*(11), 1819–1832. https://doi.org/10.1007/pl00012508

Cundy, T., Hegde, M., Naot, D., Chong, B., King, A., Wallace, R., Mulley, J., Love, D. R., Seidel, J., Fawkner, M., Banovic, T., Callon, K. E., Grey, A. B., Reid, I. R., Middleton-Hardie, C. A., & Cornish, J. (2002). A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. *Human Molecular Genetics*, *11*(18), 2119–2127. https://doi.org/10.1093/hmg/11.18.2119

Dal Toso, R., Sommer, B., Ewert, M., Herb, A., Pritchett, D. B., Bach, A., Shivers, B. D., & Seeburg, P. H. (1989). The dopamine D2 receptor: two molecular forms generated by alternative

splicing. *The EMBO Journal*, *8*(13), 4025–4034. https://doi.org/10.1002/j.1460-2075.1989.tb08585.x

Davda, D., El Azzouny, M. A., Tom, C. T. M. B., Hernandez, J. L., Majmudar, J. D., Kennedy, R. T., & Martin, B. R. (2013). Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate. *ACS Chemical Biology*, *8*(9), 1912–1917. https://doi.org/10.1021/cb400380s

Davies, M. N., Secker, A., Freitas, A. A., Mendao, M., Timmis, J., & Flower, D. R. (2007). On the hierarchical classification of G protein-coupled receptors. *Bioinformatics*, *23*(23), 3113–3118. https://doi.org/10.1093/bioinformatics/btm506

de Graaf, C., Song, G., Cao, C., Zhao, Q., Wang, M.-W., Wu, B., & Stevens, R. C. (2017). Extending the Structural View of Class B GPCRs. *Trends in Biochemical Sciences*, *42*(12), 946–960. https://doi.org/10.1016/j.tibs.2017.10.003

De Groof, T. W. M., Bobkov, V., Heukers, R., & Smit, M. J. (2019). Nanobodies: New avenues for imaging, stabilizing and modulating GPCRs. *Molecular and Cellular Endocrinology*, *484*, 15–24. https://doi.org/10.1016/j.mce.2019.01.021

De Keyser, J., Claeys, A., De Backer, J. P., Ebinger, G., Roels, F., & Vauquelin, G. (1988). Autoradiographic localization of D1 and D2 dopamine receptors in the human brain. *Neuroscience Letters*, *91*(2), 142–147. https://doi.org/10.1016/0304-3940(88)90758-6

Del Monte, F., & Agnetti, G. (2014). Protein post-translational modifications and misfolding: new concepts in heart failure. *Proteomics. Clinical Applications*, *8*(7–8), 534–542. https://doi.org/10.1002/prca.201400037

Dennis, K. M. J. H., & Heather, L. C. (2023). Post-translational palmitoylation of metabolic proteins . In *Frontiers in Physiology* (Vol. 14). https://www.frontiersin.org/articles/10.3389/fphys.2023.1122895

DeWire, S. M., Ahn, S., Lefkowitz, R. J., & Shenoy, S. K. (2007). β-Arrestins and Cell Signaling. *Annual Review of Physiology*, *69*(1), 483–510. https://doi.org/10.1146/annurev.physiol.69.022405.154749

Di Fiore, P. P., & von Zastrow, M. (2014). Endocytosis, signaling, and beyond. *Cold Spring Harbor Perspectives in Biology*, *6*(8). https://doi.org/10.1101/cshperspect.a016865

Dickson, L. J., Liu, S., & Storrie, B. (2020). Rab6 is required for rapid, cisternal-specific, intra-Golgi cargo transport. *Scientific Reports*, *10*(1), 16604. https://doi.org/10.1038/s41598-020-73276-w

DiRaddo, J. O., Miller, E. J., Hathaway, H. A., Grajkowska, E., Wroblewska, B., Wolfe, B. B., Liotta, D. C., & Wroblewski, J. T. (2014). A real-time method for measuring cAMP production modulated by Gαi/o-coupled metabotropic glutamate receptors. *The Journal of Pharmacology and Experimental Therapeutics*, *349*(3), 373–382. https://doi.org/10.1124/jpet.113.211532

Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., Butler, B. L., Binkowski, B. F., Machleidt, T., Kirkland, T. A., Wood, M. G., Eggers, C. T., Encell, L. P., & Wood, K. V. (2016). NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chemical Biology*, *11*(2), 400–408. https://doi.org/10.1021/acschembio.5b00753

Dowal, L., Yang, W., Freeman, M. R., Steen, H., & Flaumenhaft, R. (2011). Proteomic analysis of

palmitoylated platelet proteins. *Blood*, *118*(13), e62-73. https://doi.org/10.1182/blood-2011-05-353078

Downes, G. B., & Gautam, N. (1999). The G protein subunit gene families. *Genomics*, *62*(3), 544–552. https://doi.org/10.1006/geno.1999.5992

Draper, J. M., & Smith, C. D. (2009). Palmitoyl acyltransferase assays and inhibitors (Review). *Molecular Membrane Biology*, *26*(1), 5–13. https://doi.org/10.1080/09687680802683839

Drisdel, R. C., & Green, W. N. (2004). Labeling and quantifying sites of protein palmitoylation. *BioTechniques*, *36*(2), 276–285. https://doi.org/10.2144/04362rr02

Dudman, J. T., Eaton, M. E., Rajadhyaksha, A., Macías, W., Taher, M., Barczak, A., Kameyama, K., Huganir, R., & Konradi, C. (2003). Dopamine D1 receptors mediate CREB phosphorylation via phosphorylation of the NMDA receptor at Ser897-NR1. *Journal of Neurochemistry*, *87*(4), 922–934. https://doi.org/10.1046/j.1471-4159.2003.02067.x

Dunah, A. W., & Standaert, D. G. (2001). Dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors to the postsynaptic membrane. *Journal of Neuroscience*, *21*(15), 5546–5558. https://doi.org/10.1523/jneurosci.21-15-05546.2001

Duronio, R. J., Towler, D. A., Heuckeroth, R. O., & Gordon, J. I. (1989). Disruption of the yeast N-myristoyl transferase gene causes recessive lethality. *Science (New York, N.Y.)*, *243*(4892), 796–800. https://doi.org/10.1126/science.2644694

Ebersole, B., Petko, J., Woll, M., Murakami, S., Sokolina, K., Wong, V., Stagljar, I., Lüscher, B., & Levenson, R. (2015). Effect of C-Terminal S-Palmitoylation on D2 Dopamine Receptor Trafficking and Stability. *PLOS ONE*, *10*(11), e0140661. https://doi.org/10.1371/journal.pone.0140661

Edmonds, M. J., Geary, B., Doherty, M. K., & Morgan, A. (2017). Analysis of the brain palmitoyl-proteome using both acyl-biotin exchange and acyl-resin-assisted capture methods. *Scientific Reports*, *7*(1), 3299. https://doi.org/10.1038/s41598-017-03562-7

El-Ghundi, M., George, S. R., Drago, J., Fletcher, P. J., Fan, T., Nguyen, T., Liu, C., Sibley, D. R., Westphal, H., & O'Dowd, B. F. (1998). Disruption of dopamine D 1 receptor gene expression attenuates alcohol-seeking behavior. *European Journal of Pharmacology*, *353*(2–3), 149–158. https://doi.org/10.1016/S0014-2999(98)00414-2

El-Husseini, A. E.-D., Schnell, E., Dakoji, S., Sweeney, N., Zhou, Q., Prange, O., Gauthier-Campbell, C., Aguilera-Moreno, A., Nicoll, R. A., & Bredt, D. S. (2002). Synaptic strength regulated by palmitate cycling on PSD-95. *Cell*, *108*(6), 849–863. https://doi.org/10.1016/s0092-8674(02)00683-9

El-Husseini, A. E. D., & Bredt, D. S. (2002). Protein palmitoylation: A regulator of neuronal development and function. *Nature Reviews Neuroscience*, *3*(10), 791–802. https://doi.org/10.1038/nrn940

Englund, P. T. (1993). The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annual Review of Biochemistry*, *62*, 121–138. https://doi.org/10.1146/annurev.bi.62.070193.001005

Ernst, A. M., Syed, S. A., Zaki, O., Bottanelli, F., Zheng, H., Hacke, M., Xi, Z., Rivera-Molina, F.,

Graham, M., Rebane, A. A., Björkholm, P., Baddeley, D., Toomre, D., Pincet, F., & Rothman, J. E. (2018). S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi. *Developmental Cell*, *47*(4), 479-493.e7. https://doi.org/10.1016/j.devcel.2018.10.024

Fan, C. M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A., & Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell*, *81*(3), 457–465. https://doi.org/10.1016/0092-8674(95)90398-4

Faraone, S. V., & Khan, S. A. (2006). Candidate gene studies of attention-deficit/hyperactivity disorder. *Journal of Clinical Psychiatry*, *67*(SUPPL. 8), 13–20. https://doi.org/10.5455/cap.20110330

Farde, L., Nordström, A. L., Wiesel, F. A., Pauli, S., Halldin, C., & Sedvall, G. (1992). Positron emission tomographic analysis of central D1 and D2 dopamine receptor occupancy in patients treated with classical neuroleptics and clozapine. Relation to extrapyramidal side effects. *Archives of General Psychiatry*, *49*(7), 538–544. https://doi.org/10.1001/archpsyc.1992.01820070032005

Farnsworth, C. C., Gelb, M. H., & Glomset, J. A. (1990). Identification of geranylgeranyl-modified proteins in HeLa cells. *Science (New York, N.Y.)*, *247*(4940), 320–322. https://doi.org/10.1126/science.2296721

Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., & Khorana, H. G. (1996). Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science (New York, N.Y.)*, *274*(5288), 768–770. https://doi.org/10.1126/science.274.5288.768

Feinstein, T. N., Yui, N., Webber, M. J., Wehbi, V. L., Stevenson, H. P., King, J. D. J., Hallows, K. R., Brown, D., Bouley, R., & Vilardaga, J.-P. P. (2013). Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. *Journal of Biological Chemistry*, *288*(39), 27849–27860. https://doi.org/10.1074/jbc.M112.445098

Ferguson, M. A., Low, M. G., & Cross, G. A. (1985). Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to Trypanosoma brucei variant surface glycoprotein. *The Journal of Biological Chemistry*, *260*(27), 14547–14555.

Ferguson, M. A., & Williams, A. F. (1988). Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Annual Review of Biochemistry*, *57*, 285–320. https://doi.org/10.1146/annurev.bi.57.070188.001441

Ferrandon, S., Feinstein, T. N., Castro, M., Wang, B., Bouley, R., Potts, J. T., Gardella, T. J., & Vilardaga, J.-P. P. (2009). Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nature Chemical Biology*, *5*(10), 734–742. https://doi.org/10.1038/nchembio.206

Filipek, S., Stenkamp, R. E., Teller, D. C., & Palczewski, K. (2003). G protein-coupled receptor rhodopsin: a prospectus. *Annual Review of Physiology*, *65*, 851–879. https://doi.org/10.1146/annurev.physiol.65.092101.142611

Firth, T. A., & Jones, S. V. (2001). GTP-binding protein Gq mediates muscarinic-receptor-induced inhibition of the inwardly rectifying potassium channel IRK1 (Kir 2.1). *Neuropharmacology*, *40*(3), 358–365. https://doi.org/10.1016/s0028-3908(00)00161-1

Fisher, I. J., Jenkins, M. L., Tall, G. G., Burke, J. E., & Smrcka, A. V. (2020). Activation of Phospholipase C β by Gβγ and Gαq Involves C-Terminal Rearrangement to Release Autoinhibition. *Structure*, *28*(7), 810-819.e5. https://doi.org/https://doi.org/10.1016/j.str.2020.04.012

Fisone, G., Bonito-Oliva, A., & Feyder, M. (2011). Deciphering the Actions of Antiparkinsonian and Antipsychotic Drugs on cAMP/DARPP-32 Signaling . In *Frontiers in Neuroanatomy* (Vol. 5). https://www.frontiersin.org/articles/10.3389/fnana.2011.00038

Fleischhaker, C., Heiser, P., Hennighausen, K., Herpertz-Dahlmann, B., Holtkamp, K., Mehler-Wex, C., Rauh, R., Remschmidt, H., Schulz, E., & Warnke, A. (2007). Weight gain associated with clozapine, olanzapine and risperidone in children and adolescents. *Journal of Neural Transmission (Vienna, Austria : 1996)*, *114*(2), 273–280. https://doi.org/10.1007/s00702-006-0602-7

Flores-Hernández, J., Cepeda, C., Hernández-Echeagaray, E., Calvert, C. R., Jokel, E. S., Fienberg, A. A., Greengard, P., & Levine, M. S. (2002). Dopamine enhancement of NMDA currents in dissociated medium-sized striatal neurons: Role of D1 receptors and DARPP-32. *Journal of Neurophysiology*, *88*(6), 3010–3020. https://doi.org/10.1152/jn.00361.2002

Flores-hernandez, J., Hernandez, S., Snyder, G. L., Yan, Z., Fienberg, A. A., Moss, S. J., Greengard, P., Surmeier, D. J., Hernandez, S., Sny-, G. L., Yan, Z., Fienberg, A. A., Moss, S. J., Green-, P., & Surmeier, D. J. (2019). *D 1 Dopamine Receptor Activation Reduces GABA A Receptor Currents in Neostriatal Neurons Through a PKA / DARPP-32 / PP1 Signaling Cascade*.

Fong, H. K., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F., & Simon, M. I. (2006). Repetitive segmental structure of the transducin beta subunit: homology with the CDC4 gene and identification of related mRNAs. *Proceedings of the National Academy of Sciences*, *83*(7), 2162–2166. https://doi.org/10.1073/pnas.83.7.2162

Fonseca, J. P., Weinberg, Z. Y., Aslankoohi, E., & El-Samad, H. (2020). Biphasic Response of Protein Kinase A to Cyclic Adenosine Monophosphate Triggers Distinct Epithelial Phenotypes. *BioRxiv*, 747030. https://doi.org/10.1101/747030

Foord, S. M., Bonner, T. I., Neubig, R. R., Rosser, E. M., Pin, J.-P., Davenport, A. P., Spedding, M., & Harmar, A. J. (2005). International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacological Reviews*, *57*(2), 279–288. https://doi.org/10.1124/pr.57.2.5

Forrester, M. T., Hess, D. T., Thompson, J. W., Hultman, R., Moseley, M. A., Stamler, J. S., & Casey, P. J. (2011). Site-specific analysis of protein S-acylation by resin-assisted capture. *Journal of Lipid Research*, *52*(2), 393–398. https://doi.org/10.1194/jlr.D011106

Foster, S. R., Hauser, A. S., Vedel, L., Strachan, R. T., Huang, X.-P., Gavin, A. C., Shah, S. D., Nayak, A. P., Haugaard-Kedström, L. M., Penn, R. B., Roth, B. L., Bräuner-Osborne, H., & Gloriam, D. E. (2019). Discovery of Human Signaling Systems: Pairing Peptides to G Protein-Coupled Receptors. *Cell*, *179*(4), 895-908.e21. https://doi.org/10.1016/j.cell.2019.10.010

Fraering, P., Imhof, I., Meyer, U., Strub, J. M., van Dorsselaer, A., Vionnet, C., & Conzelmann, A. (2001). The GPI transamidase complex of Saccharomyces cerevisiae contains Gaa1p, Gpi8p, and Gpi16p. *Molecular Biology of the Cell*, *12*(10), 3295–3306.

https://doi.org/10.1091/mbc.12.10.3295

Frankel, J. S., & Schwartz, T. L. (2017). Brexpiprazole and cariprazine: distinguishing two new atypical antipsychotics from the original dopamine stabilizer aripiprazole. *Therapeutic Advances in Psychopharmacology*, *7*(1), 29–41. https://doi.org/10.1177/2045125316672136

Fraser, C. M., & Venter, J. C. (1982). The size of the mammalian lung β2-adrenergic receptor as determined by target size analysis and immunoaffinity chromatography. *Biochemical and Biophysical Research Communications*, *109*(1), 21–29. https://doi.org/10.1016/0006-291X(82)91560-1

Fredriksson, R., Lagerström, M. C., Lundin, L.-G. G., & Schiöth, H. B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology*, *63*(6), 1256–1272. https://doi.org/10.1124/mol.63.6.1256

Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., & Ernst, O. P. (2003). Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proceedings of the National Academy of Sciences*, *100*(5), 2290–2295. https://doi.org/10.1073/pnas.0435715100

Fujita, M., & Kinoshita, T. (2010). Structural remodeling of GPI anchors during biosynthesis and after attachment to proteins. *FEBS Letters*, *584*(9), 1670–1677. https://doi.org/10.1016/j.febslet.2009.10.079

Fukata, Y., Dimitrov, A., Boncompain, G., Vielemeyer, O., Perez, F., & Fukata, M. (2013). Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *Journal of Cell Biology*, *202*(1), 145–161. https://doi.org/10.1083/jcb.201302071

Fukata, Y., & Fukata, M. (2010). Protein palmitoylation in neuronal development and synaptic plasticity. *Nature Reviews Neuroscience*, *11*(3), 161–175. https://doi.org/10.1038/nrn2788

Fukata, Y., Murakami, T., Yokoi, N., & Fukata, M. (2016). Local Palmitoylation Cycles and Specialized Membrane Domain Organization. In *Current Topics in Membranes* (Vol. 77). Elsevier Ltd. https://doi.org/10.1016/bs.ctm.2015.10.003

Fukushima, Y., Saitoh, T., Anai, M., Ogihara, T., Inukai, K., Funaki, M., Sakoda, H., Onishi, Y., Ono, H., Fujishiro, M., Ishikawa, T., Takata, K., Nagai, R., Omata, M., & Asano, T. (2001). Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochimica et Biophysica Acta*, *1539*(3), 181–191. https://doi.org/10.1016/s0167-4889(01)00104-5

Fuxe, K., Manger, P., Genedani, S., & Agnati, L. (2006). The nigrostriatal DA pathway and Parkinson's disease. *Parkinson's Disease and Related Disorders*, 71–83. https://doi.org/10.1007/978-3-211-45295-0_13

Gacasan, S. B., Baker, D. L., & Parrill, A. L. (2017). G protein-coupled receptors: the evolution of structural insight. *AIMS Biophysics*, *4*(3), 491–527. https://doi.org/10.3934/biophy.2017.3.491

Gaillard, I., Rouquier, S., & Giorgi, D. (2004). Olfactory receptors. *Cellular and Molecular Life Sciences : CMLS*, *61*(4), 456–469. https://doi.org/10.1007/s00018-003-3273-7

Galarraga, E., Hernández-Löpez, S., Reyes, A., Barral, J., & Bargas, J. (1997). Dopamine facilitates

striatal EPSPs through an L-type Ca2+ conductance. *NeuroReport*, *8*(9–10), 2183–2186. https://doi.org/10.1097/00001756-199707070-00019

Gao, C., Sun, X., & Wolf, M. E. (2006). Activation of D1 dopamine receptors increases surface expression of AMPA receptors and facilitates their synaptic incorporation in cultured hippocampal neurons. *Journal of Neurochemistry*, *98*(5), 1664–1677. https://doi.org/10.1111/j.1471-4159.2006.03999.x

Gao, X., & Hannoush, R. N. (2018). A Decade of Click Chemistry in Protein Palmitoylation: Impact on Discovery and New Biology. *Cell Chemical Biology*, *25*(3), 236–246. https://doi.org/10.1016/j.chembiol.2017.12.002

Gao, Z.-G., Verzijl, D., Zweemer, A., Ye, K., Göblyös, A., Ijzerman, A. P., & Jacobson, K. A. (2011). Functionally biased modulation of A(3) adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. *Biochemical Pharmacology*, *82*(6), 658–668. https://doi.org/10.1016/j.bcp.2011.06.017

Gao, Z., Ni, Y., Szabo, G., & Linden, J. (1999). Palmitoylation of the recombinant human A1 adenosine receptor: Enhanced proteolysis of palmitoylation-deficient mutant receptors. *Biochemical Journal*, *342*(2), 387–395. https://doi.org/10.1042/0264-6021:3420387

Garbison, K. E., Heinz, B. A., Lajiness, M. E., & Weidner, J. R. (2015). *Phospho-ERK Assays Overview of Technology AlphaScreen SureFire ERK Assay General Background Characteristics of the AlphaScreen SureFire ERK Assay*. *Md*, 1–6.

Gauthier-Kemper, A., Igaev, M., Sündermann, F., Janning, D., Brühmann, J., Moschner, K., Reyher, H.-J., Junge, W., Glebov, K., Walter, J., Bakota, L., & Brandt, R. (2014). Interplay between phosphorylation and palmitoylation mediates plasma membrane targeting and sorting of GAP43. *Molecular Biology of the Cell*, *25*(21), 3284–3299. https://doi.org/10.1091/mbc.E13-12-0737

Gerfen, C. R., Keefe, K. A., & Gauda, E. B. (1995). D1 and D2 dopamine receptor function in the striatum: Coactivation of D1- and D2-dopamine receptors on separate populations of neurons results in potentiated immediate early gene response in D1-containing neurons. *Journal of Neuroscience*, *15*(12), 8167–8176. https://doi.org/10.1523/jneurosci.15-12-08167.1995

Gether, U. (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocrine Reviews*, *21*(1), 90–113. https://doi.org/10.1210/edrv.21.1.0390

Ghosh, E., Kumari, P., Jaiman, D., & Shukla, A. K. (2015). Methodological advances: the unsung heroes of the GPCR structural revolution. *Nature Reviews Molecular Cell Biology*, *16*(2), 69–81. https://doi.org/10.1038/nrm3933

Giang, D. K., & Cravatt, B. F. (1998). A second mammalian N-myristoyltransferase. *The Journal of Biological Chemistry*, *273*(12), 6595–6598. https://doi.org/10.1074/jbc.273.12.6595

Gibbs, J. B. (1991). Ras C-terminal processing enzymes--new drug targets? *Cell*, *65*(1), 1–4. https://doi.org/10.1016/0092-8674(91)90352-y

Giros, B., Sokoloff, P., Martres, M. P., Riou, J. F., Emorine, L. J., & Schwartz, J. C. (1989). Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature*, *342*(6252), 923–926. https://doi.org/10.1038/342923a0

Godbole, A., Lyga, S., Lohse, M. J., & Calebiro, D. (2017). Internalized TSH receptors en route to the TGN induce local Gs-protein signaling and gene transcription. *Nature Communications*, *8*(1), 443.

Goddard, A. D., & Watts, A. (2012). Regulation of G protein-coupled receptors by palmitoylation and cholesterol. In *BMC biology* (Vol. 10, p. 27). https://doi.org/10.1186/1741-7007-10-27

Gorinski, N., Bijata, M., Prasad, S., Wirth, A., Abdel Galil, D., Zeug, A., Bazovkina, D., Kondaurova, E., Kulikova, E., Ilchibaeva, T., Zareba-Koziol, M., Papaleo, F., Scheggia, D., Kochlamazashvili, G., Dityatev, A., Smyth, I., Krzystyniak, A., Wlodarczyk, J., Richter, D. W., … Ponimaskin, E. (2019). Attenuated palmitoylation of serotonin receptor 5-HT1A affects receptor function and contributes to depression-like behaviors. *Nature Communications*, *10*(1), 3924. https://doi.org/10.1038/s41467-019-11876-5

Gorvel, J.-P., Chavrier, P., Zerial, M., & Gruenberg, J. (1991). rab5 controls early endosome fusion in vitro. *Cell*, *64*(5), 915–925. https://doi.org/https://doi.org/10.1016/0092-8674(91)90316-Q

Gottwald, M. D., Bainbridge, J. L., Dowling, G. A., Aminoff, M. J., & Alldredge, B. K. (1997). New pharmacotherapy for Parkinson's disease. *The Annals of Pharmacotherapy*, *31*(10), 1205–1217. https://doi.org/10.1177/106002809703101014

Goulabchand, R., Vincent, T., Batteux, F., Eliaou, J.-F., & Guilpain, P. (2014). Impact of autoantibody glycosylation in autoimmune diseases. *Autoimmunity Reviews*, *13*(7), 742–750. https://doi.org/10.1016/j.autrev.2014.02.005

Grace, A. A. (2016). Dysregulation of the dopamine system in the pathophysiology of schizophrenia and depression. *Nature Reviews. Neuroscience*, *17*(8), 524–532. https://doi.org/10.1038/nrn.2016.57

Gray, D. L., Allen, J. A., Mente, S., O'connor, R. E., Demarco, G. J., Efremov, I., Tierney, P., Volfson, D., Davoren, J., Guilmette, E., Salafia, M., Kozak, R., & Ehlers, M. D. (2018). Impaired β-arrestin recruitment and reduced desensitization by non-catechol agonists of the D1 dopamine receptor. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-017-02776-7

Greaves, J., & Chamberlain, L. H. (2011). DHHC palmitoyl transferases: Substrate interactions and (patho)physiology. *Trends in Biochemical Sciences*, *36*(5), 245–253. https://doi.org/10.1016/j.tibs.2011.01.003

Greaves, J., Munro, K. R., Davidson, S. C., Riviere, M., Wojno, J., Smith, T. K., Tomkinson, N. C. O., & Chamberlain, L. H. (2017). Molecular basis of fatty acid selectivity in the zDHHC family of S-acyltransferases revealed by click chemistry. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(8), E1365–E1374. https://doi.org/10.1073/pnas.1612254114

Greengard, P., Allen, P. B., & Nairn, A. C. (1999). Beyond the dopamine receptor: The DARPP-32/protein phosphatase-1 cascade. *Neuron*, *23*(3), 435–447. https://doi.org/10.1016/S0896-6273(00)80798-9

Greif, G. J., Sodickson, D. L., Bean, B. P., Neer, E. J., & Mende, U. (2017). Altered Regulation of Potassium and Calcium Channels by GABA B and Adenosine Receptors in Hippocampal Neurons From Mice Lacking Gα o

. *Journal of Neurophysiology*, *83*(2), 1010–1018. https://doi.org/10.1152/jn.2000.83.2.1010

Grünewald, S., Haase, W., Reiländer, H., & Michel, H. (1996). Glycosylation, palmitoylation, and localization of the human D(2S) receptor in baculovirus-infected insect cells. *Biochemistry*, *35*(48), 15149–15161. https://doi.org/10.1021/bi9607564

Guan, X., & Fierke, C. A. (2011). Understanding protein palmitoylation: Biological significance and enzymology. *Science China Chemistry*, *54*(12), 1888–1897. https://doi.org/10.1007/s11426-011-4428-2

Guerra, F., & Bucci, C. (2016). Multiple Roles of the Small GTPase Rab7. *Cells*, *5*(3). https://doi.org/10.3390/cells5030034

Guo, H., An, S., Ward, R., Yang, Y., Liu, Y., Guo, X.-X., Hao, Q., & Xu, T.-R. (2017). Methods used to study the oligomeric structure of G-protein-coupled receptors. *Bioscience Reports*, *37*(2). https://doi.org/10.1042/BSR20160547

Gurevich, V. V., & Gurevich, E. V. (2019). GPCR signaling regulation: The role of GRKs and arrestins. *Frontiers in Pharmacology*, *10*(FEB), 1–11. https://doi.org/10.3389/fphar.2019.00125

Gurevich, V. V, & Gurevich, E. V. (2004). The molecular acrobatics of arrestin activation. *Trends in Pharmacological Sciences*, *25*(2), 105–111. https://doi.org/10.1016/j.tips.2003.12.008

Haddad, P. M., Brain, C., & Scott, J. (2014). Nonadherence with antipsychotic medication in schizophrenia: challenges and management strategies. *Patient Related Outcome Measures*, *5*, 43–62. https://doi.org/10.2147/PROM.S42735

Haider, R. S., Matthees, E. S. F., Drube, J., Reichel, M., Zabel, U., Inoue, A., Chevigné, A., Krasel, C., Deupi, X., & Hoffmann, C. (2022). β-arrestin1 and 2 exhibit distinct phosphorylation-dependent conformations when coupling to the same GPCR in living cells. *Nature Communications*, *13*(1), 5638. https://doi.org/10.1038/s41467-022-33307-8

Haltiwanger, R. S., & Lowe, J. B. (2004). Role of glycosylation in development. *Annual Review of Biochemistry*, *73*, 491–537. https://doi.org/10.1146/annurev.biochem.73.011303.074043

Han, J., Wu, P., Wang, F., & Chen, J. (2015). S-palmitoylation regulates AMPA receptors trafficking and function: A novel insight into synaptic regulation and therapeutics. *Acta Pharmaceutica Sinica B*, *5*(1), 1–7. https://doi.org/10.1016/j.apsb.2014.12.002

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell*, *57*(7), 1167–1177. https://doi.org/10.1016/0092-8674(89)90054-8

Hannoush, R. N., & Arenas-Ramirez, N. (2009). Imaging the lipidome: omega-alkynyl fatty acids for detection and cellular visualization of lipid-modified proteins. *ACS Chemical Biology*, *4*(7), 581–587. https://doi.org/10.1021/cb900085z

Hannoush, R. N., & Sun, J. (2010). The chemical toolbox for monitoring protein fatty acylation and prenylation. *Nature Chemical Biology*, *6*(7), 498–506. https://doi.org/10.1038/nchembio.388

Hanyaloglu, A. C., & von Zastrow, M. (2008). Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annual Review of Pharmacology and Toxicology*, *48*, 537–568. https://doi.org/10.1146/annurev.pharmtox.48.113006.094830

Hartman, H. L., Hicks, K. A., & Fierke, C. A. (2005). Peptide specificity of protein prenyltransferases

is determined mainly by reactivity rather than binding affinity. *Biochemistry*, *44*(46), 15314–15324. https://doi.org/10.1021/bi0509503

Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., & Gloriam, D. E. (2017). Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews. Drug Discovery*, *16*(12), 829–842. https://doi.org/10.1038/nrd.2017.178

Hawtin, S. R., Tobin, A. B., Patel, S., & Wheatley, M. (2001). Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *The Journal of Biological Chemistry*, *276*(41), 38139–38146. https://doi.org/10.1074/jbc.M106142200

Hayashi, T. (2021). Post-translational palmitoylation of ionotropic glutamate receptors in excitatory synaptic functions. *British Journal of Pharmacology*, *178*(4), 784–797. https://doi.org/10.1111/bph.15050

Hemmings, H. C., Greengard, P., Tung, H. Y. L., & Cohen, P. (1984). DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature*, *310*(5977), 503–505. https://doi.org/10.1038/310503a0

Henry, L., & Sheff, D. R. (2008). Rab8 Regulates Basolateral Secretory, But Not Recycling, Traffic at the Recycling Endosome. *Molecular Biology of the Cell*, *19*(5), 2059–2068. https://doi.org/10.1091/mbc.e07-09-0902

Hilger, D. (2018). Structure and dynamics of GPCR signaling complexes. *Nature Structural & Molecular Biology*, *25*(1), 4–12. https://doi.org/10.1038/s41594-017-0011-7

Hoare, S. R. J. (2005). Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discovery Today*, *10*(6), 417–427. https://doi.org/10.1016/S1359-6446(05)03370-2

Hoffman, G. R., Nassar, N., & Cerione, R. A. (2000). Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. *Cell*, *100*(3), 345–356. https://doi.org/10.1016/s0092-8674(00)80670-4

Hong, C.-J., Liou, Y.-J., Bai, Y. M., Chen, T.-T., Wang, Y.-C., & Tsai, S.-J. (2010). Dopamine receptor D2 gene is associated with weight gain in schizophrenic patients under long-term atypical antipsychotic treatment. *Pharmacogenetics and Genomics*, *20*(6), 359–366. https://doi.org/10.1097/FPC.0b013e3283397d06

Hoon, M. A., Adler, E., Lindemeier, J., Battey, J. F., Ryba, N. J., & Zuker, C. S. (1999). Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell*, *96*(4), 541–551. https://doi.org/10.1016/s0092-8674(00)80658-3

Horn, F., Bettler, E., Oliveira, L., Campagne, F., Cohen, F. E., & Vriend, G. (2003). GPCRDB information system for G protein-coupled receptors. *Nucleic Acids Research*, *31*(1), 294–297. https://doi.org/10.1093/nar/gkg103

Howes, O. D., & Kapur, S. (2009). The Dopamine Hypothesis of Schizophrenia: Version III—The Final Common Pathway. *Schizophrenia Bulletin*, *35*(3), 549–562. https://doi.org/10.1093/schbul/sbp006

Hu, G. M., Mai, T. L., & Chen, C. M. (2017). Visualizing the GPCR Network: Classification and

Evolution. *Scientific Reports*, *7*(1), 15495. https://doi.org/10.1038/s41598-017-15707-9

Hu, J.-L., Liu, G., Li, Y.-C., Gao, W.-J., & Huang, Y.-Q. (2010). Dopamine D1 receptor-mediated NMDA receptor insertion depends on Fyn but not Src kinase pathway in prefrontal cortical neurons. *Molecular Brain*, *3*(1), 20. https://doi.org/10.1186/1756-6606-3-20

Huang, K.-Y., Lee, T.-Y., Kao, H.-J., Ma, C.-T., Lee, C.-C., Lin, T.-H., Chang, W.-C., & Huang, H.-D. (2019). dbPTM in 2019: exploring disease association and cross-talk of post-translational modifications. *Nucleic Acids Research*, *47*(D1), D298–D308. https://doi.org/10.1093/nar/gky1074

Huang, X., Lewis, M. M., Van Scoy, L. J., De Jesus, S., Eslinger, P. J., Arnold, A. C., Miller, A. J., Fernandez-Mendoza, J., Snyder, B., Harrington, W., Kong, L., Wang, X., Sun, D., Delnomdedieu, M., Duvvuri, S., Mahoney, S. E., Gray, D. L., & Mailman, R. B. (2020). The D1/D5 Dopamine Partial Agonist PF-06412562 in Advanced-Stage Parkinson's Disease: A Feasibility Study. *Journal of Parkinson's Disease*, *10*(4), 1515–1527. https://doi.org/10.3233/JPD-202188

Hukovic, N., Panetta, R., Kumar, U., Rocheville, M., & Patel, Y. C. (1998). The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. *The Journal of Biological Chemistry*, *273*(33), 21416–21422. https://doi.org/10.1074/jbc.273.33.21416

Hurley, J. H., Cahill, A. L., Currie, K. P., & Fox, A. P. (2000). The role of dynamic palmitoylation in Ca2+ channel inactivation. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(16), 9293–9298. https://doi.org/10.1073/pnas.160589697

Hurley, M. J., & Jenner, P. (2006). What has been learnt from study of dopamine receptors in Parkinson's disease? *Pharmacology and Therapeutics*, *111*(3), 715–728. https://doi.org/10.1016/j.pharmthera.2005.12.001

Hutagalung, A. H., & Novick, P. J. (2011). Role of Rab GTPases in membrane traffic and cell physiology. *Physiological Reviews*, *91*(1), 119–149. https://doi.org/10.1152/physrev.00059.2009

Iijima, S., & Van Tol, H. H. M. (1991). © 19 9 1 Nature Publishing Group 그라첼꺼. *Nature*, *354*, 56–58.

Irannejad, R., Pessino, V., Mika, D., Huang, B., Wedegaertner, P. B., Conti, M., & von Zastrow, M. (2017). Functional selectivity of GPCR-directed drug action through location bias. *Nature Chemical Biology*, *13*(7), 799–806. https://doi.org/10.1038/nchembio.2389

Irannejad, R., Tomshine, J. C. J. R., Tomshine, J. C. J. R., Chevalier, M., Mahoney, J. P., Steyaert, J., Rasmussen, S. G. F., Sunahara, R. K., El-Samad, H., Huang, B., & Von Zastrow, M. (2013). Conformational biosensors reveal GPCR signalling from endosomes. *Nature*, *495*(7442), 534–538. https://doi.org/10.1038/nature12000

Iversen, S. D., & Iversen, L. L. (2007). Dopamine: 50 years in perspective. *Trends in Neurosciences*, *30*(5), 188–193. https://doi.org/10.1016/j.tins.2007.03.002

Jain, M. K., Nilson, A. N., Felsing, D. E., Inoue, A., & Allen, J. A. (2020). CRISPR/Cas9-mediated knockout of G proteins and β-arrestins delineates their distinct but interdependent roles in

dopamine D1 receptor signaling. *The FASEB Journal*, *34*(S1), 1. https://doi.org/https://doi.org/10.1096/fasebj.2020.34.s1.04087

James, J. R., Oliveira, M. I., Carmo, A. M., Iaboni, A., & Davis, S. J. (2006). A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. *Nature Methods*, *3*(12), 1001–1006. https://doi.org/10.1038/nmeth978

Janetzko, J., Kise, R., Barsi-Rhyne, B., Siepe, D. H., Heydenreich, F. M., Kawakami, K., Masureel, M., Maeda, S., Garcia, K. C., von Zastrow, M., Inoue, A., & Kobilka, B. K. (2022). Membrane phosphoinositides regulate GPCR-β-arrestin complex assembly and dynamics. *Cell*, *185*(24), 4560-4573.e19. https://doi.org/10.1016/j.cell.2022.10.018

Janetzko, J., Kise, R., Barsi-Ryne, B., Siepe, D. H., Heydenreich, F. M., Masureel, M., Kawakami, K., Garcia, K. C., von Zastrow, M., Inoue, A., & Kobilka, B. K. (2021). Membrane phosphoinositides stabilize GPCR-arrestin complexes and offer temporal control of complex assembly and dynamics. *BioRxiv*, 2021.10.09.463790. https://doi.org/10.1101/2021.10.09.463790

Jansen, M., & Beaumelle, B. (2022). How palmitoylation affects trafficking and signaling of membrane receptors. *Biology of the Cell*, *114*(2), 61–72. https://doi.org/https://doi.org/10.1111/boc.202100052

Jean-Charles, P.-Y., Kaur, S., & Shenoy, S. K. (2017). G Protein-Coupled Receptor Signaling Through β-Arrestin-Dependent Mechanisms. *Journal of Cardiovascular Pharmacology*, *70*(3), 142–158. https://doi.org/10.1097/FJC.0000000000000482

Jennings, B. C., & Linder, M. E. (2010). Regulation of g proteins by covalent modification. *Handbook of Cell Signaling*, *2/E*, *2*, 1629–1633. https://doi.org/10.1016/B978-0-12-374145-5.00200-X

Jennings, B. C., & Linder, M. E. (2012). DHHC protein S-acyltransferases use similar ping-pong kinetic mechanisms but display different Acyl-CoA specificities. *Journal of Biological Chemistry*, *287*(10), 7236–7245. https://doi.org/10.1074/jbc.M111.337246

Jennings, B. C., Nadolski, M. J., Ling, Y., Baker, M. B., Harrison, M. L., Deschenes, R. J., & Linder, M. E. (2009). 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. *Journal of Lipid Research*, *50*(2), 233–242. https://doi.org/10.1194/jlr.M800270-JLR200

Jensen, D. D., Godfrey, C. B., Niklas, C., Canals, M., Kocan, M., Poole, D. P., Murphy, J. E., Alemi, F., Cottrell, G. S., Korbmacher, C., Lambert, N. A., Bunnett, N. W., & Corvera, C. U. (2013). The bile acid receptor TGR5 does not interact with β-arrestins or traffic to endosomes but transmits sustained signals from plasma membrane rafts. *The Journal of Biological Chemistry*, *288*(32), 22942–22960. https://doi.org/10.1074/jbc.M113.455774

Jensen, O. N. (2004). Modification-specific proteomics: Characterization of post-translational modifications by mass spectrometry. *Current Opinion in Chemical Biology*, *8*(1), 33–41. https://doi.org/10.1016/j.cbpa.2003.12.009

Jeyifous, O., Lin, E. I., Chen, X., Antinone, S. E., Mastro, R., Drisdel, R., Reese, T. S., & Green, W. N. (2016). Palmitoylation regulates glutamate receptor distributions in postsynaptic densities

through control of PSD95 conformation and orientation. *Proceedings of the National Academy of Sciences*, *113*(52), E8482–E8491. https://doi.org/10.1073/pnas.1612963113

Jin, H., Xie, Z., George, S. R., & O'Dowd, B. F. (1999). Palmitoylation occurs at cysteine 347 and cysteine 351 of the dopamine D1 receptor. *European Journal of Pharmacology*, *386*(2–3), 305–312. https://doi.org/10.1016/S0014-2999(99)00727-X

Jin, H., Zastawny, R., George, S. R., & O'Dowd, B. F. (1997). Elimination of palmitoylation sites in the human dopamine D1 receptor does not affect receptor-G protein interaction. *European Journal of Pharmacology*, *324*(1), 109–116. https://doi.org/https://doi.org/10.1016/S0014-2999(97)00059-9

Jin, L. Q., Goswami, S., Cai, G., Zhen, X., & Friedman, E. (2003). SKF83959 selectively regulates phosphatidylinositol-linked D1 dopamine receptors in rat brain. *Journal of Neurochemistry*, *85*(2), 378–386. https://doi.org/10.1046/j.1471-4159.2003.01698.x

Johnson, K. B., Criswell, H. E., Jensen, K. F., Simson, P. E., Mueller, R. A., & Breese, G. R. (1992). Comparison of the D1-dopamine agonists SKF-38393 and A-68930 in neonatal 6-hydroxydopamine-lesioned rats: behavioral effects and induction of c-fos-like immunoreactivity. *The Journal of Pharmacology and Experimental Therapeutics*, *262*(2), 855–865.

Jose, P. A., Yamapchi, I., Eisner, G. M., Maral Mouradian, M., Felder, C. C., & Felder, R. A. (1995). Dopamine Dl Regulation of Phospholipase c. *Hypertens Res*, *18*(suppl 1), 39–42.

Juárez Olguín, H., Calderón Guzmán, D., Hernández García, E., & Barragán Mejía, G. (2016). The Role of Dopamine and Its Dysfunction as a Consequence of Oxidative Stress. *Oxidative Medicine and Cellular Longevity*, *2016*, 9730467. https://doi.org/10.1155/2016/9730467

Kahlig, K. M., Binda, F., Khoshbouei, H., Blakely, R. D., McMahon, D. G., Javitch, J. A., & Galli, A. (2005). Amphetamine induces dopamine efflux through a dopamine transporter channel. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(9), 3495–3500. https://doi.org/10.1073/pnas.0407737102

Kamiya, Y., Sakurai, A., Tamura, S., & Takahashi, N. (1978). Structure of rhodotorucine A, a novel lipopeptide, inducing mating tube formation in Rhodosporidium toruloides. *Biochemical and Biophysical Research Communications*, *83*(3), 1077–1083. https://doi.org/10.1016/0006-291x(78)91505-x

Kang, R., Wan, J., Arstikaitis, P., Takahashi, H., Huang, K., Bailey, A. O., Thompson, J. X., Roth, A. F., Drisdel, R. C., Mastro, R., Green, W. N., Yates, J. R. 3rd, Davis, N. G., & El-Husseini, A. (2008). Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature*, *456*(7224), 904–909. https://doi.org/10.1038/nature07605

Kang, Y., Zhou, X. E., Gao, X., He, Y., Liu, W., Ishchenko, A., Barty, A., White, T. A., Yefanov, O., Han, G. W., Xu, Q., de Waal, P. W., Ke, J., Tan, M. H. E., Zhang, C., Moeller, A., West, G. M., Pascal, B. D., Van Eps, N., … Xu, H. E. (2015). Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature*, *523*(7562), 561–567. https://doi.org/10.1038/nature14656

Karageorgos, V., Venihaki, M., Sakellaris, S., Pardalos, M., Kontakis, G., Matsoukas, M.-T., Gravanis, A., Margioris, A., & Liapakis, G. (2018). Current understanding of the structure and

function of family B GPCRs to design novel drugs. *Hormones*, *17*(1), 45–59. https://doi.org/10.1007/s42000-018-0009-5

Karnik, S. S., & Khorana, H. G. (1990). Assembly of functional rhodopsin requires a disulfide bond between cysteine residues 110 and 187. *The Journal of Biological Chemistry*, *265*(29), 17520–17524.

Karve, T. M., & Cheema, A. K. (2011). Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease. *Journal of Amino Acids*, *2011*.

Kaya, A. I., Perry, N. A., Gurevich, V. V, & Iverson, T. M. (2020). Phosphorylation barcode-dependent signal bias of the dopamine D1 receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(25), 14139–14149. https://doi.org/10.1073/pnas.1918736117

Kemp, J. M., & Powell, T. P. (1971). The synaptic organization of the caudate nucleus. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *262*(845), 403–412. https://doi.org/10.1098/rstb.1971.0103

Kienast, T., & Heinz, A. (2006). Dopamine and the diseased brain. *CNS & Neurological Disorders Drug Targets*, *5*(1), 109–131. http://www.ncbi.nlm.nih.gov/pubmed/16613557

Kim, H., Baek, I.-Y., & Seong, J. (2022). Genetically encoded fluorescent biosensors for GPCR research . In *Frontiers in Cell and Developmental Biology* (Vol. 10). https://www.frontiersin.org/articles/10.3389/fcell.2022.1007893

Kirchberg, K., Kim, T.-Y., Möller, M., Skegro, D., Dasara Raju, G., Granzin, J., Büldt, G., Schlesinger, R., & Alexiev, U. (2011). Conformational dynamics of helix 8 in the GPCR rhodopsin controls arrestin activation in the desensitization process. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(46), 18690–18695. https://doi.org/10.1073/pnas.1015461108

Kirchhausen, T., Macia, E., & Pelish, H. E. (2008). Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis. *Methods in Enzymology*, *438*, 77–93. https://doi.org/10.1016/S0076-6879(07)38006-3

Ko, P.-J., & Dixon, S. J. (2018). Protein palmitoylation and cancer. *EMBO Reports*, *19*(10), e46666. https://doi.org/https://doi.org/10.15252/embr.201846666

Kobe, F., Renner, U., Woehler, A., Wlodarczyk, J., Papusheva, E., Bao, G., Zeug, A., Richter, D. W., Neher, E., & Ponimaskin, E. (2008). Stimulation- and palmitoylation-dependent changes in oligomeric conformation of serotonin 5-HT1A receptors. *Biochimica et Biophysica Acta*, *1783*(8), 1503–1516. https://doi.org/10.1016/j.bbamcr.2008.02.021

Kobilka, B. (1992). *ADRENERGIC RECEPTORS PROTEIN-COUPLED RECEPTORS*. 87–114.

Koehl, A., Hu, H., Maeda, S., Zhang, Y., Qu, Q., Paggi, J. M., Latorraca, N. R., Hilger, D., Dawson, R., Matile, H., Schertler, G. F. X., Granier, S., Weis, W. I., Dror, R. O., Manglik, A., Skiniotis, G., & Kobilka, B. K. (2018). Structure of the μ-opioid receptor-G(i) protein complex. *Nature*, *558*(7711), 547–552. https://doi.org/10.1038/s41586-018-0219-7

Koh, P. O., Bergson, C., Undie, A. S., Goldman-Rakic, P. S., & Lidow, M. S. (2003). Up-regulation

of the D1 dopamine receptor-interacting protein, calcyon, in patients with schizophrenia. *Archives of General Psychiatry*, *60*(3), 311–319. https://doi.org/10.1001/archpsyc.60.3.311

Kolakowski, L. F. J. (1994). GCRDb: a G-protein-coupled receptor database. *Receptors & Channels*, *2*(1), 1–7.

Kong, M. M. C., Verma, V., O'Dowd, B. F., & George, S. R. (2011). The role of palmitoylation in directing dopamine D1 receptor internalization through selective endocytic routes. *Biochemical and Biophysical Research Communications*, *405*(3), 445–449. https://doi.org/10.1016/j.bbrc.2011.01.050

Konradi, C., Cole, R. L., Heckers, S., & Hyman, S. E. (1994). Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *Journal of Neuroscience*, *14*(9), 5623–5634. https://doi.org/10.1523/jneurosci.14-09-05623.1994

Kotowski, S. J. J., Hopf, F. W. W., Seif, T., Bonci, A., von Zastrow, M., & von Zastrow, M. (2011). Endocytosis Promotes Rapid Dopaminergic Signaling. *Neuron*, *71*(2), 278–290. https://doi.org/https://doi.org/10.1016/j.neuron.2011.05.036

Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., & Sternweis, P. C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science (New York, N.Y.)*, *280*(5372), 2109–2111. https://doi.org/10.1126/science.280.5372.2109

Kraft, K., Olbrich, H., Majoul, I., Mack, M., Proudfoot, A., & Oppermann, M. (2001). Characterization of Sequence Determinants within the Carboxyl-terminal Domain of Chemokine Receptor CCR5 that Regulate Signaling and Receptor Internalization. *Journal of Biological Chemistry*, *276*(37), 34408–34418. https://doi.org/10.1074/jbc.M102782200

Krasel, C., Bünemann, M., Lorenz, K., & Lohse, M. J. (2005). β-Arrestin Binding to the β2-Adrenergic Receptor Requires Both Receptor Phosphorylation and Receptor Activation*. *Journal of Biological Chemistry*, *280*(10), 9528–9535. https://doi.org/https://doi.org/10.1074/jbc.M413078200

Krupnick, J. G., & Benovic, J. L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annual Review of Pharmacology and Toxicology*, *38*, 289–319. https://doi.org/10.1146/annurev.pharmtox.38.1.289

Krupnick, J. G., Gurevich, V. V, & Benovic, J. L. (1997). Mechanism of quenching of phototransduction: binding competition between arrestin and transducin for phosphorhodopsin. *Journal of Biological Chemistry*, *272*(29), 18125–18131.

Kucera, A., Bakke, O., & Progida, C. (2016). The multiple roles of Rab9 in the endolysosomal system. *Communicative & Integrative Biology*, *9*(4), e1204498. https://doi.org/10.1080/19420889.2016.1204498

Kumari, P., Srivastava, A., Banerjee, R., Ghosh, E., Gupta, P., Ranjan, R., Chen, X., Gupta, B., Gupta, C., Jaiman, D., & Shukla, A. K. (2016). Functional competence of a partially engaged GPCR–β-arrestin complex. *Nature Communications*, *7*(1), 13416. https://doi.org/10.1038/ncomms13416

Kurz, M., Hummer, M., Oberbauer, H., & Fleischhacker, W. W. (1995). Extrapyramidal side effects

of clozapine and haloperidol. *Psychopharmacology*, *118*(1), 52–56. https://doi.org/10.1007/BF02245249

Kvachnina, E., Dumuis, A., Wlodarczyk, J., Renner, U., Cochet, M., Richter, D. W., & Ponimaskin, E. (2009). Constitutive Gs-mediated, but not G12-mediated, activity of the 5-hydroxytryptamine 5-HT7(a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et Biophysica Acta*, *1793*(11), 1646–1655. https://doi.org/10.1016/j.bbamcr.2009.08.008

Ladds, G., Goddard, A., Hill, C., Thornton, S., & Davey, J. (2007). Differential effects of RGS proteins on Gαq and Gα11 activity. *Cellular Signalling*, *19*(1), 103–113. https://doi.org/https://doi.org/10.1016/j.cellsig.2006.05.027

Lagerström, M. C., & Schiöth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature Reviews. Drug Discovery*, *7*(4), 339–357. https://doi.org/10.1038/nrd2518

Lan, T.-H., Kuravi, S., & Lambert, N. A. (2011). Internalization dissociates β2-adrenergic receptors. *PloS One*, *6*(2), e17361. https://doi.org/10.1371/journal.pone.0017361

Lan, T.-H., Liu, Q., Li, C., Wu, G., & Lambert, N. A. (2012). Sensitive and high resolution localization and tracking of membrane proteins in live cells with BRET. *Traffic (Copenhagen, Denmark)*, *13*(11), 1450–1456. https://doi.org/10.1111/j.1600-0854.2012.01401.x

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., … Szustakowki, J. (2001). Initial sequencing and analysis of the human genome. *Nature*, *409*(6822), 860–921. https://doi.org/10.1038/35057062

Landwehrmeyer, B., Mengod, G., & Palacios, J. M. (1993). Dopamine D3 receptor mRNA and binding sites in human brain. *Molecular Brain Research*, *18*(1–2), 187–192. https://doi.org/10.1016/0169-328X(93)90188-U

Langen, B., & Dost, R. (2011). Comparison of SHR, WKY and Wistar rats in different behavioural animal models: effect of dopamine D1 and alpha2 agonists. *Attention Deficit and Hyperactivity Disorders*, *3*(1), 1–12. https://doi.org/10.1007/s12402-010-0034-y

Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., & Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British Journal of Pharmacology*, *172*(20), 4790–4805. https://doi.org/10.1111/bph.13250

Lavine, N., Ethier, N., Oak, J. N., Pei, L., Liu, F., Trieu, P., Rebois, R. V., Bouvier, M., Hébert, T. E., & Van Tol, H. H. M. M. (2002). G Protein-coupled Receptors Form Stable Complexes with Inwardly Rectifying Potassium Channels and Adenylyl Cyclase*. *Journal of Biological Chemistry*, *277*(48), 46010–46019. https://doi.org/https://doi.org/10.1074/jbc.M205035200

Le Crom, S., Kapsimali, M., Barôme, P. O., & Vernier, P. (2003). Dopamine receptors for every species: Gene duplications and functional diversification in Craniates. *Journal of Structural and Functional Genomics*, *3*(1–4), 161–176. https://doi.org/10.1023/A:1022686622752

Le Foll, B., Gallo, A., Strat, Y. Le, Lu, L., & Gorwood, P. (2009). Genetics of dopamine receptors and drug addiction: A comprehensive review. *Behavioural Pharmacology*, *20*(1), 1–17.

https://doi.org/10.1097/FBP.0b013e3283242f05

Lebel, M., Robinson, P., & Cyr, M. (2006). : The Role of Dopamine Receptor Function in Neurodegenerative Diseases. *The Canadian Journal of Neurological Sciences*, 18–29.

Lee, C. W., Lee, K. H., Lee, S. B., Park, D., & Rhee, S. G. (1994). Regulation of phospholipase C-beta 4 by ribonucleotides and the alpha subunit of Gq. *The Journal of Biological Chemistry*, *269*(41), 25335–25338.

Lee, F. J. S., Xue, S., Pei, L., Vukusic, B., Chéry, N., Wang, Y., Wang, Y. T., Niznik, H. B., Yu, X. min, & Liu, F. (2002). Dual regulation of NMDA receptor functions by direct protein-protein interactions with the dopamine D1 receptor. *Cell*, *111*(2), 219–230. https://doi.org/10.1016/S0092-8674(02)00962-5

Lee, H., Woodman, S. E., Engelman, J. A., Volonte', D., Galbiati, F., Kaufman, H. L., Lublin, D. M., & Lisanti, M. P. (2001). Palmitoylation of Caveolin-1 at a Single Site (Cys-156) Controls Its Coupling to the c-Src Tyrosine Kinase: TARGETING OF DUALLY ACYLATED MOLECULES (GPI-LINKED, TRANSMEMBRANE, OR CYTOPLASMIC) TO CAVEOLAE EFFECTIVELY UNCOUPLES c-Src AND CAVEOLIN-1 (TYR-14)*. *Journal of Biological Chemistry*, *276*(37), 35150–35158. https://doi.org/https://doi.org/10.1074/jbc.M104530200

Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I., & Beachy, P. A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science (New York, N.Y.)*, *266*(5190), 1528–1537. https://doi.org/10.1126/science.7985023

Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lança, A. J., O'Dowd, B. F., & George, S. R. (2004). Dopamine D1 and D2 receptor co-activation generates a novel phospholipase C-mediated calcium signal. *Journal of Biological Chemistry*, *279*(34), 35671–35678. https://doi.org/10.1074/jbc.M401923200

Lefkowitz, R. J., Hausdorff, W. P., & Caron, M. G. (1990). Esensikation of the ~~ No ~ Ept ~~. *Science*, *Il*.

Lefkowitz, R. J., Pierce, K. L., & Luttrell, L. M. (2002). Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Molecular Pharmacology*, *62*(5), 971–974.

Lefkowitz, R. J., & Shenoy, S. K. (2005). Transduction of receptor signals by ß-arrestins. *Science*, *308*(5721), 512–517.

Leisman, G., & Sheldon, D. (2022). Tics and Emotions. *Brain Sciences*, *12*(2). https://doi.org/10.3390/brainsci12020242

Lemonidis, K., Salaun, C., Kouskou, M., Diez-Ardanuy, C., Chamberlain, L. H., & Greaves, J. (2017). Substrate selectivity in the zDHHC family of S-acyltransferases. *Biochemical Society Transactions*, *45*(3), 751–758. https://doi.org/10.1042/BST20160309

Levental, I., Lingwood, D., Grzybek, M., Coskun, Ü., & Simons, K. (2010). Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(51), 22050–22054. https://doi.org/10.1073/pnas.1016184107

Lévesque, D., Diaz, J., Pilon, C., Martres, M. P., Giros, B., Souil, E., Schott, D., Morgat, J. L.,

Schwartz, J. C., & Sokoloff, P. (1992). Identification, characterization, and localization of the dopamine D3 receptor in rat brain using 7-[3H]hydroxy-N,N-di-n-propyl-2-aminotetralin. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(17), 5155–5159.

Levey, A. I., Hersch, S. M., Rye, D. B., Sunahara, R. K., Niznik, H. B., Kitt, C. A., Price, D. L., Maggio, R., Brann, M. R., & Ciliax, B. J. (1993). Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(19), 8861–8865. https://doi.org/10.1073/pnas.90.19.8861

Li, M., Bermak, J. C., Wang, Z. W., & Zhou, Q. Y. (2000). Modulation of Dopamine D₂ Receptor Signaling by Actin-Binding Protein (ABP-280). *Molecular Pharmacology*, *57*(3), 446 LP – 452. https://doi.org/10.1124/mol.57.3.446

Li, M., West, J. W., Lai, Y., Scheuer, T., & Catterall, W. A. (1992). Functional modulation of brain sodium channels by cAMP-dependent phosphorylation. *Neuron*, *8*(6), 1151–1159. https://doi.org/10.1016/0896-6273(92)90135-Z

Li, Y.-X., Shao, Y.-H., & Deng, N.-Y. (2011). Improved prediction of palmitoylation sites using PWMs and SVM. *Protein and Peptide Letters*, *18*(2), 186–193. https://doi.org/10.2174/092986611794475084

Liang, X., Lu, Y., Neubert, T. A., & Resh, M. D. (2002). Mass spectrometric analysis of GAP-43/neuromodulin reveals the presence of a variety of fatty acylated species. *The Journal of Biological Chemistry*, *277*(36), 33032–33040. https://doi.org/10.1074/jbc.M204607200

Liang, Y.-L., Khoshouei, M., Radjainia, M., Zhang, Y., Glukhova, A., Tarrasch, J., Thal, D. M., Furness, S. G. B., Christopoulos, G., Coudrat, T., Danev, R., Baumeister, W., Miller, L. J., Christopoulos, A., Kobilka, B. K., Wootten, D., Skiniotis, G., & Sexton, P. M. (2017). Phase-plate cryo-EM structure of a class B GPCR-G-protein complex. *Nature*, *546*(7656), 118–123. https://doi.org/10.1038/nature22327

Lidow, M. S. (2003). Calcium signaling dysfunction in schizophrenia: a unifying approach. *Brain Research. Brain Research Reviews*, *43*(1), 70–84. https://doi.org/10.1016/s0165-0173(03)00203-0

Lidow, M. S., Roberts, A., Zhang, L., Koh, P. O., Lezcano, N., & Bergson, C. (2001). Receptor crosstalk protein, calcyon, regulates affinity state of dopamine D1 receptors. *European Journal of Pharmacology*, *427*(3), 187–193. https://doi.org/10.1016/S0014-2999(01)01265-1

Limbird, L. E., Meyts, P. D., & Lefkowitz, R. J. (1975). Beta-adrenergic receptors: evidence for negative cooperativity. *Biochemical and Biophysical Research Communications*, *64*(4), 1160–1168.

Linder, M. E., & Burr, J. G. (1988). Nonmyristoylated p60v-src fails to phosphorylate proteins of 115-120 kDa in chicken embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(8), 2608–2612. https://doi.org/10.1073/pnas.85.8.2608

Linder, M. E., & Deschenes, R. J. (2007). Palmitoylation: Policing protein stability and traffic. *Nature Reviews Molecular Cell Biology*, *8*(1), 74–84. https://doi.org/10.1038/nrm2084

Linder, M. E., Middleton, P., Hepler, J. R., Taussig, R., Gilman, A. G., & Mumby, S. M. (1993). Lipid modifications of G proteins: α Subunits are palmitoylated. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(8), 3675–3679. https://doi.org/10.1073/pnas.90.8.3675

Lindskog, M., Kim, M., Wikström, M. A., Blackwell, K. T., & Kotaleski, J. H. (2006). Transient Calcium and Dopamine Increase PKA Activity and DARPP-32 Phosphorylation. *PLOS Computational Biology*, *2*(9), e119. https://doi.org/10.1371/journal.pcbi.0020119

Liu, F., Wan, Q., Pristupa, Z. B., Yu, X. M., Wang, Y. T., & Niznik, H. B. (2000). Direct protein-protein coupling enables cross-talk between dopamine D5 and γ-aminobutyric acid A receptors. *Nature*, *403*(6767), 274–280. https://doi.org/10.1038/35002014

Liu, H., & Naismith, J. H. (2008). An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnology*, *8*, 91. https://doi.org/10.1186/1472-6750-8-91

Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., & Wüthrich, K. (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. *Science (New York, N.Y.)*, *335*(6072), 1106–1110. https://doi.org/10.1126/science.1215802

Liu, R., Wang, D., Shi, Q., Fu, Q., Hizon, S., & Xiang, Y. K. (2012). Palmitoylation regulates intracellular trafficking of β2 adrenergic receptor/arrestin/phosphodiesterase 4D complexes in cardiomyocytes. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042658

Liu, S., Paknejad, N., Zhu, L., Kihara, Y., Ray, M., Chun, J., Liu, W., Hite, R. K., & Huang, X.-Y. (2022). Differential activation mechanisms of lipid GPCRs by lysophosphatidic acid and sphingosine 1-phosphate. *Nature Communications*, *13*(1), 731. https://doi.org/10.1038/s41467-022-28417-2

Liu, W., Chun, E., Thompson, A. A., Chubukov, P., Xu, F., Katritch, V., Han, G. W., Roth, C. B., Heitman, L. H., IJzerman, A. P., Cherezov, V., & Stevens, R. C. (2012). Structural basis for allosteric regulation of GPCRs by sodium ions. *Science (New York, N.Y.)*, *337*(6091), 232–236. https://doi.org/10.1126/science.1219218

Liu, Y. F., Civelli, O., Zhou, Q. Y., & Albert, P. R. (1992). Cholera toxin-sensitive 3′, 5′-cyclic adenosine monophosphate and calcium signals of the human dopamine-D1 receptor: Selective potentiation by protein kinase A. *Molecular Endocrinology*, *6*(11), 1815–1824. https://doi.org/10.1210/mend.6.11.1282671

Lobingier, B. T., & von Zastrow, M. (2019). When trafficking and signaling mix: How subcellular location shapes G protein-coupled receptor activation of heterotrimeric G proteins. *Traffic (Copenhagen, Denmark)*, *20*(2), 130–136. https://doi.org/10.1111/tra.12634

Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., & Clapham, D. E. (1987). The βγ subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. *Nature*, *325*(6102), 321–326. https://doi.org/10.1038/325321a0

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1990). β-Arrestin: a Protein that Regulates β-adrenergic Receptor Function. *Science*, *248*(4962), 1547–1550. https://doi.org/10.1126/science.2163110

Loisel, T. P., Adam, L., Hebert, T. E., & Bouvier, M. (1996). Agonist stimulation increases the turnover rate of β2AR-bound palmitate and promotes receptor depalmitoylation. *Biochemistry*, *35*(49), 15923–15932. https://doi.org/10.1021/bi9611321

Loos, M., Pattij, T., Janssen, M. C. W., Counotte, D. S., Schoffelmeer, A. N. M., Smit, A. B., Spijker, S., & Van Gaalen, M. M. (2010). Dopamine receptor D1/D5gene expression in the medial prefrontal cortex predicts impulsive choice in rats. *Cerebral Cortex*, *20*(5), 1064–1070. https://doi.org/10.1093/cercor/bhp167

Luo, S. X., & Huang, E. J. (2016). Dopaminergic Neurons and Brain Reward Pathways: From Neurogenesis to Circuit Assembly. *The American Journal of Pathology*, *186*(3), 478–488. https://doi.org/10.1016/j.ajpath.2015.09.023

Lüscher, C., & Slesinger, P. A. (2010). Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nature Reviews Neuroscience*, *11*(5), 301–315. https://doi.org/10.1038/nrn2834

Luttrell, L. M., Daaka, Y., Della Rocca, G. J., & Lefkowitz, R. J. (1997). G Protein-coupled Receptors Mediate Two Functionally Distinct Pathways of Tyrosine Phosphorylation in Rat 1a Fibroblasts: Shc PHOSPHORYLATION AND RECEPTOR ENDOCYTOSIS CORRELATE WITH ACTIVATION OF Erk KINASES*. *Journal of Biological Chemistry*, *272*(50), 31648–31656. https://doi.org/https://doi.org/10.1074/jbc.272.50.31648

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., & Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. *Developmental Cell*, *10*(6), 839–850. https://doi.org/10.1016/j.devcel.2006.04.002

Magee, A. I., Koyama, A. H., Malfer, C., Wen, D., & Schlesinger, M. J. (1984). Release of fatty acids from virus glycoproteins by hydroxylamine. *Biochimica et Biophysica Acta*, *798*(2), 156–166. https://doi.org/10.1016/0304-4165(84)90298-8

Mahan, L. C., Burch, R. M., Monsma, F. J., & Sibley, D. R. (1990). Expression of striatal D1 dopamine receptors coupled to inositol phosphate production and Ca2+ mobilization in Xenopus oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(6), 2196–2200. https://doi.org/10.1073/pnas.87.6.2196

Mahoney, J. P., & Sunahara, R. K. (2016). Mechanistic insights into GPCR-G protein interactions. *Current Opinion in Structural Biology*, *41*, 247–254. https://doi.org/10.1016/j.sbi.2016.11.005

Main, A., & Fuller, W. (2022). Protein S-Palmitoylation: advances and challenges in studying a therapeutically important lipid modification. *The FEBS Journal*, *289*(4), 861–882. https://doi.org/https://doi.org/10.1111/febs.15781

Main, A., Robertson-Gray, O., & Fuller, W. (2021). Cyclophilin D palmitoylation and permeability transition: a new twist in the tale of myocardial ischaemia-reperfusion injury. *CARDIOVASCULAR RESEARCH*, *117*(1), 15–17. https://doi.org/10.1093/cvr/cvaa149

Mallory, D. P., Gutierrez, E., Pinkevitch, M., Klinginsmith, C., Comar, W. D., Roushar, F. J., Schlebach, J. P., Smith, A. W., & Jastrzebska, B. (2018). The Retinitis Pigmentosa-Linked Mutations in Transmembrane Helix 5 of Rhodopsin Disrupt Cellular Trafficking Regardless of Oligomerization State. *Biochemistry*, *57*(35), 5188–5201.

https://doi.org/10.1021/acs.biochem.8b00403

Manglik, A., Kobilka, B. K., & Steyaert, J. (2017). Nanobodies to Study G Protein-Coupled Receptor Structure and Function. *Annual Review of Pharmacology and Toxicology*, *57*, 19–37. https://doi.org/10.1146/annurev-pharmtox-010716-104710

Mann, M., & Jensen, O. N. (2003). Proteomic analysis of post-translational modifications. *Nature Biotechnology*, *21*(3), 255–261. https://doi.org/10.1038/nbt0303-255

Mansilla, F., Birkenkamp-Demtroder, K., Kruhøffer, M., Sørensen, F. B., Andersen, C. L., Laiho, P., Aaltonen, L. A., Verspaget, H. W., & Ørntoft, T. F. (2007). Differential expression of DHHC9 in microsatellite stable and instable human colorectal cancer subgroups. *British Journal of Cancer*, *96*(12), 1896–1903. https://doi.org/10.1038/sj.bjc.6603818

Marshall, C. J. (1993). Protein prenylation: a mediator of protein-protein interactions. *Science (New York, N.Y.)*, *259*(5103), 1865–1866. https://doi.org/10.1126/science.8456312

Martin, B. R., & Cravatt, B. F. (2009). Large-scale profiling of protein palmitoylation in mammalian cells. *Nature Methods*, *6*(2), 135–138. https://doi.org/10.1038/nmeth.1293

Martinez, V. J., Asico, L. D., Jose, P. A., & Tiu, A. C. (2020). Lipid Rafts and Dopamine Receptor Signaling. *International Journal of Molecular Sciences*, *21*(23). https://doi.org/10.3390/ijms21238909

Mattera, R., Graziano, M. P., Yatani, A., Zhou, Z., Graf, R., Codina, J., Birnbaumer, L., Gilman, A. G., & Brown, A. M. (1989). Splice variants of the alpha subunit of the G protein Gs activate both adenylyl cyclase and calcium channels. *Science (New York, N.Y.)*, *243*(4892), 804–807. https://doi.org/10.1126/science.2536957

Maurer-Stroh, S., Eisenhaber, B., & Eisenhaber, F. (2002). N-terminal N-myristoylation of proteins: prediction of substrate proteins from amino acid sequence. *Journal of Molecular Biology*, *317*(4), 541–557. https://doi.org/10.1006/jmbi.2002.5426

Maurer-Stroh, S., Koranda, M., Benetka, W., Schneider, G., Sirota, F. L., & Eisenhaber, F. (2007). Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS Computational Biology*, *3*(4), e66. https://doi.org/10.1371/journal.pcbi.0030066

Mayle, K. M., Le, A. M., & Kamei, D. T. (2012). The intracellular trafficking pathway of transferrin. *Biochimica et Biophysica Acta*, *1820*(3), 264–281. https://doi.org/10.1016/j.bbagen.2011.09.009

Maziarz, M., Park, J. C., Leyme, A., Marivin, A., Garcia-Lopez, A., Patel, P. P., & Garcia-Marcos, M. (2020). Revealing the Activity of Trimeric G-proteins in Live Cells with a Versatile Biosensor Design. *Cell*, *182*(3), 770-785.e16. https://doi.org/10.1016/j.cell.2020.06.020

McCluskey, A., Daniel, J. A., Hadzic, G., Chau, N., Clayton, E. L., Mariana, A., Whiting, A., Gorgani, N. N., Lloyd, J., Quan, A., Moshkanbaryans, L., Krishnan, S., Perera, S., Chircop, M., von Kleist, L., McGeachie, A. B., Howes, M. T., Parton, R. G., Campbell, M., … Robinson, P. J. (2013). Building a better dynasore: the dyngo compounds potently inhibit dynamin and endocytosis. *Traffic (Copenhagen, Denmark)*, *14*(12), 1272–1289. https://doi.org/10.1111/tra.12119

McCutcheon, R. A., Abi-Dargham, A., & Howes, O. D. (2019). Schizophrenia, Dopamine and the

Striatum: From Biology to Symptoms. *Trends in Neurosciences*, *42*(3), 205–220. https://doi.org/10.1016/j.tins.2018.12.004

McCutcheon, R. A., Krystal, J. H., & Howes, O. D. (2020). Dopamine and glutamate in schizophrenia: biology, symptoms and treatment. *World Psychiatry : Official Journal of the World Psychiatric Association (WPA)*, *19*(1), 15–33. https://doi.org/10.1002/wps.20693

McIlhinney, R. A., & McGlone, K. (1996). Immunocytochemical characterization and subcellular localization of human myristoyl-CoA: protein N-myristoyltransferase in HeLa cells. *Experimental Cell Research*, *223*(2), 348–356. https://doi.org/10.1006/excr.1996.0090

McIlhinney, R. A., McGlone, K., & Willis, A. C. (1993). Purification and partial sequencing of myristoyl-CoA:protein N-myristoyltransferase from bovine brain. *The Biochemical Journal*, *290 (Pt 2*(Pt 2), 405–410. https://doi.org/10.1042/bj2900405

McIntyre, R. S., McCann, S. M., & Kennedy, S. H. (2001). Antipsychotic metabolic effects: weight gain, diabetes mellitus, and lipid abnormalities. *Canadian Journal of Psychiatry. Revue Canadienne de Psychiatrie*, *46*(3), 273–281. https://doi.org/10.1177/070674370104600308

Messa, P., Alfieri, C., & Brezzi, B. (2008). Cinacalcet: pharmacological and clinical aspects. *Expert Opinion on Drug Metabolism & Toxicology*, *4*(12), 1551–1560. https://doi.org/10.1517/17425250802587017

Mihara, K., Kondo, T., Suzuki, A., Yasui-Furukori, N., Ono, S., Sano, A., Koshiro, K., Otani, K., & Kaneko, S. (2003). Relationship between functional dopamine D2 and D3 receptors gene polymorphisms and neuroleptic malignant syndrome. *American Journal of Medical Genetics*, *117B*(1), 57–60. https://doi.org/10.1002/ajmg.b.10025

Min, C., Zheng, M., Zhang, X., Guo, S., Kwon, K.-J., Shin, C. Y., Kim, H.-S., Cheon, S. H., & Kim, K.-M. (2015). N-linked Glycosylation on the N-terminus of the dopamine D2 and D3 receptors determines receptor association with specific microdomains in the plasma membrane. *Biochimica et Biophysica Acta*, *1853*(1), 41–51. https://doi.org/10.1016/j.bbamcr.2014.09.024

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., & Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiological Reviews*, *78*(1), 189–225. https://doi.org/10.1152/physrev.1998.78.1.189

Missale, C., Russel Nash, S., Robinson, S. W., Jaber, M., & Caron, M. G. (1998). Dopamine receptors: From structure to function. *Physiological Reviews*, *78*(1), 189–225. https://doi.org/10.1152/physrev.1998.78.1.189

Mitchell, D. A., Mitchell, G., Ling, Y., Budde, C., & Deschenes, R. J. (2010). Mutational analysis of Saccharomyces cerevisiae Erf2 reveals a two-step reaction mechanism for protein palmitoylation by DHHC enzymes. *Journal of Biological Chemistry*, *285*(49), 38104–38114. https://doi.org/10.1074/jbc.M110.169102

Mizuno, N., & Itoh, H. (2009). Functions and Regulatory Mechanisms of Gq-Signaling Pathways. *Neurosignals*, *17*(1), 42–54. https://doi.org/10.1159/000186689

Mo, X. L., & Fu, H. (2016). BRET: Nanoluc-based bioluminescence resonance energy transfer platform to monitor protein-protein interactions in live cells. *Methods in Molecular Biology*, *1439*, 263–271. https://doi.org/10.1007/978-1-4939-3673-1_17

Moffett, S., Rousseau, G., Lagacé, M., & Bouvier, M. (2001). The palmitoylation state of the β2-adrenergic receptor regulates the synergistic action of cyclic AMP-dependent protein kinase and β-adrenergic receptor kinase involved in its phosphorylation and desensitization. *Journal of Neurochemistry*, *76*(1), 269–279. https://doi.org/10.1046/j.1471-4159.2001.00005.x

Molinoff, P. B., & Axelrod, J. (1971). Biochemistry of catecholamines. *Annual Review of Biochemistry*, *40*, 465–500. https://doi.org/10.1146/annurev.bi.40.070171.002341

Montag, C., Jurkiewicz, M., & Reuter, M. (2012). The role of the catechol-O-methyltransferase (COMT) gene in personality and related psychopathological disorders. *CNS & Neurological Disorders Drug Targets*, *11*(3), 236–250. https://doi.org/10.2174/187152712800672382

Moo, E. Von, van Senten, J. R., Bräuner-Osborne, H., & Møller, T. C. (2021). Arrestin-Dependent and -Independent Internalization of G Protein-Coupled Receptors: Methods, Mechanisms, and Implications on Cell Signaling. *Molecular Pharmacology*, *99*(4), 242–255. https://doi.org/10.1124/molpharm.120.000192

Moritz, A. E., Madaras, N. S., Rankin, M. L., Inbody, L. R., & Sibley, D. R. (2023). Delineation of G Protein-Coupled Receptor Kinase Phosphorylation Sites within the D(1) Dopamine Receptor and Their Roles in Modulating β-Arrestin Binding and Activation. *International Journal of Molecular Sciences*, *24*(7). https://doi.org/10.3390/ijms24076599

Müller, T. D., Finan, B., Bloom, S. R., D'Alessio, D., Drucker, D. J., Flatt, P. R., Fritsche, A., Gribble, F., Grill, H. J., Habener, J. F., Holst, J. J., Langhans, W., Meier, J. J., Nauck, M. A., Perez-Tilve, D., Pocai, A., Reimann, F., Sandoval, D. A., Schwartz, T. W., … Tschöp, M. H. (2019). Glucagon-like peptide 1 (GLP-1). *Molecular Metabolism*, *30*, 72–130. https://doi.org/10.1016/j.molmet.2019.09.010

Mumby, S. M., Heukeroth, R. O., Gordon, J. I., & Gilman, A. G. (1990). G-protein alpha-subunit expression, myristoylation, and membrane association in COS cells. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(2), 728–732. https://doi.org/10.1073/pnas.87.2.728

Munday, A. D., & López, J. A. (2007). Posttranslational Protein Palmitoylation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*(7), 1496–1499. https://doi.org/10.1161/atvbaha.106.136226

Murphy, B. J., Rossie, S., De Jongh, K. S., & Catterall, W. A. (1993). Identification of the sites of selective phosphorylation and dephosphorylation of the rat brain Na+ channel α subunit by cAMP-dependent protein kinase and phosphoprotein phosphatases. *Journal of Biological Chemistry*, *268*(36), 27355–27362.

Murray, A. M., Ryoo, H. L., Gurevich, E., & Joyce, J. N. (1994). Localization of dopamine D3 receptors to mesolimbic and D2 receptors to mesostriatal regions of human forebrain. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(23), 11271–11275. https://doi.org/10.1073/pnas.91.23.11271

Nadolski, M. J., & Linder, M. E. (2007). Protein lipidation. *The FEBS Journal*, *274*(20), 5202–5210. https://doi.org/10.1111/j.1742-4658.2007.06056.x

Namkung, Y., Le Gouill, C., Lukashova, V., Kobayashi, H., Hogue, M., Khoury, E., Song, M.,

Bouvier, M., & Laporte, S. A. (2016a). Monitoring G protein-coupled receptor and β-arrestin trafficking in live cells using enhanced bystander BRET. *Nature Communications*, *7*, 12178. https://doi.org/10.1038/ncomms12178

Namkung, Y., Le Gouill, C., Lukashova, V., Kobayashi, H., Hogue, M., Khoury, E., Song, M., Bouvier, M., & Laporte, S. A. S. A. S. A. (2016b). Monitoring G protein-coupled receptor and β-arrestin trafficking in live cells using enhanced bystander BRET. *Nature Communications*, *7*(1), 12178. https://doi.org/10.1038/ncomms12178

Naranjo, A. N., Chevalier, A., Cousins, G. D., Ayettey, E., McCusker, E. C., Wenk, C., & Robinson, A. S. (2015). Conserved disulfide bond is not essential for the adenosine A2A receptor: Extracellular cysteines influence receptor distribution within the cell and ligand-binding recognition. *Biochimica et Biophysica Acta*, *1848*(2), 603–614. https://doi.org/10.1016/j.bbamem.2014.11.010

Nash, C. A., Wei, W., Irannejad, R., & Smrcka, A. V. (2019). Golgi localized β1-adrenergic receptors stimulate Golgi PI4P hydrolysis by PLCε to regulate cardiac hypertrophy. *ELife*, *8*. https://doi.org/10.7554/eLife.48167

Naumenko, V. S., & Ponimaskin, E. (2018). Palmitoylation as a Functional Regulator of Neurotransmitter Receptors. *Neural Plasticity*, *2018*, 5701348. https://doi.org/10.1155/2018/5701348

Nehmé, R., Carpenter, B., Singhal, A., Strege, A., Edwards, P. C., White, C. F., Du, H., Grisshammer, R., & Tate, C. G. (2017). Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PloS One*, *12*(4), e0175642. https://doi.org/10.1371/journal.pone.0175642

Nestler, E. J., & Carlezon, W. A. (2006). The Mesolimbic Dopamine Reward Circuit in Depression. *Biological Psychiatry*, *59*(12), 1151–1159. https://doi.org/10.1016/j.biopsych.2005.09.018

Neve, K. A., Seamans, J. K., & Trantham-Davidson, H. (2004). Dopamine Receptor Signaling. *Journal of Receptor and Signal Transduction Research*, *24*(3), 165–205. https://doi.org/10.1081/lrst-200029981

Ng, G. Y. K. K., O'Dowd, B. F., Caron, M., Dennis, M., Brann, M. R., & George, S. R. (1994). Phosphorylation and Palmitoylation of the Human D2L Dopamine Receptor in Sf9 Cells. *Journal of Neurochemistry*, *63*(5), 1589–1595. https://doi.org/https://doi.org/10.1046/j.1471-4159.1994.63051589.x

Ng, G. Y. K., Mouillac, B., George, S. R., Caron, M., Dennis, M., Bouvier, M., & O'Dowd, B. F. (1994). Desensitization, phosphorylation and palmitoylation of the human dopamine D1 receptor. *European Journal of Pharmacology: Molecular Pharmacology*, *267*(1), 7–19.

Nguyen, A. H., Thomsen, A. R. B., Cahill, T. J. 3rd, Huang, R., Huang, L.-Y., Marcink, T., Clarke, O. B., Heissel, S., Masoudi, A., Ben-Hail, D., Samaan, F., Dandey, V. P., Tan, Y. Z., Hong, C., Mahoney, J. P., Triest, S., Little, J. 4th, Chen, X., Sunahara, R., … Lefkowitz, R. J. (2019). Structure of an endosomal signaling GPCR-G protein-β-arrestin megacomplex. *Nature Structural & Molecular Biology*, *26*(12), 1123–1131. https://doi.org/10.1038/s41594-019-0330-y

Nichols, B. (2003). Caveosomes and endocytosis of lipid rafts. *Journal of Cell Science*, *116*(Pt 23),

4707–4714. https://doi.org/10.1242/jcs.00840

Nicola, S. M., & Malenka, R. C. (1998). Modulation of synaptic transmission by dopamine and norepinephrine in ventral but not dorsal striatum. *Journal of Neurophysiology*, *79*(4), 1768–1776. https://doi.org/10.1152/jn.1998.79.4.1768

Nishi, A., Bibb, J. A., Snyder, G. L., Higashi, H., Nairn, A. C., & Greengard, P. (2000). Amplification of dopaminergic signaling by a positive feedback loop. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(23), 12840–12845. https://doi.org/10.1073/pnas.220410397

Noda, K., Saad, Y., Graham, R. M., & Karnik, S. S. (1994). The high affinity state of the beta 2-adrenergic receptor requires unique interaction between conserved and non-conserved extracellular loop cysteines. *The Journal of Biological Chemistry*, *269*(9), 6743–6752.

Ntwasa, M., Egerton, M., & Gay, N. J. (1997). Sequence and expression of Drosophila myristoyl-CoA: protein N-myristoyl transferase: evidence for proteolytic processing and membrane localisation. *Journal of Cell Science*, *110 (Pt 2*, 149–156. https://doi.org/10.1242/jcs.110.2.149

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, *287*(5785), 795–801. https://doi.org/10.1038/287795a0

O'Brien, P. J., & Zatz, M. (1984). Acylation of bovine rhodopsin by [3H]palmitic acid. *The Journal of Biological Chemistry*, *259*(8), 5054–5057.

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., & Bouvier, M. (1989). Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *The Journal of Biological Chemistry*, *264*(13), 7564–7569.

Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., & Barak, L. S. (2000). Differential affinities of visual arrestin, βarrestin1, and βarrestin2 for G protein-coupled receptors delineate two major classes of receptors. *Journal of Biological Chemistry*, *275*(22), 17201–17210.

Oddi, S., Dainese, E., Sandiford, S., Fezza, F., Lanuti, M., Chiurchiù, V., Totaro, A., Catanzaro, G., Barcaroli, D., De Laurenzi, V., Centonze, D., Mukhopadhyay, S., Selent, J., Howlett, A. C., & Maccarrone, M. (2012). Effects of palmitoylation of Cys(415) in helix 8 of the CB(1) cannabinoid receptor on membrane localization and signalling. *British Journal of Pharmacology*, *165*(8), 2635–2651. https://doi.org/10.1111/j.1476-5381.2011.01658.x

Oddi, S., Stepniewski, T. M., Totaro, A., Selent, J., Scipioni, L., Dufrusine, B., Fezza, F., Dainese, E., & Maccarrone, M. (2017). Palmitoylation of cysteine 415 of CB(1) receptor affects ligand-stimulated internalization and selective interaction with membrane cholesterol and caveolin 1. *Biochimica et Biophysica Acta. Molecular and Cell Biology of Lipids*, *1862*(5), 523–532. https://doi.org/10.1016/j.bbalip.2017.02.004

Oddi, S., Totaro, A., Scipioni, L., Dufrusine, B., Stepniewski, T. M., Selent, J., Maccarrone, M., & Dainese, E. (2018). Role of palmitoylation of cysteine 415 in functional coupling CB(1) receptor to Gα(i2) protein. *Biotechnology and Applied Biochemistry*, *65*(1), 16–20. https://doi.org/10.1002/bab.1575

Ohno, Y., Kihara, A., Sano, T., & Igarashi, Y. (2006). Intracellular localization and tissue-specific

distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1761*(4), 474–483. https://doi.org/10.1016/j.bbalip.2006.03.010

Ohtsubo, K., & Marth, J. D. (2006). Glycosylation in cellular mechanisms of health and disease. *Cell*, *126*(5), 855–867. https://doi.org/10.1016/j.cell.2006.08.019

Oldham, W. M., & Hamm, H. E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews. Molecular Cell Biology*, *9*(1), 60–71. https://doi.org/10.1038/nrm2299

Olsen, R. H. J., DiBerto, J. F., English, J. G., Glaudin, A. M., Krumm, B. E., Slocum, S. T., Che, T., Gavin, A. C., McCorvy, J. D., Roth, B. L., & Strachan, R. T. (2020). TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. *Nature Chemical Biology*, *16*(8), 841–849. https://doi.org/10.1038/s41589-020-0535-8

Pacheco, M. A., & Jope, R. S. (2002). Comparison of [3H]Phosphatidylinositol and [3H]Phosphatidylinositol 4,5-Bisphosphate Hydrolysis in Postmortem Human Brain Membranes and Characterization of Stimulation by Dopamine D1 Receptors. *Journal of Neurochemistry*, *69*(2), 639–644. https://doi.org/10.1046/j.1471-4159.1997.69020639.x

Pal, S., & Chattopadhyay, A. (2019). Extramembranous Regions in G Protein-Coupled Receptors: Cinderella in Receptor Biology? *The Journal of Membrane Biology*, *252*(4), 483–497. https://doi.org/10.1007/s00232-019-00092-3

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., & Miyano, M. (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (New York, N.Y.)*, *289*(5480), 739–745. https://doi.org/10.1126/science.289.5480.739

Panchalingam, S., & Undie, A. S. (2001). SKF83959 exhibits biochemical agonism by stimulating [35S]GTPγS binding and phosphoinositide hydrolysis in rat and monkey brain. *Neuropharmacology*, *40*(6), 826–837. https://doi.org/10.1016/S0028-3908(01)00011-9

Papoucheva, E., Dumuis, A., Sebben, M., Richter, D. W., & Ponimaskin, E. G. (2004). The 5-hydroxytryptamine(1A) receptor is stably palmitoylated, and acylation is critical for communication of receptor with Gi protein. *The Journal of Biological Chemistry*, *279*(5), 3280–3291. https://doi.org/10.1074/jbc.M308177200

Park, D., Jhon, D. Y., Lee, C. W., Lee, K. H., & Rhee, S. G. (1993). Activation of phospholipase C isozymes by G protein beta gamma subunits. *The Journal of Biological Chemistry*, *268*(7), 4573–4576.

Parker, M. S., Wong, Y. Y., & Parker, S. L. (2008). An ion-responsive motif in the second transmembrane segment of rhodopsin-like receptors. *Amino Acids*, *35*(1), 1–15. https://doi.org/10.1007/s00726-008-0637-6

Parthier, C., Reedtz-Runge, S., Rudolph, R., & Stubbs, M. T. (2009). Passing the baton in class B GPCRs: peptide hormone activation via helix induction? *Trends in Biochemical Sciences*, *34*(6), 303–310. https://doi.org/10.1016/j.tibs.2009.02.004

Patwardhan, A., Cheng, N., & Trejo, J. (2021). Post-translational modifications of g protein–coupled

receptors control cellular signaling dynamics in space and time. *Pharmacological Reviews*, *73*(1), 120–151. https://doi.org/10.1124/pharmrev.120.000082

Paulick, M. G., & Bertozzi, C. R. (2008). The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry*, *47*(27), 6991–7000. https://doi.org/10.1021/bi8006324

Pavlos, N. J., & Friedman, P. A. (2017). GPCR Signaling and Trafficking: The Long and Short of It. *Trends in Endocrinology & Metabolism*, *28*(3), 213–226. https://doi.org/10.1016/j.tem.2016.10.007

Peeters, M. C., van Westen, G. J. P., Li, Q., & IJzerman, A. P. (2011). Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends in Pharmacological Sciences*, *32*(1), 35–42. https://doi.org/10.1016/j.tips.2010.10.001

Pei, Z., Xiao, Y., Meng, J., Hudmon, A., & Cummins, T. R. (2016). Cardiac sodium channel palmitoylation regulates channel availability and myocyte excitability with implications for arrhythmia generation. *Nature Communications*, *7*(May), 1–13. https://doi.org/10.1038/ncomms12035

Peinemann, A., Schuller, S., Pohl, C., Jahn, T., Weindl, A., & Kassubek, J. (2005). Executive dysfunction in early stages of Huntington's disease is associated with striatal and insular atrophy: A neuropsychological and voxel-based morphometric study. *Journal of the Neurological Sciences*, *239*(1), 11–19. https://doi.org/10.1016/j.jns.2005.07.007

Peitzsch, R. M., & McLaughlin, S. (1993). Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins. *Biochemistry*, *32*(39), 10436–10443. https://doi.org/10.1021/bi00090a020

Peng, G. E., Pessino, V., Huang, B., & von Zastrow, M. (2021). Spatial decoding of endosomal cAMP signals by a metastable cytoplasmic PKA network. *Nature Chemical Biology*, *17*, 558–566.

Peng, Y., McCorvy, J. D., Harpsøe, K., Lansu, K., Yuan, S., Popov, P., Qu, L., Pu, M., Che, T., Nikolajsen, L. F., Huang, X.-P., Wu, Y., Shen, L., Bjørn-Yoshimoto, W. E., Ding, K., Wacker, D., Han, G. W., Cheng, J., Katritch, V., … Liu, Z.-J. (2018). No Title. *Cell*, *172*(4), 719-730.e14. https://doi.org/10.1016/j.cell.2018.01.001

Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F., & Bachelerie, F. (2001). Palmitoylation-dependent Control of Degradation, Life Span, and Membrane Expression of the CCR5 Receptor. *Journal of Biological Chemistry*, *276*(34), 31936–31944. https://doi.org/10.1074/jbc.M104013200

Perry, S. J., Baillie, G. S., Kohout, T. A., McPhee, I., Magiera, M. M., Ang, K. L., Miller, W. E., McLean, A. J., Conti, M., Houslay, M. D., & Lefkowitz, R. J. (2002). Targeting of cyclic AMP degradation to β2-adrenergic receptors by β-arrestins. *Science*, *298*(5594), 834–836. https://doi.org/10.1126/science.1074683

Pfeffer, S. R. (2013). Rab GTPase regulation of membrane identity. *Current Opinion in Cell Biology*, *25*(4), 414–419. https://doi.org/https://doi.org/10.1016/j.ceb.2013.04.002

Pfeil, E. M., Brands, J., Merten, N., Vögtle, T., Vescovo, M., Rick, U., Albrecht, I.-M., Heycke, N.,

Kawakami, K., Ono, Y., Ngako Kadji, F. M., Hiratsuka, S., Aoki, J., Häberlein, F., Matthey, M., Garg, J., Hennen, S., Jobin, M.-L., Seier, K., … Kostenis, E. (2020). Heterotrimeric G Protein Subunit Gαq Is a Master Switch for Gβγ-Mediated Calcium Mobilization by Gi-Coupled GPCRs. *Molecular Cell*, *80*(6), 940-954.e6. https://doi.org/https://doi.org/10.1016/j.molcel.2020.10.027

Philippe, J. M., & Jenkins, P. M. (2019). Spatial organization of palmitoyl acyl transferases governs substrate localization and function. *Molecular Membrane Biology*, *35*(1), 60–75. https://doi.org/10.1080/09687688.2019.1710274

Pin, J.-P., Kniazeff, J., Liu, J., Binet, V., Goudet, C., Rondard, P., & Prézeau, L. (2005). Allosteric functioning of dimeric class C G-protein-coupled receptors. *The FEBS Journal*, *272*(12), 2947–2955. https://doi.org/10.1111/j.1742-4658.2005.04728.x

Pitcher, J., Caron, M. G., Lefkowitz, R. J., Lohse, M. J., & Codina, J. (1992). Desensitization of the Isolated β2-Adrenergic Receptor by β-Adrenergic Receptor Kinase, cAMP-Dependent Protein Kinase, and Protein Kinase C Occurs Via Distinct Molecular Mechanisms. *Biochemistry*, *31*(12), 3193–3197. https://doi.org/10.1021/bi00127a021

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., & Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature*, *374*(6520), 363–366. https://doi.org/10.1038/374363a0

Porter, J. A., Young, K. E., & Beachy, P. A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science (New York, N.Y.)*, *274*(5285), 255–259. https://doi.org/10.1126/science.274.5285.255

Posner, B. I., & Laporte, S. A. (2010). Cellular signalling: Peptide hormones and growth factors. *Progress in Brain Research*, *181*, 1–16. https://doi.org/10.1016/S0079-6123(08)81001-1

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., & Sealfon, S. C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. *DNA and Cell Biology*, *11*(1), 1–20. https://doi.org/10.1089/dna.1992.11.1

Promega. (2015). NanoBRET ™ Protein : Protein Interaction System. *Tm439*, *4*, 1–28.

Puri, N. M., Romano, G. R., Lin, T.-Y., Mai, Q. N., & Irannejad, R. (2022). The organic cation transporter 2 regulates dopamine D1 receptor signaling at the Golgi apparatus. *ELife*, *11*, e75468. https://doi.org/10.7554/eLife.75468

Qaddumi, W. N., & Jose, P. A. (2021). The Role of the Renal Dopaminergic System and Oxidative Stress in the Pathogenesis of Hypertension. In *Biomedicines* (Vol. 9, Issue 2). https://doi.org/10.3390/biomedicines9020139

Qanbar, R., & Bouvier, M. (2003). Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacology & Therapeutics*, *97*(1), 1–33. https://doi.org/10.1016/s0163-7258(02)00300-5

Rahmeh, R., Damian, M., Cottet, M., Orcel, H., Mendre, C., Durroux, T., Sharma, K. S., Durand, G., Pucci, B., Trinquet, E., Zwier, J. M., Deupi, X., Bron, P., Banères, J.-L., Mouillac, B., & Granier, S. (2012). Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. *Proceedings of the National Academy of Sciences of the United*

States of America, *109*(17), 6733–6738. https://doi.org/10.1073/pnas.1201093109

Raju, R. V, Kalra, J., & Sharma, R. K. (1994). Purification and properties of bovine spleen N-myristoyl-CoA protein:N-myristoyltransferase. *The Journal of Biological Chemistry*, *269*(16), 12080–12083.

Ramazi, S., Allahverdi, A., & Zahiri, J. (2020). Evaluation of post-translational modifications in histone proteins: a review on histone modification defects in developmental and neurological disorders. *Journal of Biosciences*, *45*, 1–29.

Rangel-Barajas, C., Silva, I., García-Ramírez, M., Sánchez-Lemus, E., Floran, L., Aceves, J., Erlij, D., & Florán, B. (2008). 6-OHDA-induced hemiparkinsonism and chronic l-DOPA treatment increase dopamine D1-stimulated [3H]-GABA release and [3H]-cAMP production in substantia nigra pars reticulata of the rat. *Neuropharmacology*, *55*(5), 704–711. https://doi.org/10.1016/j.neuropharm.2008.06.002

Rao, S., Cunningham, D., de Gramont, A., Scheithauer, W., Smakal, M., Humblet, Y., Kourteva, G., Iveson, T., Andre, T., Dostalova, J., Illes, A., Belly, R., Perez-Ruixo, J. J., Park, Y. C., & Palmer, P. A. (2004). Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *22*(19), 3950–3957. https://doi.org/10.1200/JCO.2004.10.037

Rasmussen, S. G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., DeVree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S., Schnapp, A., Konetzki, I., Sunahara, R. K., Gellman, S. H., Pautsch, A., Steyaert, J., Weis, W. I., & Kobilka, B. K. (2011). Structure of a nanobody-stabilized active state of the β2 adrenoceptor. *Nature*, *469*(7329), 175–180. https://doi.org/10.1038/nature09648

Rasmussen, S. G. F., Choi, H.-J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R. P., Sanishvili, R., Fischetti, R. F., Schertler, G. F. X., Weis, W. I., & Kobilka, B. K. (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature*, *450*(7168), 383–387. https://doi.org/10.1038/nature06325

Reid, H. M., & Kinsella, B. T. (2007). Palmitoylation of the TPbeta isoform of the human thromboxane A2 receptor. Modulation of G protein: effector coupling and modes of receptor internalization. *Cellular Signalling*, *19*(5), 1056–1070. https://doi.org/10.1016/j.cellsig.2006.12.001

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., & Yao, X. (2008). CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Engineering, Design & Selection : PEDS*, *21*(11), 639–644. https://doi.org/10.1093/protein/gzn039

Renner, U., Glebov, K., Lang, T., Papusheva, E., Balakrishnan, S., Keller, B., Richter, D. W., Jahn, R., & Ponimaskin, E. (2007). Localization of the mouse 5-hydroxytryptamine(1A) receptor in lipid microdomains depends on its palmitoylation and is involved in receptor-mediated signaling. *Molecular Pharmacology*, *72*(3), 502–513. https://doi.org/10.1124/mol.107.037085

Ricci, A., Mignini, F., Tomassoni, D., & Amenta, F. (2006). Dopamine receptor subtypes in the human pulmonary arterial tree. *Autonomic and Autacoid Pharmacology*, *26*(4), 361–369.

https://doi.org/10.1111/j.1474-8673.2006.00376.x

Ring, A. M., Manglik, A., Kruse, A. C., Enos, M. D., Weis, W. I., Garcia, K. C., & Kobilka, B. K. (2013). Adrenaline-activated structure of β2-adrenoceptor stabilized by an engineered nanobody. *Nature*, *502*(7472), 575–579.

Rogers, T. B. (1984). High affinity angiotensin II receptors in myocardial sarcolemmal membranes. Characterization of receptors and covalent linkage of 125I-angiotensin II to a membrane component of 116,000 daltons. *The Journal of Biological Chemistry*, *259*(13), 8106–8114.

Rose, J. J., Taylor, J. B., Shi, J., Cockett, M. I., Jones, P. G., & Hepler, J. R. (2000). RGS7 is palmitoylated and exists as biochemically distinct forms. *Journal of Neurochemistry*, *75*(5), 2103–2112. https://doi.org/10.1046/j.1471-4159.2000.0752103.x

Ross, A. B., Langer, J. D., & Jovanovic, M. (2021). Proteome Turnover in the Spotlight: Approaches, Applications, and Perspectives. *Molecular & Cellular Proteomics : MCP*, *20*, 100016. https://doi.org/10.1074/mcp.R120.002190

Ross, E. M., & Wilkie, T. M. (2000). *GTP ASE -A CTIVATING P ROTEINS FOR H ETEROTRIMERIC G P ROTEINS : Regulators of G Protein Signaling (RGS) and RGS-Like Proteins*. 795–827.

Roth, A. F., Wan, J., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., Phinney, B. S., Yates, J. R., & Davis, N. G. (2006). Global Analysis of Protein Palmitoylation in Yeast. *Cell*, *125*(5), 1003–1013. https://doi.org/10.1016/j.cell.2006.03.042

Rudnick, D. A., McWherter, C. A., Rocque, W. J., Lennon, P. J., Getman, D. P., & Gordon, J. I. (1991). Kinetic and structural evidence for a sequential ordered Bi Bi mechanism of catalysis by Saccharomyces cerevisiae myristoyl-CoA:protein N-myristoyltransferase. *The Journal of Biological Chemistry*, *266*(15), 9732–9739.

Rutherford, K., & Daggett, V. (2010). Polymorphisms and disease: Hotspots of inactivation in methyltransferases. *Trends in Biochemical Sciences*, *35*(10), 531–538. https://doi.org/10.1016/j.tibs.2010.04.003

Ryšlavá, H., Doubnerová, V., Kavan, D., & Vaněk, O. (2013). Effect of posttranslational modifications on enzyme function and assembly. *Journal of Proteomics*, *92*, 80–109. https://doi.org/10.1016/j.jprot.2013.03.025

Sadana, R., & Dessauer, C. W. (2009). Physiological roles for G protein-regulated adenylyl cyclase isoforms: Insights from knockout and overexpression studies. *NeuroSignals*, *17*(1), 5–22. https://doi.org/10.1159/000166277

Sadeghi, H. M., Innamorati, G., Dagarag, M., & Birnbaumer, M. (1997). Palmitoylation of the V2 vasopressin receptor. *Molecular Pharmacology*, *52*(1), 21–29. https://doi.org/10.1124/mol.52.1.21

Sakamoto, K., Karelina, K., & Obrietan, K. (2011). CREB: a multifaceted regulator of neuronal plasticity and protection. *Journal of Neurochemistry*, *116*(1), 1–9. https://doi.org/10.1111/j.1471-4159.2010.07080.x

Sakurai, N., & Utsumi, T. (2006). Posttranslational N-myristoylation is required for the anti-apoptotic activity of human tGelsolin, the C-terminal caspase cleavage product of human gelsolin. *The*

Journal of Biological Chemistry, *281*(20), 14288–14295. https://doi.org/10.1074/jbc.M510338200

Santini, E., Valjent, E., Usiello, A., Carta, M., Borgkvist, A., Girault, J.-A., Herve, D., Greengard, P., & Fisone, G. (2007). Critical Involvement of cAMP/DARPP-32 and Extracellular Signal-Regulated Protein Kinase Signaling in L-DOPA-Induced Dyskinesia. *Journal of Neuroscience*, *27*(26), 6995–7005. https://doi.org/10.1523/JNEUROSCI.0852-07.2007

Sarantis, K., Matsokis, N., & Angelatou, F. (2009). Synergistic interactions of dopamine D1 and glutamate NMDA receptors in rat hippocampus and prefrontal cortex: Involvement of ERK1/2 signaling. *Neuroscience*, *163*(4), 1135–1145. https://doi.org/10.1016/j.neuroscience.2009.07.056

Sauvageau, E., & Lefrancois, S. (2019). A beginner's guide to bioluminescence resonance energy transfer (BRET). *The Biochemist*, *41*(6), 36–40. https://doi.org/10.1042/BIO04106036

Schiöth, H. B., & Fredriksson, R. (2005). The GRAFS classification system of G-protein coupled receptors in comparative perspective. *General and Comparative Endocrinology*, *142*(1), 94–101. https://doi.org/https://doi.org/10.1016/j.ygcen.2004.12.018

Schlesinger, M. J., Magee, A. I., & Schmidt, M. F. (1980). Fatty acid acylation of proteins in cultured cells. *The Journal of Biological Chemistry*, *255*(21), 10021–10024.

Schmidt, M. F. G., & Schlesinger, M. J. (1979). Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein. *Cell*, *17*(4), 813–819. https://doi.org/10.1016/0092-8674(79)90321-0

Schneier, F. R., Liebowitz, M. R., Abi-Dargham, A., Zea-Ponce, Y., Lin, S. H., & Laruelle, M. (2000). Low dopamine D 2 receptor binding potential in social phobia. *American Journal of Psychiatry*, *157*(3), 457–459. https://doi.org/10.1176/appi.ajp.157.3.457

Scholl, D. J., & Wells, J. N. (2000). Serine and alanine mutagenesis of the nine native cysteine residues of the human A(1) adenosine receptor. *Biochemical Pharmacology*, *60*(11), 1647–1654. https://doi.org/10.1016/s0006-2952(00)00474-3

Schubert, B., VanDongen, A. M., Kirsch, G. E., & Brown, A. M. (1989). Beta-adrenergic inhibition of cardiac sodium channels by dual G-protein pathways. *Science (New York, N.Y.)*, *245*(4917), 516–519. https://doi.org/10.1126/science.2547248

Schülein, R., Liebenhoff, U., Müller, H., Birnbaumer, M., & Rosenthal, W. (1996). Properties of the human arginine vasopressin V2 receptor after site-directed mutagenesis of its putative palmitoylation site. *The Biochemical Journal*, *313 (Pt 2*(Pt 2), 611–616. https://doi.org/10.1042/bj3130611

Schwartz, J.-C., Diaz, J., Bordet, R., Griffon, N., Perachon, S., Pilon, C., Ridray, S., & Sokoloff, P. (1998). Functional implications of multiple dopamine receptor subtypes: the D1/D3 receptor coexistence1Published on the World Wide Web on 24 November 1997.1. *Brain Research Reviews*, *26*(2–3), 236–242. https://doi.org/10.1016/s0165-0173(97)00046-5

Sekar, R., Singh, K., Arokiaraj, A. W. R., & Chow, B. K. C. (2016). Pharmacological Actions of Glucagon-Like Peptide-1, Gastric Inhibitory Polypeptide, and Glucagon. *International Review of Cell and Molecular Biology*, *326*, 279–341. https://doi.org/10.1016/bs.ircmb.2016.05.002

Selvakumar, P., Lakshmikuttyamma, A., Shrivastav, A., Das, S. B., Dimmock, J. R., & Sharma, R. K. (2007). Potential role of N-myristoyltransferase in cancer. *Progress in Lipid Research*, *46*(1), 1–36. https://doi.org/10.1016/j.plipres.2006.05.002

Seno, K., & Hayashi, F. (2017). Palmitoylation is a prerequisite for dimerization-dependent raftophilicity of rhodopsin. *The Journal of Biological Chemistry*, *292*(37), 15321–15328. https://doi.org/10.1074/jbc.M117.804880

Seykora, J. T., Myat, M. M., Allen, L. A., Ravetch, J. V, & Aderem, A. (1996). Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *The Journal of Biological Chemistry*, *271*(31), 18797–18802. https://doi.org/10.1074/jbc.271.31.18797

Shaye, H., Ishchenko, A., Lam, J. H., Han, G. W., Xue, L., Rondard, P., Pin, J.-P., Katritch, V., Gati, C., & Cherezov, V. (2020). Structural basis of the activation of a metabotropic GABA receptor. *Nature*, *584*(7820), 298–303. https://doi.org/10.1038/s41586-020-2408-4

Shearer, L. J., & Petersen, N. O. (2019). Distribution and Co-localization of endosome markers in cells. *Heliyon*, *5*(9), e02375. https://doi.org/https://doi.org/10.1016/j.heliyon.2019.e02375

Shpakov, A. O. (2023). Allosteric Regulation of G-Protein-Coupled Receptors: From Diversity of Molecular Mechanisms to Multiple Allosteric Sites and Their Ligands. In *International Journal of Molecular Sciences* (Vol. 24, Issue 7). https://doi.org/10.3390/ijms24076187

Sibley, D. R., & Monsma, F. J. (1992). Molecular biology of dopamine receptors. *Trends in Pharmacological Sciences*, *13*, 61–69. https://doi.org/https://doi.org/10.1016/0165-6147(92)90025-2

Simon, M. I., Strathmann, M. P., & Gautam, N. (1991). Diversity of G proteins in signal transduction. *Science (New York, N.Y.)*, *252*(5007), 802–808. https://doi.org/10.1126/science.1902986

Smith, R. D., & Goldin, A. L. (1997). Phosphorylation at a single site in the rat brain sodium channel is necessary and sufficient for current reduction by protein kinase A. *Journal of Neuroscience*, *17*(16), 6086–6093. https://doi.org/10.1523/jneurosci.17-16-06086.1997

Smrcka, A. V, & Sternweis, P. C. (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein alpha and beta gamma subunits. *The Journal of Biological Chemistry*, *268*(13), 9667–9674.

Snyder, G. L., Allen, P. B., Fienberg, A. A., Valle, C. G., Huganir, R. L., Nairn, A. C., & Greengard, P. (2000). Regulation of phosphorylation of the GluR1 AMPA receptor in the neostriatum by dopamine and psychostimulants in vivo. *Journal of Neuroscience*, *20*(12), 4480–4488. https://doi.org/10.1523/jneurosci.20-12-04480.2000

Snyder, G. L., Fienberg, A. A., Huganir, R. L., & Greengard, P. (1998). A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (M(r) 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor. *Journal of Neuroscience*, *18*(24), 10297–10303. https://doi.org/10.1523/JNEUROSCI.18-24-10297.1998

Sokoloff, P., Giros, B., Martres, M. P., Bouthenet, M. L., & Schwartz, J. C. (1990). Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature*, *347*(6289), 146–151. https://doi.org/10.1038/347146a0

Solís, O., Garcia-Montes, J. R., González-Granillo, A., Xu, M., & Moratalla, R. (2017). Dopamine D3 Receptor Modulates l-DOPA-Induced Dyskinesia by Targeting D1 Receptor-Mediated Striatal Signaling. *Cerebral Cortex (New York, N.Y. : 1991)*, *27*(1), 435–446. https://doi.org/10.1093/cercor/bhv231

Sondek, J., & Siderovski, D. P. (2001). Gγ-like (GGL) domains: New frontiers in G-protein signaling and β-propeller scaffolding. *Biochemical Pharmacology*, *61*(11), 1329–1337. https://doi.org/10.1016/S0006-2952(01)00633-5

Sposini, S., Jean-Alphonse, F. G., Ayoub, M. A., Oqua, A., West, C., Lavery, S., Brosens, J. J., Reiter, E., & Hanyaloglu, A. C. (2017). Integration of GPCR Signaling and Sorting from Very Early Endosomes via Opposing APPL1 Mechanisms. *Cell Reports*, *21*(10), 2855–2867. https://doi.org/10.1016/j.celrep.2017.11.023

Sprang, S. (2001). GEFs: master regulators of G-protein activation. *Trends in Biochemical Sciences*, *26*(4), 266–267. https://doi.org/https://doi.org/10.1016/S0968-0004(01)01818-7

Sriram, K., & Insel, P. A. (2018). G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs? *Molecular Pharmacology*, *93*(4), 251–258. https://doi.org/10.1124/mol.117.111062

Stagkourakis, S., Dunevall, J., Taleat, Z., Ewing, A. G., & Broberger, C. (2019). Dopamine Release Dynamics in the Tuberoinfundibular Dopamine System. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *39*(21), 4009–4022. https://doi.org/10.1523/JNEUROSCI.2339-18.2019

Staufenbiel, M. (1987). Ankyrin-bound fatty acid turns over rapidly at the erythrocyte plasma membrane. *Molecular and Cellular Biology*, *7*(8), 2981–2984. https://doi.org/10.1128/mcb.7.8.2981

Staus, D. P., Hu, H., Robertson, M. J., Kleinhenz, A. L. W., Wingler, L. M., Capel, W. D., Latorraca, N. R., Lefkowitz, R. J., & Skiniotis, G. (2020). Structure of the M2 muscarinic receptor-β-arrestin complex in a lipid nanodisc. *Nature*, *579*(7798), 297–302. https://doi.org/10.1038/s41586-020-1954-0

Stoffel, R. H., Randall, R. R., Premont, R. T., Lefkowitz, R. J., & Inglese, J. (1994). Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *The Journal of Biological Chemistry*, *269*(45), 27791–27794.

Strumillo, M., & Beltrao, P. (2015). Towards the computational design of protein post-translational regulation. *Bioorganic & Medicinal Chemistry*, *23*(12), 2877–2882. https://doi.org/10.1016/j.bmc.2015.04.056

Stryer, L. (1986). Cyclic GMP Cascade of Vision. *Annual Review of Neuroscience*, *9*(1), 87–119. https://doi.org/10.1146/annurev.ne.09.030186.000511

Sugars, J. M., Cellek, S., Manifava, M., Coadwell, J., & Ktistakis, N. T. (1999). Fatty acylation of phospholipase D1 on cysteine residues 240 and 241 determines localization on intracellular membranes. *The Journal of Biological Chemistry*, *274*(42), 30023–30027. https://doi.org/10.1074/jbc.274.42.30023

Sun, W.-L., Quizon, P. M., & Zhu, J. (2016). Molecular Mechanism: ERK Signaling, Drug

Addiction, and Behavioral Effects. *Progress in Molecular Biology and Translational Science*, *137*, 1–40. https://doi.org/10.1016/bs.pmbts.2015.10.017

Surmeier, D. J., Bargas, J., Hemmings, H. C., Nairn, A. C., & Greengard, P. (1995). Modulation of calcium currents by a D1 dopaminergic protein kinase/phosphatase cascade in rat neostriatal neurons. *Neuron*, *14*(2), 385–397. https://doi.org/10.1016/0896-6273(95)90294-5

Surmeier, D. J., Eberwine, J., Wilson, C. J., Cao, Y., Stefani, A., & Kitai, S. T. (1992). Dopamine receptor subtypes colocalize in rat striatonigral neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(21), 10178–10182. https://doi.org/10.1073/pnas.89.21.10178

Surmeier, D. J., & Kitai, S. T. (1993). D1 and D2 dopamine receptor modulation of sodium and potassium currents in rat neostriatal neurons. *Progress in Brain Research*, *99*(C), 309–324. https://doi.org/10.1016/S0079-6123(08)61354-0

Surmeier, D. J., Song, W. J., & Yan, Z. (1996). Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *Journal of Neuroscience*, *16*(20), 6579–6591. https://doi.org/10.1523/jneurosci.16-20-06579.1996

Sutkeviciute, I., & Vilardaga, J.-P. (2020). Structural insights into emergent signaling modes of G protein-coupled receptors. *The Journal of Biological Chemistry*, *295*(33), 11626–11642. https://doi.org/10.1074/jbc.REV120.009348

Suzuki, N., Nakamura, S., Mano, H., & Kozasa, T. (2003). Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(2), 733–738. https://doi.org/10.1073/pnas.0234057100

Swarthout, J. T., Lobo, S., Farh, L., Croke, M. R., Greentree, W. K., Deschenes, R. J., & Linder, M. E. (2005). DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. *The Journal of Biological Chemistry*, *280*(35), 31141–31148. https://doi.org/10.1074/jbc.M504113200

Szakadáti, G., Tóth, A. D., Oláh, I., Erdélyi, L. S., Balla, T., Várnai, P., Hunyady, L., & Balla, A. (2015). Investigation of the fate of type I angiotensin receptor after biased activation. *Molecular Pharmacology*, *87*(6), 972–981. https://doi.org/10.1124/mol.114.097030

Szalai, B., Hoffmann, P., Prokop, S., Erdélyi, L., Várnai, P., & Hunyady, L. (2014). Improved methodical approach for quantitative BRET analysis of G protein coupled receptor dimerization. *PLoS ONE*, *9*(10), 1–11. https://doi.org/10.1371/journal.pone.0109503

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., & Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Developmental Cell*, *11*(6), 791–801. https://doi.org/10.1016/j.devcel.2006.10.003

Takeda, J., & Kinoshita, T. (1995). GPI-anchor biosynthesis. *Trends in Biochemical Sciences*, *20*(9), 367–371. https://doi.org/10.1016/s0968-0004(00)89078-7

Takei, K., Yoshida, Y., & Yamada, H. (2005). Regulatory mechanisms of dynamin-dependent endocytosis. *Journal of Biochemistry*, *137*(3), 243–247. https://doi.org/10.1093/jb/mvi052

Tanaka, K., Nagayama, Y., Nishihara, E., Namba, H., Yamashita, S., & Niwa, M. (1998).

Palmitoylation of human thyrotropin receptor: slower intracellular trafficking of the palmitoylation-defective mutant. *Endocrinology*, *139*(2), 803–806. https://doi.org/10.1210/endo.139.2.5911

Tanimura, A., Pancani, T., Lim, S. A. O., Tubert, C., Melendez, A. E., Shen, W., & Surmeier, D. J. (2018). Striatal cholinergic interneurons and Parkinson's disease. *The European Journal of Neuroscience*, *47*(10), 1148–1158. https://doi.org/10.1111/ejn.13638

Taylor, J. S., Reid, T. S., Terry, K. L., Casey, P. J., & Beese, L. S. (2003). Structure of mammalian protein geranylgeranyltransferase type-I. *The EMBO Journal*, *22*(22), 5963–5974. https://doi.org/10.1093/emboj/cdg571

Tewari, R., West, S. J., Shayahati, B., & Akimzhanov, A. M. (2020). Detection of protein s-acylation using acyl-resin assisted capture. *Journal of Visualized Experiments*, *2020*(158), 1–6. https://doi.org/10.3791/61016

Thomas, J. R., Dwek, R. A., & Rademacher, T. W. (1990). Structure, biosynthesis, and function of glycosylphosphatidylinositols. *Biochemistry*, *29*(23), 5413–5422. https://doi.org/10.1021/bi00475a001

Thomsen, A. R. B., Plouffe, B., Cahill, T. J., Shukla, A. K., Tarrasch, J. T., Dosey, A. M., Kahsai, A. W., Strachan, R. T., Pani, B., Mahoney, J. P., Huang, L., Breton, B., Heydenreich, F. M., Sunahara, R. K., Skiniotis, G., Bouvier, M., & Lefkowitz, R. J. (2016). GPCR-G Protein-β-Arrestin Super-Complex Mediates Sustained G Protein Signaling. *Cell*, *166*(4), 907–919. https://doi.org/10.1016/j.cell.2016.07.004

Tian, X., Kang, D. S., & Benovic, J. L. (2014). β-arrestins and G protein-coupled receptor trafficking. *Handbook of Experimental Pharmacology*, *219*, 173–186. https://doi.org/10.1007/978-3-642-41199-1_9

Tiu, A. C., Yang, J., Asico, L. D., Konkalmatt, P., Zheng, X., Cuevas, S., Wang, X., Lee, H., Mazhar, M., Felder, R. A., Jose, P. A., & Villar, V. A. M. (2020). Lipid rafts are required for effective renal D(1) dopamine receptor function. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *34*(5), 6999–7017. https://doi.org/10.1096/fj.201902710RR

Tiulpakov, A., White, C. W., Abhayawardana, R. S., See, H. B., Chan, A. S., Seeber, R. M., Heng, J. I., Dedov, I., Pavlos, N. J., & Pfleger, K. D. G. G. (2016). Mutations of Vasopressin Receptor 2 Including Novel L312S Have Differential Effects on Trafficking | Molecular Endocrinology | Oxford Academic. *Molecular Endocrinology*, *30*(8), 889–904. https://doi.org/10.1210/me.2016-1002

Tobin, A. B., & Wheatley, M. (2004). G-protein-coupled receptor phosphorylation and palmitoylation. *Methods in Molecular Biology (Clifton, N.J.)*, *259*, 275–281. https://doi.org/10.1385/1-59259-754-8:275

Topinka, J. R., & Bredt, D. S. (1998). N-terminal palmitoylation of PSD-95 regulates association with cell membranes and interaction with K+ channel K(v)1.4. *Neuron*, *20*(1), 125–134. https://doi.org/10.1016/S0896-6273(00)80440-7

Tota, M. R., & Strader, C. D. (1990). Characterization of the binding domain of the beta-adrenergic

receptor with the fluorescent antagonist carazolol. Evidence for a buried ligand binding site. *Journal of Biological Chemistry*, *265*(28), 16891–16897. https://doi.org/https://doi.org/10.1016/S0021-9258(17)44846-0

Towler, D. A., Eubanks, S. R., Towery, D. S., Adams, S. P., & Glaser, L. (1987). Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *The Journal of Biological Chemistry*, *262*(3), 1030–1036.

Traut, T. W. (1994). Physiological concentrations of purines and pyrimidines. *Molecular and Cellular Biochemistry*, *140*(1), 1–22. https://doi.org/10.1007/BF00928361

Tréfier, A., Musnier, A., Landomiel, F., Bourquard, T., Boulo, T., Ayoub, M. A., León, K., Bruneau, G., Chevalier, M., Durand, G., Blache, M. C., Inoue, A., Fontaine, J., Gauthier, C., Tesseraud, S., Reiter, E., Poupon, A., & Crépieux, P. (2018). G protein-dependent signaling triggers a β-arrestin-scaffolded p70S6K/ rpS6 module that controls 5′TOP mRNA translation. *FASEB Journal*, *32*(3), 1154–1169. https://doi.org/10.1096/fj.201700763R

Tsukamoto, T., Li, X., Morita, H., Minowa, T., Aizawa, T., Hanagata, N., & Demura, M. (2013). Role of S-Palmitoylation on IFITM5 for the Interaction with FKBP11 in Osteoblast Cells. *PLOS ONE*, *8*(9), e75831. https://doi.org/10.1371/journal.pone.0075831

Tsvetanova, N. G., & von Zastrow, M. (2014). Spatial encoding of cyclic AMP signaling specificity by GPCR endocytosis. *Nature Chemical Biology*, *10*(12), 1061–1065. https://doi.org/10.1038/nchembio.1665

Ulengin-Talkish, I., Parson, M. A. H., Jenkins, M. L., Roy, J., Shih, A. Z. L., St-Denis, N., Gulyas, G., Balla, T., Gingras, A.-C., Várnai, P., Conibear, E., Burke, J. E., & Cyert, M. S. (2021). Palmitoylation targets the calcineurin phosphatase to the phosphatidylinositol 4-kinase complex at the plasma membrane. *Nature Communications*, *12*(1), 6064. https://doi.org/10.1038/s41467-021-26326-4

Undieh, A. S. (2010). Pharmacology of signaling induced by dopamine D(1)-like receptor activation. *Pharmacology & Therapeutics*, *128*(1), 37–60. https://doi.org/10.1016/j.pharmthera.2010.05.003

Uribe, A., Zariñán, T., Pérez-Solis, M. A., Gutiérrez-Sagal, R., Jardón-Valadez, E., Piñeiro, A., Dias, J. A., & Ulloa-Aguirre, A. (2008). Functional and structural roles of conserved cysteine residues in the carboxyl-terminal domain of the follicle-stimulating hormone receptor in human embryonic kidney 293 cells. *Biology of Reproduction*, *78*(5), 869–882. https://doi.org/10.1095/biolreprod.107.063925

Urs, N. M., Daigle, T. L., & Caron, M. G. (2011). A dopamine D1 receptor-dependent β-arrestin signaling complex potentially regulates morphine-induced psychomotor activation but not reward in mice. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *36*(3), 551–558. https://doi.org/10.1038/npp.2010.186

Utsumi, T., Sakurai, N., Nakano, K., & Ishisaka, R. (2003). C-terminal 15 kDa fragment of cytoskeletal actin is posttranslationally N-myristoylated upon caspase-mediated cleavage and targeted to mitochondria. *FEBS Letters*, *539*(1–3), 37–44. https://doi.org/10.1016/s0014-5793(03)00180-7

van der Vusse, G. J., van Bilsen, M., Glatz, J. F. C., Hasselbaink, D. M., & Luiken, J. J. F. P. (2002). Critical steps in cellular fatty acid uptake and utilization. *Molecular and Cellular Biochemistry*, *239*(1–2), 9–15. https://doi.org/10.1007/978-1-4419-9270-3_2

Van Eps, N., Altenbach, C., Caro, L. N., Latorraca, N. R., Hollingsworth, S. A., Dror, R. O., Ernst, O. P., & Hubbell, W. L. (2018). G(i)- and G(s)-coupled GPCRs show different modes of G-protein binding. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(10), 2383–2388. https://doi.org/10.1073/pnas.1721896115

Vartak, N., Papke, B., Grecco, H. E., Rossmannek, L., Waldmann, H., Hedberg, C., & Bastiaens, P. I. H. (2014). The autodepalmitoylating activity of APT maintains the spatial organization of palmitoylated membrane proteins. *Biophysical Journal*, *106*(1), 93–105. https://doi.org/10.1016/j.bpj.2013.11.024

Veit, M. (2012). Palmitoylation of virus proteins. *Biology of the Cell*, *104*(9), 493–515. https://doi.org/10.1111/boc.201200006

Venkatesan, S., Rose, J. J., & Lodge, R. (2002). Distinct Mechanisms of Agonist-induced Endocytosis for Human Chemokine Receptors CCR5 and CXCR4. *Molecular Biology of the Cell*, *13*(November), 4100–4109. https://doi.org/10.1091/mbc.E02

Vermeulen, R. J., Drukarch, B., Sahadat, M. C., Goosen, C., Wolters, E. C., & Stoof, J. C. (1994). The dopamine D1 agonist SKF 81297 and the dopamine D2 agonist LY 171555 act synergistically to stimulate motor behavior of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned parkinsonian rhesus monkeys. *Movement Disorders : Official Journal of the Movement Disorder Society*, *9*(6), 664–672. https://doi.org/10.1002/mds.870090613

Vilardaga, J. P., Bünemann, M., Feinstein, T. N., Lambert, N., Nikolaev, V. O., Engelhardt, S., Lohse, M. J., & Hoffmann, C. (2009). Minireview: GPCR and G proteins: Drug efficacy and activation in live cells. *Molecular Endocrinology*, *23*(5), 590–599. https://doi.org/10.1210/me.2008-0204

Villar, V. A. M., Cuevas, S., Zheng, X., & Jose, P. A. (2016). Localization and signaling of GPCRs in lipid rafts. *Methods in Cell Biology*, *132*, 3–23. https://doi.org/10.1016/bs.mcb.2015.11.008

Vizurraga, A., Adhikari, R., Yeung, J., Yu, M., & Tall, G. G. (2020). Mechanisms of adhesion G protein-coupled receptor activation. *The Journal of Biological Chemistry*, *295*(41), 14065–14083. https://doi.org/10.1074/jbc.REV120.007423

Voyno-Yasenetskaya, T. A., Faure, M. P., Ahn, N. G., & Bourne, H. R. (1996). Galpha12 and Galpha13 regulate extracellular signal-regulated kinase and c-Jun kinase pathways by different mechanisms in COS-7 cells. *The Journal of Biological Chemistry*, *271*(35), 21081–21087. https://doi.org/10.1074/jbc.271.35.21081

Voyno-Yasenetskaya, T., Conklin, B. R., Gilbert, R. L., Hooley, R., Bourne, H. R., & Barber, D. L. (1994). G alpha 13 stimulates Na-H exchange. *The Journal of Biological Chemistry*, *269*(7), 4721–4724.

Wan, Q., Okashah, N., Inoue, A., Nehme, R., Carpenter, B., Tate, C. G., & Lambert, N. A. (2018). Mini G protein probes for active G protein– coupled receptors (GPCRs) in live cells. *Journal of Biological Chemistry*, *293*(19), 7466–7473. https://doi.org/10.1074/jbc.RA118.001975

Wang, E. A., Cepeda, C., & Levine, M. S. (2012). Cognitive Deficits in Huntington's Disease: Insights from Animal Models. *Current Translational Geriatrics and Experimental Gerontology Reports*, *1*(1), 29–38. https://doi.org/10.1007/s13670-011-0005-y

Wang, H. Y., Undie, A. S., & Friedman, E. (1995). Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: Possible role in dopamine-mediated inositol phosphate formation. *Molecular Pharmacology*, *48*(6), 988–994.

Wang, M., Datta, D., Enwright, J., Galvin, V., Yang, S.-T., Paspalas, C., Kozak, R., Gray, D. L., Lewis, D. A., & Arnsten, A. F. T. (2019). A novel dopamine D1 receptor agonist excites delay-dependent working memory-related neuronal firing in primate dorsolateral prefrontal cortex. *Neuropharmacology*, *150*, 46–58. https://doi.org/10.1016/j.neuropharm.2019.03.001

Wang, M., Jiang, Y., & Xu, X. (2015). A novel method for predicting post-translational modifications on serine and threonine sites by using site-modification network profiles. *Molecular BioSystems*, *11*(11), 3092–3100. https://doi.org/10.1039/c5mb00384a

Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V, Sharpless, K. B., & Finn, M. G. (2003). Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *Journal of the American Chemical Society*, *125*(11), 3192–3193. https://doi.org/10.1021/ja021381e

Wang, Y.-C., Peterson, S. E., & Loring, J. F. (2014). Protein post-translational modifications and regulation of pluripotency in human stem cells. *Cell Research*, *24*(2), 143–160.

Wanka, L., Babilon, S., Kaiser, A., Mörl, K., & Beck-Sickinger, A. G. (2018). Different mode of arrestin-3 binding at the human Y1 and Y2 receptor. *Cellular Signalling*, *50*, 58–71. https://doi.org/https://doi.org/10.1016/j.cellsig.2018.06.010

Wedegaertner, P. B. (1998). Lipid modifications and membrane targeting of G alpha. *Biological Signals and Receptors*, *7*(2), 125–135. https://doi.org/10.1159/000014538

Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., & Bourne, H. R. (1993). Palmitoylation is required for signaling functions and membrane attachment of G(q)α and G(s)α. *Journal of Biological Chemistry*, *268*(33), 25001–25008.

Weinstein, L. S., Liu, J., Sakamoto, A., Xie, T., & Chen, M. (2004). Minireview: GNAS: Normal and abnormal functions. *Endocrinology*, *145*(12), 5459–5464. https://doi.org/10.1210/en.2004-0865

Weis, W. I., & Kobilka, B. K. (2018). The Molecular Basis of G Protein–Coupled Receptor Activation. *Annual Review of Biochemistry*, *87*(1), 897–919. https://doi.org/10.1146/annurev-biochem-060614-033910

Whorton, M. R., Bokoch, M. P., Rasmussen, S. G. F., Huang, B., Zare, R. N., Kobilka, B., & Sunahara, R. K. (2007). A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(18), 7682–7687. https://doi.org/10.1073/pnas.0611448104

Wilcox, C., Hu, J. S., & Olson, E. N. (1987). Acylation of proteins with myristic acid occurs cotranslationally. *Science (New York, N.Y.)*, *238*(4831), 1275–1278. https://doi.org/10.1126/science.3685978

Wilden, U. (1995). Duration and Amplitude of the Light-Induced cGMP Hydrolysis in Vertebrate

Photoreceptors Are Regulated by Multiple Phosphorylation of Rhodopsin and by Arrestin Binding. *Biochemistry*, *34*(4), 1446–1454. https://doi.org/10.1021/bi00004a040

Wilden, U., Hall, S. W., & Kühn, H. (1986). Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proceedings of the National Academy of Sciences*, *83*(5), 1174–1178. https://doi.org/10.1073/pnas.83.5.1174

Williams, D. M., Nawaz, A., & Evans, M. (2020). Drug Therapy in Obesity: A Review of Current and Emerging Treatments. *Diabetes Therapy : Research, Treatment and Education of Diabetes and Related Disorders*, *11*(6), 1199–1216. https://doi.org/10.1007/s13300-020-00816-y

Winfield, I., Barkan, K., Routledge, S., Robertson, N. J., Harris, M., Jazayeri, A., Simms, J., Reynolds, C. A., Poyner, D. R., & Ladds, G. (2022). The Role of ICL1 and H8 in Class B1 GPCRs; Implications for Receptor Activation. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/fendo.2021.792912

Wishart, D. S., Feunang, Y. D., Guo, A. C., Lo, E. J., Marcu, A., Grant, J. R., Sajed, T., Johnson, D., Li, C., Sayeeda, Z., Assempour, N., Iynkkaran, I., Liu, Y., Maciejewski, A., Gale, N., Wilson, A., Chin, L., Cummings, R., Le, D., … Wilson, M. (2018). DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Research*, *46*(D1), D1074–D1082. https://doi.org/10.1093/nar/gkx1037

Wong, A. H. C., Buckle, C. E., & Van Tol, H. H. M. (2000). Polymorphisms in dopamine receptors: What do they tell us? *European Journal of Pharmacology*, *410*(2–3), 183–203. https://doi.org/10.1016/S0014-2999(00)00815-3

Wood, E. J. (2006). Posttranslational Modification of Proteins: Expanding Nature's Inventory. *Bambed*, *34*(6), 461–462.

Wu, F., Yang, L., Hang, K., Laursen, M., Wu, L., Han, G. W., Ren, Q., Roed, N. K., Lin, G., Hanson, M. A., Jiang, H., Wang, M.-W., Reedtz-Runge, S., Song, G., & Stevens, R. C. (2020). Full-length human GLP-1 receptor structure without orthosteric ligands. *Nature Communications*, *11*(1), 1272. https://doi.org/10.1038/s41467-020-14934-5

Xiang, Y., Rybin, V. O., Steinberg, S. F., & Kobilka, B. (2002). Caveolar localization dictates physiologic signaling of beta 2-adrenoceptors in neonatal cardiac myocytes. *The Journal of Biological Chemistry*, *277*(37), 34280–34286. https://doi.org/10.1074/jbc.M201644200

Xiao, X., Tang, J.-J., Peng, C., Wang, Y., Fu, L., Qiu, Z.-P., Xiong, Y., Yang, L.-F., Cui, H.-W., He, X.-L., Yin, L., Qi, W., Wong, C. C. L., Zhao, Y., Li, B.-L., Qiu, W.-W., & Song, B.-L. (2017). Cholesterol Modification of Smoothened Is Required for Hedgehog Signaling. *Molecular Cell*, *66*(1), 154-162.e10. https://doi.org/https://doi.org/10.1016/j.molcel.2017.02.015

Xie, Y., Zheng, Y., Li, H., Luo, X., He, Z., Cao, S., Shi, Y., Zhao, Q., Xue, Y., Zuo, Z., & Ren, J. (2016). GPS-Lipid: a robust tool for the prediction of multiple lipid modification sites. *Scientific Reports*, *6*, 28249. https://doi.org/10.1038/srep28249

Xu, P., Huang, S., Krumm, B. E., Zhuang, Y., Mao, C., Zhang, Y., Wang, Y., Huang, X.-P., Liu, Y.-F., He, X., Li, H., Yin, W., Jiang, Y., Zhang, Y., Roth, B. L., & Xu, H. E. (2022). Structural Genomics of the Human Dopamine Receptor System. *BioRxiv*, 2022.10.09.511478.

https://doi.org/10.1101/2022.10.09.511478

Xu, Y., & Chou, K.-C. (2016). Recent Progress in Predicting Posttranslational Modification Sites in Proteins. *Current Topics in Medicinal Chemistry*, *16*(6), 591–603. https://doi.org/10.2174/1568026615666150819110421

Xue, Y., Chen, H., Jin, C., Sun, Z., & Yao, X. (2006). *NBA-Palm : prediction of palmitoylation site implemented in Naïve Bayes algorithm*. *10*. https://doi.org/10.1186/1471-2105-7-458

Yan, Z., Hsieh-Wilson, L., Feng, J., Tomizawa, K., Allen, P. B., Fienberg, A. A., Nairn, A. C., & Greengard, P. (1999). Protein phosphatase 1 modulation of neostriatal AMPA channels: Regulation by DARPP-32 and spinophilin. *Nature Neuroscience*, *2*(1), 13–17. https://doi.org/10.1038/4516

Yan, Z., Song, W. J., & Surmeier, D. J. (1997). D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-insensitive pathway. *Journal of Neurophysiology*, *77*(2), 1003–1015. https://doi.org/10.1152/jn.1997.77.2.1003

Yang, D., Zhou, Q., Labroska, V., Qin, S., Darbalaei, S., Wu, Y., Yuliantie, E., Xie, L., Tao, H., Cheng, J., Liu, Q., Zhao, S., Shui, W., Jiang, Y., & Wang, M.-W. (2021). G protein-coupled receptors: structure- and function-based drug discovery. *Signal Transduction and Targeted Therapy*, *6*(1), 7. https://doi.org/10.1038/s41392-020-00435-w

Yang, H.-S., Sun, N., Zhao, X., Kim, H. R., Park, H.-J., Kim, K.-M., & Chung, K. Y. (2019). Role of Helix 8 in Dopamine Receptor Signaling. *Biomolecules & Therapeutics*, *27*(6), 514–521. https://doi.org/10.4062/biomolther.2019.026

Yang, S. N. (2000). Sustained enhancement of AMPA receptor- and NMDA receptor-mediated currents induced by dopamine D1/D5 receptor activation in the hippocampus: an essential role of postsynaptic Ca2+. *Hippocampus*, *10*(1), 57–63. https://doi.org/10.1002/(SICI)1098-1063(2000)10:1<57::AID-HIPO6>3.0.CO;2-0

Yang, Y., Lee, S.-M., Imamura, F., Gowda, K., Amin, S., & Mailman, R. B. (2021). D1 dopamine receptors intrinsic activity and functional selectivity affect working memory in prefrontal cortex. *Molecular Psychiatry*, *26*(2), 645–655. https://doi.org/10.1038/s41380-018-0312-1

Yang, Y., Lewis, M. M., Huang, X., Dokholyan, N. V, & Mailman, R. B. (2022). Dopamine D(1) receptor-mediated β-arrestin signaling: Insight from pharmacology, biology, behavior, and neurophysiology. *The International Journal of Biochemistry & Cell Biology*, *148*, 106235. https://doi.org/10.1016/j.biocel.2022.106235

Yatani, A., Mattera, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M., & Birnbaumer, L. (1988). The G protein-gated atrial K+ channel is stimulated by three distinct Gi alpha-subunits. *Nature*, *336*(6200), 680–682. https://doi.org/10.1038/336680a0

Yeste-Velasco, M., Linder, M. E., & Lu, Y. J. (2015). Protein S-palmitoylation and cancer. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1856*(1), 107–120. https://doi.org/10.1016/j.bbcan.2015.06.004

Young, C. E., & Yang, C. R. (2004). Dopamine D1/D5 Receptor Modulates State-Dependent Switching of Soma-Dendritic Ca2+ Potentials via Differential Protein Kinase A and C

Activation in Rat Prefrontal Cortical Neurons. *Journal of Neuroscience*, *24*(1), 8–23. https://doi.org/10.1523/JNEUROSCI.1650-03.2004

Zha, J., Weiler, S., Oh, K. J., Wei, M. C., & Korsmeyer, S. J. (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science (New York, N.Y.)*, *290*(5497), 1761–1765. https://doi.org/10.1126/science.290.5497.1761

Zhang, B., Li, S., & Shui, W. (2022). Post-Translational Modifications of G Protein-Coupled Receptors Revealed by Proteomics and Structural Biology. *Frontiers in Chemistry*, *10*, 843502. https://doi.org/10.3389/fchem.2022.843502

Zhang, H., Kong, Q., Wang, J., Jiang, Y., & Hua, H. (2020). Complex roles of cAMP–PKA–CREB signaling in cancer. *Experimental Hematology & Oncology*, *9*(1), 32. https://doi.org/10.1186/s40164-020-00191-1

Zhang, M. M., & Hang, H. C. (2017). Protein S-palmitoylation in cellular differentiation. *Biochemical Society Transactions*, *45*(1), 275–285. https://doi.org/10.1042/BST20160236

Zhang, R., & Xie, X. (2012). Tools for GPCR drug discovery. *Acta Pharmacologica Sinica*, *33*(3), 372–384. https://doi.org/10.1038/aps.2011.173

Zhang, X., & Kim, K.-M. (2016). Palmitoylation of the carboxyl-terminal tail of dopamine D4 receptor is required for surface expression, endocytosis, and signaling. *Biochemical and Biophysical Research Communications*, *479*(2), 398–403. https://doi.org/https://doi.org/10.1016/j.bbrc.2016.09.094

Zhang, X., Le, H. T., Zhang, X., Zheng, M., Choi, B.-G., & Kim, K.-M. (2016). Palmitoylation on the carboxyl terminus tail is required for the selective regulation of dopamine D2 versus D3 receptors. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1858*(9), 2152–2162. https://doi.org/https://doi.org/10.1016/j.bbamem.2016.06.021

Zhang, Y., Hoon, M. A., Chandrashekar, J., Mueller, K. L., Cook, B., Wu, D., Zuker, C. S., & Ryba, N. J. P. (2003). Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell*, *112*(3), 293–301. https://doi.org/10.1016/s0092-8674(03)00071-0

Zhang, Y., Qin, Z., Sun, W., Chu, F., & Zhou, F. (2021). Function of Protein S-Palmitoylation in Immunity and Immune-Related Diseases. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.661202

Zhang, Z., Li, X., Yang, F., Chen, C., Liu, P., Ren, Y., Sun, P., Wang, Z., You, Y., Zeng, Y.-X., & Li, X. (2021). DHHC9-mediated GLUT1 S-palmitoylation promotes glioblastoma glycolysis and tumorigenesis. *Nature Communications*, *12*(1), 5872. https://doi.org/10.1038/s41467-021-26180-4

Zheng, B., DeRan, M., Li, X., Liao, X., Fukata, M., & Wu, X. (2013). 2-Bromopalmitate analogues as activity-based probes to explore palmitoyl acyltransferases. *Journal of the American Chemical Society*, *135*(19), 7082–7085. https://doi.org/10.1021/ja311416v

Zheng, H., Pearsall, E. A., Hurst, D. P., Zhang, Y., Chu, J., Zhou, Y., Reggio, P. H., Loh, H. H., & Law, P.-Y. (2012). Palmitoylation and membrane cholesterol stabilize μ-opioid receptor homodimerization and G protein coupling. *BMC Cell Biology*, *13*, 6.

https://doi.org/10.1186/1471-2121-13-6

Zhou, F., Xue, Y., Yao, X., & Xu, Y. (2006). CSS-Palm: palmitoylation site prediction with a clustering and scoring strategy (CSS). *Bioinformatics (Oxford, England)*, *22*(7), 894–896. https://doi.org/10.1093/bioinformatics/btl013

Zhou, X. E., He, Y., de Waal, P. W., Gao, X., Kang, Y., Van Eps, N., Yin, Y., Pal, K., Goswami, D., White, T. A., Barty, A., Latorraca, N. R., Chapman, H. N., Hubbell, W. L., Dror, R. O., Stevens, R. C., Cherezov, V., Gurevich, V. V, Griffin, P. R., … Xu, H. E. (2017). Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. *Cell*, *170*(3), 457-469.e13. https://doi.org/10.1016/j.cell.2017.07.002

Zhu, J., & Reith, M. E. A. (2008). Role of the dopamine transporter in the action of psychostimulants, nicotine, and other drugs of abuse. *CNS & Neurological Disorders Drug Targets*, *7*(5), 393–409. https://doi.org/10.2174/187152708786927877

Zhu, M.-Y., & Juorio, A. V. (1995). Aromatic l-amino acid decarboxylase: Biological characterization and functional role. *General Pharmacology: The Vascular System*, *26*(4), 681–696. https://doi.org/https://doi.org/10.1016/0306-3623(94)00223-A

Zmuda, F., & Chamberlain, L. H. (2020). Regulatory effects of post-translational modifications on zDHHC S-acyltransferases. *The Journal of Biological Chemistry*, *295*(43), 14640–14652. https://doi.org/10.1074/jbc.REV120.014717

Zverina, E. A., Lamphear, C. L., Wright, E. N., & Fierke, C. A. (2012). Recent advances in protein prenyltransferases: substrate identification, regulation, and disease interventions. *Current Opinion in Chemical Biology*, *16*(5–6), 544–552. https://doi.org/10.1016/j.cbpa.2012.10.015

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