Basis and molecular consequences of interactions between adrenomedullin and ACKR3/CXCR4

Pietro Cocchiara

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ABSTRACT

C-X-C-motif chemokine Receptor 4 (CXCR4) and Atypical Chemokine Receptor 3 (ACKR3) are two 7-transmembrane domains receptors widely expressed in the body and predominantly found in immune cells. These two receptors are often studied together due to their common ligand C-X-C-motif chemokine Ligand 12 (CXCL12) and its implication in several patho-physiological processes, such as embryo differentiation, cancer development and cancer cell proliferation, and the regulation of immune system responses to inflammatory stimuli. In the past, several non-chemokine ligands have been identified to be able to bind and exert various functions once bound to these receptors. In this thesis, the role of adrenomedullin (ADM), a non-chemokine peptide with pro-angiogenic effects, was explored. Since this small peptide is considered a putative ligand for ACKR3, the effects of the interactions between this ligand and ACKR3 were explored in Chapter 3. Indeed, a possible interaction was observed in xCELLigence Real-Time Cell Analysis (RTCA) with adrenomedullin influencing the activation of the receptor. However, this activation is still functionally poorly understood, since in β-arrestin recruitment the concentrations required for this interaction to happen were over physiological levels. Chapter 4 characterized the effects of the possible interaction between CXCR4 and adrenomedullin. Since in the past, the heterodimerization between CXCR4 and ACKR3 was shown, it was investigated whether adrenomedullin would have elicited any response upon stimulation of CXCR4 with this ligand to better clarify the role of this ligand in regulating the CXCR4-CXCL12-ACKR3 axis. Several approaches were used to study G protein signalling activation, β-arrestin recruitment and phosphorylation of CXCR4, for which adrenomedullin acted as a negative allosteric modulator affecting the efficacy of CXCL12 in activating G protein signalling, and as a biased antagonist towards CXCR4, showing a preference for blocking the G protein signalling pathway. Finally, in Chapter 5 a preliminary study of the GRK-mediated phosphorylation of both receptors was carried out, by using a small antagonist to block GRKs phosphorylation and NanoBiT technology to study the recruitment of these kinases to these receptors upon ligand-mediated activation. These approaches showed that GRK2/3/5/6 have an important role in phosphorylating the receptor, although the outcomes were cell-type and assay-dependent. However, this study opens the possibility of clarifying how the phosphorylation state affects the pharmacology of these receptors and to validate an effective "barcoding" of the receptors. In this thesis, it was demonstrated that adrenomedullin may represent an interesting starting point for developing new therapeutics targeting CXCR4 and ACKR3, although important structural studies are still required to highlight the outcomes of the interactions here described.

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Caspar, B., **Cocchiara, P.,** Melet, A., Van Emelen, K., Van der Aa, A., Milligan, G., & Herbeuval, J. P. (2022). CXCR4 as a novel target in immunology: moving away from typical antagonists. Future drug discovery, 4(2), FDD77. https://doi.org/10.4155/fdd-2022-0007

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AUTHOR'S DECLARATION

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not previously been submitted for a degree or diploma at the University of Glasgow or at any other institution.

Signature : ……...

Name : Pietro Cocchiara Date: 12th January 2024

LIST OF ABBREVIATIONS

Aminoacid letter codes:

A: Alanine

R: Arginine

N: Asparagine

D: Aspartic acid

C: Cysteine

E: Glutamic acid

Q: Glutamine

G: Glycine

H: Histidine

I: Isoleucine

L: Leucine

K: Lysine

M: Methionine

F: Phenylalanine

P: Proline

S: Serine

T: Threonine

W: Tryptophan

Y: Tyrosine

V: Valine

7TM: 7 transmembrane domains

α-SNAP: Alpha soluble NSF attachment protein

α1-AR: α1 adrenergic receptor

AC: adenylyl cyclase

ACKR#: atypical chemokine receptor #

ADCYAP1R1/PAC1: adenylate cyclase activating polypeptide receptor

ADM: adrenomedullin

ADP: adenosine diphosphate

aGPCR: adhesion G protein-coupled receptor

Akt: Ak mouse strain thymoma

AMD3100: plerixafor

AMR#: adrenomedullin receptor #

AP-2: adaptor protein complex 2

AT1A receptor: angiotensin 1A receptor

ATP: adenosine triphosphate

BAM22: Bovine adrenal medulla 22

BCA: bicinchoninic acid

bGFG: basic fibroblast growth factor

BLAST: Basic Local Alignment Search Tool

BN-PAGE: blue native polyacrylamide gel electrophoresis

BRET: bioluminescence resonance energy transfer

BSA: bovine serum albumin

Btk: Bruton tyrosine kinases

C-tail: carboxyl tail

C-terminus: carboxyl terminus

CALCR: calcitonin receptor

CALCRL: calcitonin-like receptor

cAMP: cyclic adenosine monophosphate

CaSR: calcium sensing receptor

CB1 receptor: cannabinoid type 1 receptor

CCL# : C-C chemokine ligand type #

CCR#: C-C chemokine receptor type #

CD#: cluster differentiation #

CI: cell index

CRHR#: corticotropin-releasing hormone receptor #

CRISPR-Cas9: r clustered regularly interspaced short palindromic repeats Cas9

CRS1/2: chemokine recognition site 1/2

CXCL12: CXC motif chemokine ligand 12

CXCR#: C-X-C chemokine receptor type #

DAG: diacylglycerol

DAMPs: damage-associated molecular patterns

DMSO: Dimethylsulfoxide

DMEM: Dulbecco's modified Eagle's medium

DNA: desoxyribonucleic acid

dNTPs: deoxyribonucleotides

Dox: doxycycline

DRY motif: Aspartate-Arginine-Tyrosine motif

DRYLAIV: Aspartate-Arginine-Tyrosine-Leucine-Alanine-Isoleucine-Valine motif

DRYLSIT: Aspartate-Arginine-Tyrosine-Leucine-Serine-Isoleucin-Threonine

DTT: dithiothreitol

E1/2/3: enzyme1/2/3

EC_{80}: effective concentration 80%

ECL: enhanced chemiluminescence5

ECL#: extracellular loop #

EDTA: Ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

eUb: extracellular ubiquitin

eYFP: enhanced yellow fluorescent protein

FBS: foetal bovine serum

FDA: Food and Drug Administration Organization

FZD#: frizzled receptor #

G protein: Guanosine nucleotide-binding protein

$GABA_{A/B}$: gamma-aminobutyric acid receptor A and B

GAIN: GPCR autoproteolysis-inducing domain

GAP1: general amino acids permease 1

GASP: G protein-coupled receptor-associated sorting protein

GCGR: glucagon receptor

GDP: guanosine diphosphate

GFP: green fluorescent protein

GHRHR: growth hormone- releasing hormone receptor

GIPR: gastric inhibitory polypeptide receptor

GLP#R: glucagon-like peptide # receptor

Gp120: glycoprotein 120

GPCR: G protein-coupled receptor

GPRC6A: G protein-coupled receptor family C group 6 member A

GPS: GPCRs proteolytic site

GRAFS: Glutamate, Rhodopsin, Adhesion, Frizzled/Tas2 and Secretin receptors

GRK#: G protein-coupled receptors kinase #

GTP: guanosine triphosphate

H8: eighth helix

HBD3: human ß-defensin-3

HBSS: Hank's balance salt solution

HEK cells: Human Embryonic Kidney cells

HHV8: Human Herpesvirus 8

HIF-1α: hypoxia-induced factor-1α

HIV: human immunodeficiency virus

HMGB1 protein: high mobility group box 1 protein

HMW: high molecular weight

HRP: horseradish peroxidase

HTRF: homogeneous time-resolved fluorescence

I-TAC: interferon-inducible T-cell alpha chemoattractant

IC_{50}: inhibitory concentration 50%

ICL#: intracellular loop #

IgG: immunoglobulin G

IL#: interleukin #

IP3: inositol 1,4,5-triphosphate

IRDye: Infra-Red Dye

IT1t: isothiourea-1t

IUPHAR: International Union of Basic and Clinical Pharmacology

JAK2: Janus Kinase 2

KO mice: knockout mice

KSHV: Kaposi's sarcoma Herpes virus

LB: Luria-Bertani

LgBiT: large part of binary technology

LPS: lipopolysaccharide

M-tropic: macrophages tropic

M2R: muscarinic 2 receptor

M2 macrophages: alternatively activated macrophages

MAPK: Mitogen-activated protein kinases

mEGFP: monomeric enhanced green fluorescent protein

mGlu1R: metabotropic glutamate receptor 1

mGlu5R: metabotropic glutamate receptor 5

MIF: macrophage migration inhibitory factor

MOPS: 3-(N-morpholino)propanesulfonic acid

M_r: relative molecular weight

mRNA: messenger ribonucleic acid

N-terminus: amino terminus

NanoBiT: Nanoluciferase Binary Technology

NF-κB: Nuclear Factor κB

NG108-15: Neuroblastoma x glioma hybrid 108-15

NRF-1: nuclear respiratory factor-1

NSF protein: N-ethylmaleimide-sensitive fusion protein

p38: protein 38

PAMP: pro-adrenomedullin N-terminal 20 peptide

PAMPs: pathogen-associated molecular pattern molecules

PBS: phosphate-buffered saline solution

pcDNA5/FRT/TO: plasmid construct DNA 5/ FRT site/ Tetracyclin on

PCR: polymerase chain reaction

pDC: plasmacytoid dendritic cells

PDZ: postsynaptic density protein of 95 kDa (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (Zo-1)

PEI: poly-ethylene-imide

PGCs: primordial germ cells

PI3K: Phosphatidyl-Inositol 3 Kinase

PH domain: pleckstrin homology domain

PIP2: phosphatidylino-sitol-4,5 bisphosphate

PLC-ß: phospholipase C type ß

PKA: protein kinase A

PKC: protein kinase C

POL6326: balixafortide

proADM: proadrenomedullin

PTHR#: parathyroid hormone receptor #

PTX: pertussis toxin

R: receptor in inactive state

R*: receptor in active state

R5 virus: CCR5-dependent HIV virus

RAMP#: receptor activity-modifying protein #

RAS-GAPs: Ras-specific GTPase-activating proteins

RFFESH motif: Arginine-Phenylalanine-Phenylalanine-Glutamate-Serine-Histidine motif

RGS: regulator of G protein signalling

RhoGEF: Rho-guanine nucleotide exchange factor

Rluc: renilla luciferase

RTCA: real time cell analysis

SCTR: secretin receptor

SDF-1: stromal-derived factor-1

SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SLE: systemic lupus erythematosus

SI variants: syncytium-inducing variants

SmBiT: small part of binary technology

SMO: smoothened receptors

SNARE proteins: SNAP REceptors proteins

Src: v-src avian sarcoma viral oncogene homolog

STAM1: signal-transducing adaptor molecule 1

STAT3: Signal Transducer and Activator of Transcription 3

T-tropic: T cells tropic

T2R: Tas2 receptor

T4 lysozyme: phage T4 lysozyme

TAE: Tris-acetate-EDTA

TAS1R1-3: taste 1 receptor member 1 – 3

TBS(T): Tris-buffered saline solution (+ tween20)

TE: Tris-EDTA

TG-0054: burixafor

T_h: T helper cells

TLRs: Toll-like receptor

TM#: transmembrane domain #

TNFα/β: tumour necrosis factor α/β

Tregs: regulatory T cells

U87 cells: Uppsala 87 cells

vCCL#: viral CC motif chemokine ligand #

VEGF: vascular endothelial growth factor

VFD: Venus fly domain

vMIP #: viral Macrophage Inflammatory protein #

vIPR#: vasoactive intestinal peptide receptors

WHIM syndrome: Warts, Hypogammaglobulinemia, Infections, and Myelokathexis syndrome

Wnt: wingless-related integration site

X4 virus: CXCR4-dependent HIV virus

X4P-001: mavorixafor

ZAP70: zeta-chain-associated protein kinase 70

CHAPTER 1 – Introduction

1.1 Introduction to the family of G protein-coupled receptors (GPCRs)

G protein-coupled receptors, also known with the acronym GPCRs, form the biggest group of cell membrane receptor proteins and the biggest group of drug targets because of their important role in regulation of several physiological processes and in development of several pathological conditions and diseases. They regulate a wide range of cellular processes and control several intracellular systems and these processes are mediated by hormones, neurotransmitters, metabolites, and pathogens. Even physiological processes such as olfaction, vision and taste are regulated and mediated by different GPCRs (Rosenbaum et al, 2009). Therefore, it is not surprising how GPCRs became the major targets for the pharmaceutical industry to treat different types of diseases, as reflected by the fact that 35% of all FDA-approved drugs act on a GPCR (Sriram and Insel, 2018). Although a huge number of receptors are part of this group, these proteins share a general common structure, which is characterized by a protein chain crossing the plasma membrane with seven α-helical domains. These helices are connected one to another by the presence of three intracellular and three extracellular loop domains which alternate across the full sequence; moreover, at their extremities they also present an extracellular N-terminus, and an intracellular C-terminus. Even though this highly similar conserved structure represents a simplified description of their structure, from this main feature GPCRs derived their name of seven-transmembrane domain receptors, indicated with the acronym 7TM.

1.2 Structure of GPCRs

In the past years, the development of effective crystallographic approaches allowed us to unveil the structure of many GPCRs and helped in the identification of the sites where the ligands can bind their cognate receptors to exert their function. Having knowledge of GPCRs structure and ligand effects had an impact in hypothesizing and uncovering the several mechanisms of actions of these ligands and in characterizing these receptors' functioning and signalling. It is widely described in literature that GPCRs show highly conserved features from

an evolutionary line which are present in the structure of many of these proteins. One of the most frequent features to underline is the length of ECL2 (ExtraCellular Loop 2), the longest extracellular loop in the majority of known GPCRs (Kmiecik et al., 2014). Moreover, cysteine residues belonging to ECL2 (such as Cys45.50 and Cys3.25, according to Ballesteros and Weinstein residues numbering) (Ballesteros and Weinstein, 1995) are involved in the formation of disulphide bridges with residues in TM3 (TransMembrane domain 3), which mostly contribute to receptor stability (Venkatakrishnan et al., 2013, Nicoli et al., 2022).

The transmembrane region could be considered as the core of a GPCR, and it has a big role in facilitating the communication between the ligand-binding pocket complex and the intracellular proteins located downstream of the receptor itself which may start relevant signalling cascades (Katritch et al., 2013). In some cases, the occurrence of an eighth helix (H8) was observed in some GPCRs. The orientation of this helix is parallel to the plasma membrane and a short linker separate this domain from TM7: this helix, together with the ICLs (IntraCellular Loops), importantly regulates the receptor activation and the interaction with cellular signalling effectors (Wess et al., 2008). Additionally, there are specific regions of GPCRs, such as the carboxyl terminus, the intracellular loop located in between TM5 and TM6, and the amino terminus region, which show a great level of variability. In particular, the N-terminus side of GPCRs represents the domain with the highest variability level, with a crucial role in signalling regulation of these receptors (Kobilka et al., 2007).

1.3 Classification systems of the different groups of GPCRs

Even though GPCRs share a typical structure, several differences in the past have been described for many of them and this is the reason why they have been divided into different families. Differences can be related to the structure and the amino acid sequence of their non-transmembrane domains, the type of ligands interacting with these receptors to exert their functions, the expression level of these proteins, the type of cellular response generated as result of signalling pathways that are activated when they are stimulated with a ligand, and their role in regulation of physiological processes and development of diseases. Because of all this heterogeneity that has been observed for these receptors, in the

past years several classification systems have been adopted. The two most used systems of classification are the ABCDEF system (Attwood and Findlay, 1994) and the GRAFS system (Fredriksson et al., 2003). The first is highly comprehensive, because it counts all the known GPCRs that are present both in vertebrates and invertebrates; a limit of this system is that some families do not have representatives in humans. The GRAFS classification resulted from using an extensive bioinformatic approach on human data known at that time. This system allowed us to divide all the human GPCRs into 5 families: Glutamate, Rhodopsin, Adhesion, Frizzled/Tas2 and Secretin receptors, the first letter of which family names form the acronym GRAFS itself (Fredriksson et al., 2003). All these categories show different features that will be explained in the next paragraphs, and they are also graphically reported in **Fig 1.1**. In this thesis, the classifications system adopted is the GRAFS system, and, to follow, a deeper and more detailed description of the different subfamilies has been reported.

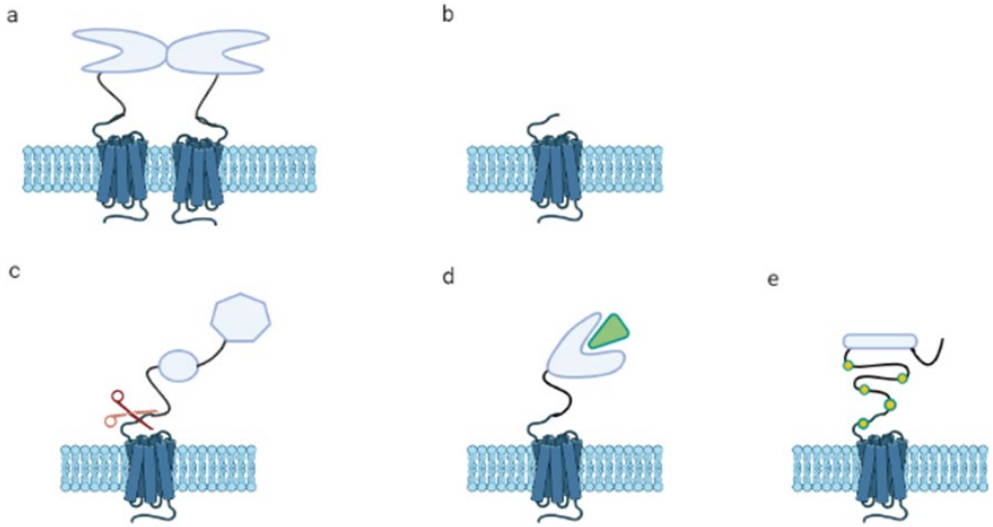


Fig 1.1: GPCRs classification and their structural features (a-e). Glutamate-like receptors (Fig. 1.1A) are functional only as dimers, and have long N-terminus region where a Venus Flytrap Domain (VFD) forms the binding domain for the agonist. Rhodopsin-like receptors (**Fig. 1.1B**) are the most known GPCRs, whose peculiarities reside in their primary structure. Adhesion receptors (**Fig. 1.1C**) have different motifs in their long N-terminus to allow cell to cell adhesion and a proteolytic cleavage site (GPS) upstream of TM1. Frizzled/Tas2 receptors (**Fig. 1.1D**) differ for functions and ligands that they bind, and they have in common some conserved motifs in their primary structures. Secretin-like receptors (**Fig. 1.1E**) N-terminal, ECL1 and ECL2 domains have several conserved cysteines, responsible for a more constrained extracellular region. Dark blue indicates the receptor, light blue the membrane, green is the agonist, red is cleavage sites. Yellow circles are conserved cysteines in EC domains. Figure created on BioRender.com.

1.3.1 Metabotropic Glutamate-like receptors

Metabotropic glutamate receptors, the $GABA_B1/2$ receptors, the extracellular calcium-sensing receptor (CaSR), odorant receptors in fish, the pheromone receptors, sweet and umami taste receptors (TAS1R1-3), GPCR Class C Group 6 Member A (GPRC6A) and seven orphan receptors and corresponds to Class C of the ABCDEF classification (Yang et al, 2021). Differently from other GPCRs, the members of this group are functional only when they form dimers, with a mechanism of action in which one monomer binds the ligand, and a consequential conformational change makes the other monomer able to initiate the relevant signalling pathways (Brauner-Osborne et al., 2007). Moreover, another key feature of members of this group is the presence of an extended N-terminus (between 280-580 residues), forming a two lobes area referred to as Venus Flytrap Domain (VFD): upon binding of an orthosteric ligand in the area comprised between the two lobes, these can move towards one another (**Fig. 1.1A**) (Cao et al., 2009).

1.3.2 Rhodopsin-like receptors

The most important and common feature of receptors belonging to this subfamily is the presence for almost all of them of a characteristic DRY motif in their primary structure, at the connection point between the transmembrane domain three (TM3) and the intracellular loop two (IL2) (**Fig. 1.1B**).

Receptors of rhodopsin-like group are generally characterized by an overall elliptic shape. The reason behind this conformation is that the transmembrane domain helices of members of rhodopsin subfamily have an irregular shape because of conformational changes caused by Gly–Pro residues (Teller et al., 2001). Moreover, the transmembrane domain seven (TM7) is characterised by a NSxxNPxxY motif that may play a role in conformational switch of the receptors. As said before, only a few receptors do not have these features, but they have been included anyway in this family because of their phylogenic profile which shows resemblance with receptors of this category (Fredriksson et al., 2003). The binding site for an orthosteric ligand is usually located within the extracellular region of the transmembrane domains bundle (Gacasan et al., 2017).

1.3.3 Adhesion receptors

Adhesion GPCRs (aGPCRs) are characterized by having an especially extended amino terminal side, known as N-Terminal Fragment, which is cleavable from the membrane spanning domain (also called as C-Terminal Fragment) by proteolysis (**Fig. 1.1C**) (Monk et al., 2015). The first region contains key domains to allow interactions between cells and between cells and matrix (Langenhan et al., 2013). Another common feature shared by members of this family is the presence of the GPCR proteolytic site (GPS) immediately upstream of TM1, which is contained in a GPCR autoproteolysis-inducing (GAIN) domain (Liebscher et al., 2013). This domain composed by around 40 residues is highly conserved and contains cysteine and tryptophan residues (Krasnoperov et al., 1999) essential for autoproteolysis events occurring for these GPCRs.

1.3.4 Frizzled/Tas2 receptors

As the name of this family indicates, frizzled receptors (FZD1-10), smoothened receptors (SMO) and twenty-five Tas2 (T2R) receptors are included in this group of receptors. Even though the members of this category are apparently different, they show common consensus sequences that allow us to include them in the same group, such as motifs IFL in TM2, SFLL in TM5 and SxKTL in TM7 (Lagerström and Schiöth, 2008) (**Fig. 1.1D**). Another aspect of this group is that the ligands binding to these receptors vary in terms of nature and function: for example, Wnt ligands are involved in embryo development (Zhang et al., 2018), while T2R ligands are molecules related to taste sensing (Haraguchi et al., 2018).

1.3.5 Secretin-like receptors

The members of this family derive their name from the rat Secretin Receptor (SCTR), the first member to be discovered (Ishihara et al., 1991). This group includes vasoactive intestinal peptide receptors (vIPR1, vIPR2), glucagon-like peptide receptors (GLP1R, GLP2R), adenylate cyclase activating polypeptide receptor (PAC1/ADCYAP1R1), growth hormone-releasing hormone receptor (GHRHR), calcitonin and calcitonin-like receptors (CALCR, CALCRL), gastric inhibitory polypeptide receptor (GIPR), secretin receptor (SCTR), corticotropin-releasing hormone receptors (CRHR1, CRHR2), glucagon receptor (GCGR), and

parathyroid hormone receptors (PTHR1, PTHR2) (Lagerström and Schiöth, 2008; Fredriksson et al., 2003). The receptors included in this group present conserved cysteine residues in the first and second extracellular loops (ECL1 and ECL2), which help in stabilizing the structure of the receptor by forming bridges between them or with less conserved cysteines placed in the N-terminal domain (**Fig. 1.1E**) (Lagerström and Schiöth, 2008). Moreover, the endogenous ligands of this class of receptors are generally peptides sharing high amino acid identity (Fredriksson et al., 2003).

1.4 Function of a GPCR: signalling

The regulation of GPCR functioning passes through a finely intricated mechanisms involving several agents called ligands. GPCRs can bind different ligands which differ among them for structure, chemical nature, size, biological effects they generate. The specificity and reversibility of this binding can be different and must be considered separately for each GPCR and each ligand. As a result of the ligand promoted stimulation of the GPCR, the receptor can be activated or inactivated, and the activation may enhance or diminish a particular cell function. During the activation phase, the extracellular domains and the non-cytosolic regions of TM domains form a space for the ligand allocation, referred to as orthosteric binding pocket, allowing its interaction with the receptor at different levels (Zhang et al., 2015). Because of ligand binding, the receptor undergoes conformational changes, especially at the level of the ICLs, and this conformational change promotes the binding of several intracellular effectors (Nygaard et al., 2009). In general, ICL2 and ICL3 have been reported to be the G protein-interacting sites of most of GPCRs (Wess, 1997).

Because of the huge numbers of effects associated with GPCRs signalling, these receptors evolved several and different signalling pathways that can be originated by the interaction of GPCRs with specific ligands and effectors, leading to various signalling cascades and generating different biological effects because of these interactions. These pathways are going to be explained in more detail in the next paragraphs and chapters.

Among the canonical ligands with a more simple mode of action, certain ligands are instead called “biased”, because upon receptor binding, they stabilize the receptor in a determined conformation which promotes a selective activation of a

specific signalling pathway: they can promote a specific G protein-coupling, initiate β-arrestin-mediated signalling, or influence receptor internalization or recycling to plasma membrane (Urban et al., 2007; Tan et al., 2018).

Therefore, it is not surprising that biased ligands can be quite relevant for characterizing GPCRs pharmacology; indeed, they have great potential in drug discovery allowing a better specificity of responses and a reduction of on-target side effects generated by undesired activation of pathways that could be deleterious (Whalen et al., 2011).

1.4.1 Concepts of pharmacology: ligands

As previously specified, the signalling of a GPCR can depend on ligands effects and functions. Ligands can work in different and specific ways, and because of this reason, they have been classified according to the behaviour they have toward the cognate GPCRs and the effect that they have on them. An agonist is a drug, a small molecule, or a chemical, that shows affinity for the target receptor to which it binds and intrinsic efficacy, since an agonist can change the receptor activity to produce a response. Agonists are divided into full agonist and partial agonists, where the distinction is related to their intrinsic efficacy and structural differences. A full agonist generates the maximal response allowed for in a ligand-receptor system, while a partial agonist produces a submaximal response. However, even full agonists can differ among them, since a full agonist can produce a maximum response even by binding low proportion of receptor (this is the case of what in pharmacology is referred to as “spare receptor”) (Berg and Clarke, 2018).

A different type of ligand in pharmacology which are important for their role in drug discovery is represented by the antagonists. These are small molecules, drugs, chemicals that possess affinity but no intrinsic efficacy towards the receptor they bind to; therefore, they can bind to the target receptor without producing a response. According to the affinity of the antagonists, which can occupy a variable fraction of the receptor population, these ligands can reduce the probability of occupancy of the receptors by agonists with a corresponding reduction in response generated upon receptor stimulation.

In addition to the traditional concepts of agonist and antagonists, there are other types of ligands that have been described in pharmacology. For example, Costa and Herz (1989), in their experiments with delta opioid receptors in NG108-15

neuroblastoma cells, found out that many antagonists decreased the constitutive receptor-stimulated GTPase activity, so they re-classified these ligands as "inverse" agonists, because of the opposite effect they had compared to agonists of these receptors. These ligands have different levels of negative intrinsic efficacy (the ability to decrease the activity of a receptor), that can result in strong and weak (partial) inverse agonisms (Berg and Clarke, 2018).

The role and the functioning of inverse agonists can be explained by the 2-state model (Del Castillo and Katz, 1957), according to which in a physiological condition GPCRs can exist in an equilibrium between two different states: an inactive state, indicated with "R", unable to signal, and an active state, indicated with "R*", that can instead actively signal. An agonist tends to have higher affinity for R* than R, enriching the proportion of receptors in active state and causing a depletion of the inactive forms. This results in an increment in signalling. An inverse agonist, instead, because of its higher affinity for R, causes an enrichment of the inactive form of receptors, at the expense of the active state forms. As a result, the number of active receptors diminishes, and this leads to decreased signalling. On the other side, an antagonist binds with equal affinity to both states and the equilibrium existing between the two conformations is not altered upon its action. The change of the response of the receptor, in this case, does not depend on the effect of the antagonist itself, but on the occupancy level the antagonist has towards the receptor, affecting its binding to agonists and inverse agonists and, by this way, modulating the receptor signalling (Berg and Clarke, 2018). It is clear the importance that the inverse agonists can have in a drug discovery perspective, because they can allow a better modulation of the receptor functions with the aim of improving the therapeutic effect of new drugs to develop.

Finally, another interesting category of ligands that recently acquired growing importance in pharmacology is composed by biased ligands (agonists mostly, but even some antagonists have been reported (Hitchinson et al., 2018). We speak about biased (ant)agonism when a ligand can activate/block a specific type of GPCRs signalling cascade, as the β-arrestin-recruitment-mediated pathways or G protein-related pathways, but not both pathways simultaneously (Andresen, 2011). Biased agonists are often and simplistically described as agonists activating only one type of signalling pathways while inhibiting the other ones. So, GRK/β-arrestin biased agonists will appear to be antagonists when only

examining G protein-mediated signalling. The discovery of this group of ligands opens new modalities of treating several diseases and, therefore, some of them are already used or been tested to use in clinics, in a hope of improving the health status of patients in a more efficient and targeted way.

1.4.2 Orthosteric and allosteric ligands

Ligands binding specific GPCRs are not acting all in the same way and they can be classified into orthosteric and allosteric ligands. Orthosteric ligands or drugs bind their cognate receptors in a region defined as a receptor's active site. In case of an orthosteric antagonist, this competes with the natural substrate or ligand for the same binding site. The blockade of the binding site depends on their affinity to the active site surface. Indeed, if their affinity is high, they can displace the endogenous ligand and this displacement results in receptor's signalling blockade. On the other hand, allosteric ligands or drugs bind elsewhere on the protein surface, with a variability that depends on the structure of the receptor, the structure of the molecule and the chemical interactions occurring when the bound complex is formed (Digby et al., 2010; Mohr et al., 2010). Allosteric ligands affect protein activity by changing the conformation at the second binding site and this is why the two types of ligands have different effects: while orthosteric ligands stop protein activity entirely, this does not happen for allosteric ligands which can modulate the activity of the receptor in different levels because they do not compete with the endogenous ligands for the same binding site (Nussinov and Tsai, 2012). Allosteric drugs are appreciated in pharmacology because of the advantages they have: in fact, they can offer a less disruptive way to influence the functioning of a pathway and, additionally, they are likely to have fewer side effects, which make them more likable in the current drug market (Digby et al., 2010).

1.4.3 G proteins

As the name already suggests, generally GPCRs are in most of the cases associated with G protein coupling. G proteins are heterotrimeric guanine nucleotide-binding proteins with a regulatory function, which work in a double step mechanism which is regulated by the bound nucleotide. Therefore, they

usually considered dual molecular switches (Gilman, 1987; Simon et al., 1991; Syrovatkina et al., 2016).

These complexes directly transmit the signals from activated GPCRs. The heterotrimeric structure of G proteins consists of three different subunits called α, β and γ subunits. β and γ subunits are strictly connected in exerting their function and therefore they are considered as a single functional subunit. Instead, G_α subunit can be different, and therefore G proteins are generally classified according to their G_α subunit.

An inactive G protein is found as a heterotrimeric complex with a molecule of guanosine diphosphate (GDP) bound to this complex: once the agonist binds the receptor, the G protein is recruited by the GPCR, and this causes a substitution of GDP with a molecule of GTP (guanosine triphosphate). Upon GDP to GTP substitution, the G_α subunit dissociates from the $G_{\beta\gamma}$ complex, and each subunit functions as a signalling effector, stimulating different signalling pathways. Since the functioning of these proteins is finely regulated, the intrinsic GTPase activity of G_α subunit terminates the signalling promoted by G_α subunit itself, with a negative feedback regulation, hydrolysing the bound GTP to GDP.

Therefore, regulators of G protein signalling proteins (RGS proteins) are activated with the important role of binding the G_α subunit in order to accelerate the low GTPase activity of G_α subunit. On the other side, the $G_{\beta\gamma}$ signalling is terminated when the heterodimer reassociates with Gα·GDP complex. At this point, the cycle of G protein activation is terminated, and it can restart from the beginning (Milligan and Kostenis, 2006).

The typical G protein signalling cascade, with the consequential activation of different cellular signalling pathways, which has been described in this paragraph is graphically represented in **Fig. 1.2** (generated on BioRender.com).

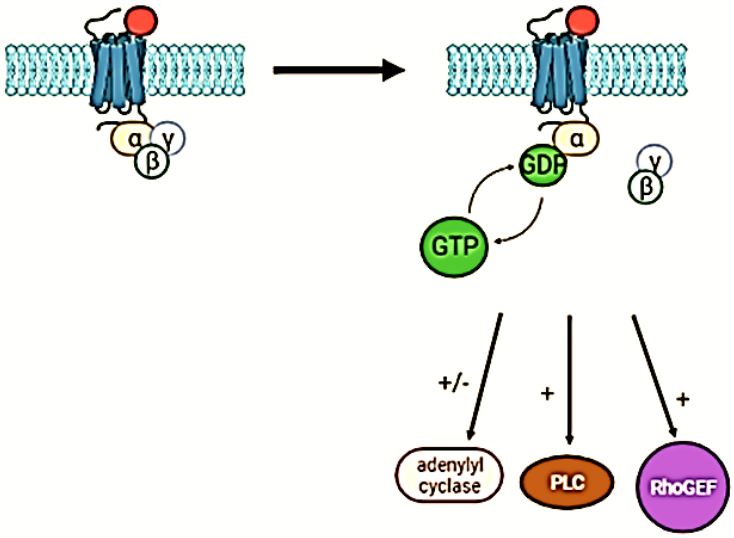


Figure 1.2.: G protein signalling pathways. Upon agonist binding, GPCRs are activated with the dissociation of G protein in G_{α} and $G_{\beta\gamma}$ subunits. A GDP molecule is exchanged with GTP that is hydrolysed to GDP, starting several signalling pathways (adenylyl cyclase up- or down-regulation, PLC stimulation and RhoGEF activation). Dark blue represents the receptor, light blue represents the membrane, red represents the ligand. Created on BioRender.com.

1.4.3.1 Gα subunit

Several types of G proteins exist and they are classified into four different families according to differences in their G_{α} subunits: $G_{\alpha s}$ (up-regulators of adenylyl cyclase, including $G_{\alpha s}$ and $G_{\alpha olf}$), $G_{\alpha i}$ (inhibitor of adenylyl cyclase, including $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha i/o}$, $G_{\alpha i/t}$, $G_{\alpha i/g}$ and $G_{\alpha i/z}$), $G_{\alpha q}$ (phospholipase C β, or PLC-β, activators which contains the members $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15/16}$) and $G_{12/13}$ (Rho-guanine nucleotide exchange factor, or RhoGEFs, activators) (Simon et al., 1991, Downes and Gautman, 1999). **Fig. 1.2** shows the different cellular effectors that are activated upon G protein activation, depending on the type of G_{α} subunit.

$G_{\alpha s}$ (where "s" stands for stimulation) is found in the majority of cell types, instead the expression of $G_{\alpha olf}$ (where "olf" indicates olfaction) has only been in olfactory sensory neurons. $G_{\alpha s}$ stimulates an enzyme called adenylyl cyclase (AC) which promotes the conversion of ATP (adenosine triphospate) to second messenger cAMP (cyclic adenosine monophosphate): incremented levels of this molecule activate protein kinase A (PKA), important for the regulation of GPCRs phosphorylation, and other cAMP-dependent cellular effectors.

$G_{\alpha i}$ family is the largest and most diverse family ("i" means inhibition) and members of this group have been found in many categories of cells. $G_{\alpha i/o}$ exist in two different spliced variants identified as type A and type B. The members of the inhibitory family have the main function of inhibiting the adenylyl cyclase, and, therefore, decreasing the intracellular cAMP levels (Wettschureck and Offermanns, 2005). $G_{\alpha i/t}$ ("t" indicates transducin) also has been found in two different isoforms, indicated as 1 and 2. The first was found only in the eye rod cells, while the expression of the second was detected in the eye cone cells. $G_{\alpha i/g}$ ("g" indicates gustducin) is found in taste receptor cells, while $G_{\alpha i/z}$ is expressed in neuronal tissues and in platelets (Syrovatkina et al., 2016).

About the third group, $G_{\alpha q}$ and $G_{\alpha 11}$ are ubiquitously expressed, while $G_{\alpha 14}$ is specifically found in kidney, lung, and liver and $G_{\alpha 15/16}$ is specifically expressed in hematopoietic cells (Syrovatkina et al., 2016). Differently from other G proteins, $G_{\alpha q/11}$ proteins activate an enzyme called phospholipase C type β (indicated by using the acronym PLC-*β*). This has the important role of cleaving the phosphatidylinositol 4,5 bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). The IP3 binds the IP3 receptors located on the endoplasmic reticulum, causing intracellular calcium release, important to regulate many biological effects, while the DAG activates protein kinase C (Goo, 2001).

The last group, $G_{12/13}$, is widely expressed in many types of cells. G_{13} can directly increase the activity of different effectors, such as the p115RhoGEF, PDZ-RhoGEF (PDZ domain-containing Rho guanine nucleotide exchange factor) and leukemia-associated RhoGEF by recruiting them to the membrane and binding them (Kozasa et al., 1998), G_{12} can interact with Gap1 (General Amino acid permease 1), rasGAP, *α*-SNAP and p120-catenin, while both can interact with Bruton tyrosine kinases (Btk) and cadherins (Syrovatkina et al., 2016).

1.4.3.2 Gβγ subunit

G_{β} has five isoforms whose expression is widespread across the body, with the only exception of the $G_{\beta 5}$ that is only expressed in brain cells. G_{γ} subunit presents eleven isoforms ($G_{\gamma 1\text{-}5}$ and $G_{\gamma 7\text{-}12}$) (Syrovatkina et al., 2016). The $G_{\beta\gamma}$ heterodimer, once the G protein is activated and the heterotrimeric complex disassemble, can start signalling cascades, regulating AC and PLC-*β* activity, the inwardly

rectifying potassium channel, and the voltage-gated calcium channels (Khan et al., 2013; Capoferri, 2020).

1.4.4 G protein-independent pathways

Many other effectors, different from G proteins, have been described as able to interact with GPCRs in the cytosolic side of the membrane. The idea that two different phenomena could contribute to signalling of GPCRs, either in a G protein-dependent or G protein-independent, has been described by Rajagopal et al. (2005). There are two main G protein-independent signal transducers currently known in literature, and they will be described in the following paragraphs of this chapter. These transducers are called arrestins and G protein-coupled receptors kinases (GRKs).

1.4.4.1 Arrestins

Arrestins are cytoplasmatic soluble proteins with an important role in the regulation of GPCRs downstream signal transduction. These adaptor proteins were reported to be important players of a two-step mechanism of action that represents a fundamental regulation process of GPCRs activity in rhodopsin (Wilden et al., 1986) and in the β-adrenergic system (Lohse et al., 1990). Arrestins are involved in several aspects of receptor's physiology, but one of the most important is receptor desensitization: after the GRKs kinases (G protein coupled Receptor Kinases) phosphorylate specific serine and threonine residues which are located at the level of the receptor's ILs and C-terminus, the GPCR is subject to a conformational change causing the recruitment of arrestins to the receptor. This interaction between arrestins and GPCRs has the result of removing these receptors from the plasma membrane and uncoupling these receptors from G proteins which are, therefore, inactivated (Komolov and Benovic, 2018; Zhuo et al., 2022).

The interactions between these adaptor proteins and GPCRs leads to the activation of different signalling pathways, such as ERK (Extracellular signal-Regulated Kinase) phosphorylation (Kumari et al., 2017), Akt (Ak mouse strain thymoma) ser/thr-kinase, Src (v-src avian sarcoma viral oncogene homolog) tyrosine-kinase, NF-κB (Nuclear Factor κB) and PI3K (Phosphatidyl-Inositol 3 Kinase) (Smith

and Rajagopal, 2016). Arrestins are also connected to the internalization process of the GPCRs, since they mediate the internalization of the receptor *via* clathrin (Goodman et al., 1996) and *via* AP2 (Laporte et al., 1999).

The arrestin family has four members in mammals: arrestin1 (called visual or rod arrestin in some species, and previously called S-antigen or 48 kDa protein), arrestin2 (also known as β-arrestin or β-arrestin1), arrestin3 (β-arrestin2) and arrestin4 (cone arrestin or X-arrestin) (Gurevich EV and Gurevich VV, 2006). In terms of structure and functions executed, these proteins are classified into two subgroups: visual or sensory (arrestin1 and arrestin4) and non-visual (arrestin2 and arrestin3) (Gurevich EV and Gurevich VV, 2006).

1.4.4.2 GRKs

GRKs are a group of seven different soluble (except for GRK5 and GRK6 which are membrane tethered, as explained later in details) proteins whose main function is to phosphorylate Ser or Thr residues within the intracellular loops and/or carboxyl-terminal tail (C-tail) of GPCRs upon agonist-promoted activation of the receptor to promote the receptor desensitisation.

GRK1 and GRK7 are localized in the eye. Indeed, GRK1 is expressed in retinal rods, while GRK7 expression has been detected in cones cells. GRK4 expression has been reported only in cerebellar, testicular and kidney tissues. Moreover, GRK1 and GRK7 presents a prenylation motif at their C-termini, which makes these GRKs constitutively localized to the membrane (Klaasse et al., 2008; Reiter and Lefkowitz, 2006).

On the contrary, GRK 2, 3, 5 and 6 are ubiquitously expressed (Pitcher et al., 1998). GRK2 and GRK3 localize to the membrane and thanks to the pleckstrin homology (PH) domain they can bind the $G_{\beta\gamma}$ subunit of the G proteins. The binding of this subunit recruits GRK2/3 to the membrane, where the receptors reside, to make the signalling shut off (Haga K. and Haga T., 1992; Pitcher et al. 1992; Pitcher et al., 1995).

GRK4/5/6 do not present in their structure either the PH domain or the C-terminal prenylation as described for other GRKs (Gurevich et al., 2012). Instead, the association to the plasma membrane for these GRKs occurs *via* palmitoylation of specific C-terminal cysteines (Cys^{561}, Cys^{562}, Cys^{565}) and/or *via* an amphipathic helix interacting with the membrane phospholipids (Gurevich et al., 2012;

Gurevich V.V. and Gurevich E.V., 2019; Bianchi and Mezzapelle, 2020). These features make them constitutively localized to the plasma membrane.

1.5 Regulation of GPCRs

The multitude of cellular functions regulated by GPCRs signalling is finely orchestrated thanks to an elegant and developed negative feedback system to counteract the agonist stimulation that can be sustained in time. Desensitisation and internalisation are the most common ways through which GPCRs can regulate their signalling. The former prevents negative effects that long exposure to an agonist could have on cells expressing cognate GPCRs, while the latter consist of a physical removal of both ligand-bound and unbound GPCRs that usually results in a lower amount of receptor on the surface (Magalhaes et al., 2012; Rajagopal and Shenoy, 2018).

1.5.1 Desensitisation

This phenomenon is defined as the spontaneous decline in the response to a continuous application of an agonist, or to repeated applications or doses (Ferguson et al., 1998; Neubig et al., 2003). Almost every GPCR so far undergoes desensitisation, and therefore understanding the mechanisms of desensitization of the receptor can have an impact on a clinical practice. In fact, if this phenomenon verifies, the long-time exposure to some drugs can decrease their efficacy in the long-term period, and therefore, by solving this problem, it would be possible to improve greatly the treatment of patients suffering with chronic diseases.

Historically, desensitization has been divided into two forms: when the loss of GPCRs subtype is caused by agonist-dependent responses, this kind of desensitization is usually referred to as homologous. Instead, with heterologous desensitization we usually refer to a more generalized effect in which loss agonist responsiveness for more receptors subtypes is observed even in the absence of agonist occupation of the other receptors (Hosey, 1999; Kelly et al., 2007). This phenomenon occurs when two elements, GRKs and arrestins, act coordinately (Drake et al., 2006), exerting an important function in coordinating processes such as receptor endocytosis and ubiquitination, by acting on regulatory proteins fundamental for these processes (Rajagopal and Shenoy, 2018).

Generally, since the phosphorylation started upon GRKs action is not sufficient by itself for G protein uncoupling, cells developed a system in which *β*-arrestin recruitment become fundamental to potentiate this process and terminate G protein-mediated signalling: in fact, arrestins act to sterically hinder the receptor, and this step prevents further interactions of the GPCR with G proteins. GRKs and *β*-arrestin can be modulated by further accessory proteins such as tubulin, cytoskeletal proteins, and regulators of G proteins (RGS) (Szénási et al., 2023).

The desensitization phenomenon can be subdivided into several sub-phases: firstly, there is the receptor phosphorylation, that leads to the *β*-arrestin recruitment; after this step, the receptor undergoes internalisation, and this step is crucial to decide the destiny of the receptor itself: indeed, it can be recycled to the membrane with consequent re-sensitisation or it can be degraded via lysosomes, causing a receptor down-regulation (Shankaran et al., 2007). As a result of these processes previously described, there is an overall loss of the receptor quantity at a cellular level and, therefore, the response of receptors to their own ligands is more limited, although some internalised receptors still retain the ability of signalling from the endosome (Feinstein et al., 2013); moreover, *β*-arrestins proteins themselves have been reported in some cases as able to act as important mediators of G protein-independent signalling pathways (Smith and Rajagopal, 2016).

1.5.2 Phosphorylation of GPCRs

Upon ligand binding and activation, most GPCRs localized on the plasma membrane are phosphorylated at serine, threonine, and tyrosine located on intracellular loops (particularly the intracellular loop 3, or ICL) or C-terminal tails. The phosphorylation pattern of these residues can differ in many cases, and it depends on the type of ligand bound and to the expression system in which the receptor is found (Patwardhan et al., 2021).

GPCRs phosphorylation is mediated by mainly by two classes of serine/threonine kinases: this group includes the second messenger-dependent kinases (such as protein kinase A, or PKA, and protein kinase C, or PKC) and second messenger-independent kinases (as for example GRKs) (Lefkowitz, 1998). The phosphorylation mediated by the former type of kinases does not require ligand binding as a condition and it directly uncouples the receptors from G proteins,

leading to heterologous desensitization (Hausdorff et al., 1990). Instead, phosphorylation caused by GRKs depends on the stimulation of the receptor promoted by an agonist and is followed by the recruitment of arrestins to the receptor sterically inhibiting G protein coupling, starting homologous desensitization (Pitcher et al., 1992; Tobin et al., 2008; Gurevich et al., 2012).
Because of these different phosphorylation-related events in distinct sites of these receptors, recently a new concept of "barcode" has been hypothesized for GPCRs. These "barcodes", specific for each receptor and strictly depending on phosphorylation patterns of GPCRs, can dictate the different downstream signalling cascades of these receptors, leading to different physiological functions exerted by these receptors (Butcher et al., 2011). These "barcodes" can be determined also by different kinases cooperating in phosphorylating specific sites of the receptors, to regulate their signalling. This heterogeneity of the phosphorylation patterns involves even the dynamics by which different sites are phosphorylated in a specific sequence, in which one site allows and favours the phosphorylation of the other, increasing the complexity of the concept of "barcode" (Kim et al., 2004).

1.5.3 Down-regulation

One of the steps of the desensitization process is represented by GPCR down-regulation, that occurs when receptors eventually undergo proteolysis, having as outcome a general quantitative decrease of receptors that are able to signal. Going through this process, GPCRs are disassembled when they reach the lysosomes, and their mRNA level often decreases. To regulate this mechanism, an important protein called ubiquitin, is attached via covalent bonds to a GPCR by an enzymatic cascade involving the sequential activity of three enzymes, referred to as E1, E2, and E3. This promotes receptor degradation following a progressive path from early to late endosomes or maturing vesicles (Kennedy and Marchese, 2015). Ubiquitination can be reversible, and it is carried out by a family of enzymes called ubiquitin-specific proteinases, involved in the de-ubiquitination of the receptor. In fact, they finely regulate the removal of ubiquitin sequences from targeted receptors, in a process which can also take place when cells need to decide whether the receptor must be recycled to the cell membrane or degraded,

by this way determining the destiny of proteins. (Baek, 2003; Pal and Donato, 2014).

1.5.4 Recycling and re-sensitisation

The re-sensitization of GPCRs is a phenomenon that occurs when the receptors, after their internalization, are not degraded via lysosomes. Instead, they are dephosphorylated, and they are sent to the plasma membrane, to be ready to start again its cycle. The re-sensitization needs both the receptor's dephosphorylation and additionally its dissociation from the β-arrestin (Krupnick and Benovic, 1998). The former process occurs in vesicles characterized by an acidic pH, to allow the dissociation of the ligand from the receptor and the dephosphorylation process (Krueger et al., 1997; Sibley et al., 1986) and it has been proven that the internalization before re-sensitisation is a mandatory step by using mutants defective of the ability to undergo endocytosis (Luttrell and Lefkowitz, 2002).

1.5.5 Internalization

Internalisation of a GPCR causes a general decrease in the proportion of receptors located on the plasma membrane, with the result of a lower response generated upon ligand binding: because of this important role, it is considered one of the key processes of GPCR life cycle regulation (Ferguson, 2001). However, even though these two concepts are often considered connected and strictly dependent on each other, there are cases when the internalization-dependent loss of receptor does not alter its overall function, because these phenomena can also occur independently (Koenig and Edwardson, 1997). Moreover, some cases have been recently reported in which some GPCRs can be still active upon internalization and hold an effective signalling (Feinstein et al.,2013; Thomsen et al., 2016).

Many mechanisms of internalization have been characterized, which can differ according to the cell type used for the assay and to the experimental conditions adopted. One of the main reasons behind the variability in the mode of internalization adopted by different GPCRs is the presence in many receptors of different internalisation motifs in their sequence, allowing a big variety of mechanisms through which the receptors can be internalised. Some examples are the dileucine motif recognized by several clathrin adaptors participating in the

trafficking of other membrane proteins, the interaction motifs for G-protein-coupled receptor-associated sorting protein (GASP), N-ethylmaleimide-sensitive fusion protein (NSF) and PDZ domain interaction motifs (Gurevich V.V. and Gurevich E.V., 2006). Arrestins and trafficking proteins compete for these sites, increasing the complexity of the mechanism.

1.5.5.1 Agonist-dependent internalization

Within minutes once a ligand has bound and activated a receptor, the internalization of GPCRs starts the full cascade in which activated receptors undergo phosphorylation and β-arrestins recruitment. A key mediator of this process is the adaptor protein complex 2 (AP-2), whose role is facilitated by the agonist-receptor interactions, which increase the accessibility of this protein to GPCRs C-tail domain. This is an important mediator for the formation of clathrin-coated pits, facilitating the interactions between GPCRs and clathrin monomers (Kohout and Lefkowitz, 2003). However, differently from this first way just discussed, GPCRs can be internalised through the formation of non-coated membrane invaginations named caveolae (Nichols and Lippincott-Schwartz, 2001), associated with a family of cholesterol-binding proteins named caveolins.

1.5.5.2 Agonist-independent internalization

Many different types of GPCRs showed their ability to internalize independently on the agonist effect, such as angiotensin 1A (AT1AR) receptor, cannabinoid type 1 (CB1) receptor, α1-adrenergic receptor, chemokine receptor 4 (CXCR4), metabotropic glutamate receptor 1 and 5 (mGlu1R and mGlu5R), M2R muscarinic receptor and thyrotropin receptor (Xu et al., 2007). Sometimes considered as a constitutive internalization mechanism, this phenomenon is slower compared to the previous form of internalization which depends on the action of an agonist (Drake et al., 2006). This type of internalization can occur both clathrin-dependently and -independently (Fourgeaud et al., 2003).

1.6 GPCR quaternary structures

GPCRs exist, bind their cognate ligands, and exert their function either as monomers or as dimers and higher-order oligomers. They can form stable and transient homo-oligomeric and hetero-oligomeric macromolecular structures, that can become central for cell surface delivery and the mechanisms of G protein binding and activation (Milligan, 2007). The oligomerization of proteins can affect many aspects of the receptor's life cycle, such as the ontogeny of the receptor. In fact, the oligomerization can work as a quality control at the level of the endoplasmic reticulum (ER) during the protein synthesis, by masking specific retention signals or hydrophobic patches that would retain the proteins in the ER (Reddy & Corley, 1998; Terrillon and Bouvier, 2004). When on the cell surface, these receptors can form oligomeric complexes strongly regulated by several cellular effectors and by the microenvironment in which the complex is found. There are still many studies ongoing that are investigating oligomerization can be regulated by protomers activation or inhibition, with different hypothesis that not always agree in a univocal way. In fact, many studies suggest that a ligand can stimulate the activation of the single protomer and, therefore, the oligomerization of the receptor (Kroeger et al., 2001), or, on the contrary, inhibit the receptor oligomerization (Latif et al., 2002). According to other studies, instead, the homodimerization and heterodimerization are constitutive processes that are not modulated by ligand binding (Canals et al., 2003).

GPCR heterodimerization could have a role in determining the pharmacological diversity of a ligand binding these receptors. Indeed, the positive or negative ligand-binding cooperativity and the increased or decreased potency of a ligand that occur in the presence of different receptors has been correlated to receptor heterodimeric complexes formation for many GPCRs (Galvez et al., 2001; Ward et al., 2011). Additionally, Galvez et al., in their work, also underlined the crucial function of the oligomerization of GPCRs in their signal transduction (Galvez et al., 2001). The last aspect to consider about GPCR oligomerization is that several works proposed receptor heterodimerization as capable of influencing the agonist-promoted GPCRs endocytosis. In this case, it would be sufficient that a single protomer is stimulated to induce the internalization of the two receptors together (Terrillon and Bouvier, 2004).

According to our current knowledge about GPCR oligomerization, TM1 regulates the interaction between dimers, while it has been reported the importance of TM4

and TM5 for interactions established among monomers (Fotiadis et al., 2004). Historically, it was firstly thought that oligomerization of these receptors was essential for the interaction and binding of G proteins. In fact, some works displayed a model in which rhodopsin interacted with transducin G protein and it was observed the necessity of a dimeric form of receptor to accommodate the G protein. In fact, the three subunits of this G protein were too big to be allocated on the monomeric cytoplasmatic area of rhodopsin receptor (Fotiadis et al., 2006). Since then, the importance of oligomerization of GPCRs has been taken into account more and more, especially because even though many experimental methods have been developed to investigate this aspect of GPCRs, still many point stay undetermined or not fully understood, suggesting that further studies and approaches need to be developed in order to characterize the mechanisms by which the oligomerization occurs but also the structural, functional and physiological relevance this process can have.

Indeed, quaternary structures of GPCRs have been reported to be important in mechanisms that have not still clarified and related to receptors' biased signalling (Paradis et al., 2022). Paradis et al. (2022) showed in their work that a biased switch is present at the dimer interface of several GPCRs that selectively regulates the activation of G protein versus β-arrestin-mediated pathways. Because these switches are mostly extracellularly located, they could be druggable sites with a strategical importance for drug discovery. Therefore, this information makes even more important the need of understanding in a clearer way the oligomerization of GPCRs, that could be helpful in a therapeutic perspective with an advantage in developing more effective treatments against diseases.

1.7 Chemokines and chemokine receptors

Chemokines represent a class of secreted proteins with a size in the range of 8-12 kDa, members belonging to the large group of cytokines, that have a specific effect on the interactions and communications between cells. Among the different types of cytokines, they are defined as chemoattractant cytokines and, therefore, their main role consists of the regulation of migration of immune cells across the body. As ligands, they can signal by binding several GPCRs which, because of their ability to bind chemokines to exert their function, are called chemokine receptors. Chemokines are mostly known for their role in stimulating the cell

migration of immune cells, especially leukocytes (Hughes and Nibbs, 2018). Therefore, these molecules play a fundamental role in physiological processes like the development and homeostasis of the immune system and are involved in all protective or destructive immune and inflammatory responses. Other processes in which these proteins are involved are organogenesis, haematopoiesis, and immunomodulation.

From a pathological point of view, they play a critical role in inflammation, allergy, autoimmune diseases, many types of cancer, and in innate immunity, including processes such as leukocyte recruitment, a full delivery of antigen-presenting cells to the effector cells of the adaptive immune system and the maturation of lymphocyte themselves (Chen et al., 2018; Hughes and Nibbs, 2018). They are secreted by many types of immune cells with various mechanisms (Arango and Descoteaux, 2014; Tecchio et al., 2014; Moon et al., 2014; Davoine and Lacy, 2014). For examples, macrophages lack secretory granules and rely on a constitutive secretory pathway that engages recycling endosomes for the secretion of cytokines, including tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-10 (Lacy, 2015). Also, mast cell secretion of cytokines occurs through a trafficking mechanism that is similar to the SNAREs system used by macrophages, while the way neutrophils produce and secrete cytokines and chemokines is still not fully clear (Lacy, 2015). Since one of their most important functions is represented by the regulation of immune cell migration, they represent encouraging drug targets to allow the treatment of different diseases originated from a dysfunction of the immune system. These pathological conditions include, among others, chronic obstructive pulmonary disease, multiple sclerosis, rheumatoid arthritis, HIV-1 infection, and cancer (Bongers et al., 2010; Adlere et al., 2019).

Chemokine ligands are classified according to differences in their primary structure into four subfamilies: CC, CXC, XC and CX_3C. The classification is based on the first cysteine in their sequence found, beginning to count from their N-terminus, fundamental to form disulphide bridges which have an essential function in stabilizing a chemokine's structure. This residue can be followed by a second cysteine residue directly (CC), spaced by a random amino acid (CXC), spaced by three amino acids (CX_3C), or can lack a proximal second cysteine (XC) (Capoferri, 2020). Additionally, it is possible to distinguish in a simplistic way homeostatic from inflammatory chemokines; the former type is responsible for

basal leukocyte migration, the latter are produced under pathological conditions as consequence of pro-inflammatory stimuli and take part actively in the inflammatory response by attracting immune cells to the site of inflammation. (Zlotnik et al., 2011; Zlotnik and Yoshie, 2012). The GPCRs that have chemokines as ligands are referred to as chemokine receptors, and they belong to the family of rhodopsin-like receptors. From a cytogenetic point of view, these receptors are located in clusters on different chromosomes, and this seems to suggest a common ancestral origin: this might be due to several local gene duplications combined with genome duplications (Fredriksson et al., 2003). A more recent classification of chemokine receptors divides these proteins into two macro-groups, referred to as "typical" and "atypical". This classification is based on whether the individual receptors are able to elicit a G protein-mediated response, with the atypical receptors unable to do so. Typical chemokine receptors are divided into different groups according to the class of chemokine ligands they bind: CCR bind CC chemokines, CXCR bind CXC chemokines, XCR bind XC chemokines and CX3CR bind CX3C chemokines (Bachelerie et al., 2014) (**Table 1.1**).

1.7.1 Chemokine ligands

IUPHAR currently recognizes 45 human chemokines ligads (Bachelerie et al., 2014). They present different features that allow us to distinguish them: CC and CXC chemokines generally have a 6-10 amino acids long N-terminal domain preceding the first structural cysteine, followed by a long loop (N-loop), a 3_{10} helix, three β-strands and a C-terminal α-helix 20-30 amino acids long highly disordered structurally (Rajarathnam et al., 1995; Allen et al., 2007). The structural arrangement typical of these subfamilies of chemokine ligands is conserved but the sequence identity results to be highly divergent. The XC chemokine group contains only two members, lymphotactin-α and -β (also referred to as XCL1 and XCL2), while the only CX_3C chemokine, called fractalkine (CX_3CL1), is membrane bound (Zlotnik and Yoshie, 2012).

CXC chemokine have a crucial role in inflammation-related processes and angiogenesis, since they finely regulate both processes interacting with their respective cognate receptors expressed by endothelial cells (Romagnani et al., 2004, Bikfalvi and Billottet, 2020, Gorbachev and Fairchild, 2014). Therefore, as

a rule, it is accepted that CXC chemokines affect tumour growth by modulating angiogenesis and/or recruitment of effector or regulatory immune cells.

As an additional division of CXC chemokines, they can be classified as ELR^+ and ELR^- subgroups, because of the presence and absence, respectively, of a three amino acid Glu-Leu-Arg motif located in the N-terminal region before the first conserved cysteine (Ishimoto et al., 2023). Moreover, ELR^+ CXC chemokines are grouped on chromosome 4 in humans and are mostly important for the chemoattraction of neutrophils and, therefore, they contribute to wound repair, by attracting neutrophils and inducing degranulation and the respiratory burst. This type of chemokine ligand is produced and secreted thanks to the stimuli promoted by pro-inflammatory cytokines such as IL-1, TNF-α or microbic PAMPs (Baggiolini, 2001). Also ELR^- CXC chemokines facilitate the process of wound repair.

The class of CC chemokines is the largest group of chemokines and signals through different CC chemokine receptors with the main function of recruiting monocytes and macrophages, immune cells involved both in acute and chronic inflammation, and diseases such as atherosclerosis, rheumatoid arthritis, and adipose inflammation (Charo and Ransohoff, 2006). In addition to creating either tumour-suppressing conditions (such as recruitment of effector T cells) or tumour-promoting conditions (such as recruitment of Tregs, M2-type macrophages) in the tumour microenvironment, some CC chemokines promote tumour expansion by mediating tumour cell invasion or migration to distant sites (Gorbachev and Fairchild, 2014). Previous studies in both mice and humans have shown that CCL1 is critical for metastasis formation in tumour-draining lymph nodes by recruiting CCR8-expressing melanoma or breast carcinoma cells into lymph nodes (Das et al., 2013).

Due to their single conserved cysteine residue, XC chemokines exist in two different conformations in equilibrium with one another, in which the monomeric state resembles the conventional chemokine fold, while the other conformation is a four-stranded β-sheet, which forms when the usually extended N-terminal regions pairs with the third β-strand in the sheet (Sonay Kuloglu et al., 2002; Capoferri, 2020). CX_3CL1 is a mucin-like membrane protein with an extracellular chemokine domain working as an adhesion molecule; this domain can detach from the membrane anchor upon protease action, and act like a secreted chemokine (Wojdasiewicz et al., 2014).

Receptor	Ligand	Receptor	Ligand	Receptor	Ligand
CXCR1	CXCL5 CXCL6 CXCL8	CCR1	CCL3 CCL4 CCL5 CCL7 CCL8 CCL13 CCL14 CCL15 CCL16 CCL23	XCR1	XCL1 XCL2
CXCR2	CXCL1 CXCL2 CXCL3 CXCL5 CXCL6 CXCL7 CXCL8	CCR2	CCL2 CCL5 CCL7 CCL8 CCL13 CCL16	CX_3CR1	CX_3CL1
CXCR3	CXCL4 CXCL4L1 CXCL9 CXCL10 CXCL11	CCR3	CCL4 CCL5 CCL7 CCL11 CCL13 CCL15 CCL24 CCL26 CCL28	ACKR1	CCL2 CCL5 CCL7 CCL11 CCL13 CCL14 CCL17 CXCL5 CXCL6 CXCL8 CXCL11
CXCR4	CXCL12	CCR4	CCL17 CCL22	ACKR2	CCL2 CCL3 CCL4 CCL5 CCL7 CCL8 CCL11 CCL13 CCL14 CCL17 CCL22
CXCR5	CXCL13	CCR5	CCL3 CCL4 CCL5 CCL7 CCL14 CCL16	ACKR3	CXCL11 CXCL12
CXCR6	CXCL16	CCR6	CCL20	ACKR4	CCL19 CCL21 CCL25
???	CXCL14 CXCL17	CCR7	CCL19 CCL21	ACKR5	CCL19
		CCR8	CCL18		
		CCR9	CCL25		
		CCR10	CCL27 CCL28		

Table 1.1: Classification of chemokine receptors and their cognate ligands

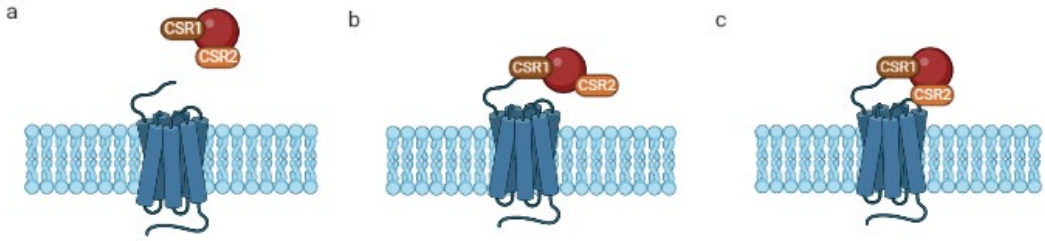


Fig.1.3: Two-step recognition binding model. (Fig. 1.3a) The chemokine ligand shows a CSR1 region and a CSR2 region to allow the interaction with the receptor. (**Fig. 1.3b**) The first step is the interaction between the chemokine receptor N-terminus with the CRS1 of the chemokine ligand. This leads to the docking of chemokine ligand CRS2 (**Fig. 1.3c**) into the chemokine receptor 7TM region, allowing the latter to transmit the signal intracellularly. Image created on Biorender.com

1.7.2 CXCR4 and its pathophysiological role

Human CXC-motif chemokine Receptor 4 (hCXCR4) is one of the best characterized GPCRs within the chemokine receptor family, due to its important role in several biological processes such as embryonic development, haematopoietic cell trafficking, mature leukocyte homing and angiogenesis (Wang and Knaut, 2014). When mutated and not in physiological conditions, this receptor shows an important role in leading the process to develop different immune system-related pathologies, such as the congenital immune deficiency WHIM (Warts, Hypogammaglobulinemia, Infections, and Myelokathexis syndrome). Moreover, this receptor is known to act as a co-receptor for T-tropic HIV-1 protein gp120, therefore favouring infection by HIV-1 (Balabanian et al., 2004), and additionally its expression is found to be upregulated in different neoplastic conditions (Vela et al., 2015; Balkwill, 2004b), including Waldenström macroglobulinaemia.

$G_{\alpha i/o}$ family represents the predominant family of G proteins through which hCXCR4 signal, even though it was proved by further studies that this receptor is able to recruit $G_{\alpha 13}$ (Tan et al., 2006; Yagi et al., 2011; Kumar et al., 2011), $G_{\alpha q}$ (Soede et al., 2001) and β-arrestins. Since the preferential G protein activated upon ligand stimulation are members of the inhibitory family, as result of the receptor activation upon agonist stimulation, an enzyme called adenylyl cyclase is inhibited. This enzyme is necessary to produce an important second messenger

called cyclic AMP (cAMP), which is reduced intracellularly as consequence of the agonist-mediated activation of hCXCR4, and therefore, cAMP-dependent protein kinase activity is decreased or blocked.

$G_{\alpha 13}$ protein signalling was initially identified during Jurkat T cell migration (immortalised cell line derived from a T-cells acute lymphoblastic leukemia), which expressed CXCR4 and where Rho GTPase and Rac protein (activated via $G_{\alpha i}$ protein) were activated (Tan et al., 2006), as well as in CXCR4-induced metastatic basal-like breast cancer cells (Yagi et al., 2011).

The $G_{\alpha q}$ protein activate PLC-β (phospholipase C type β), which degrades phosphatidylinositol-4,5 bisphosphate (PIP2) to form intracellular messengers linked to the elevation of cytosolic Ca^{2+} levels and increase in protein kinase C (PKC) activity. This signalling pathway was reported for hCXCR4 only upon ligand-mediated stimulation only in dendritic cells and granulocytes (Soede et al., 2001), showing once again the important of cellular context for GPCR signalling functioning.

hCXCR4 can signal even through G protein-independent pathways and the most important and well characterized is the β-arrestin recruitment, which importantly regulates receptor internalization, recycling to the membrane and receptor degradation via lysosomes. Additionally, this signalling pathway has a role in termination of G protein signalling and may affect the chemotaxis of hCXCR4. Both β-arrestin1 and 2 are recruited once kinases called GRKs phosphorylate specific serine, threonine and tyrosine residues located in the ICL3 and in the C-terminus side of GPCRs upon agonist-promoted stimulation, starting the formation of clathrin-coated pits.

The carboxy-terminal domain of hCXCR4 determines the binding of β-arrestin to this receptor, so it does not surprise the importance of this region for the receptor's functionality. For example, in the case of the congenital disease named WHIM syndrome, the origin of this syndrome has been connected to a truncation of CXCR4 C-tail, since patients have cells that cannot downregulate hCXCR4 expression on the membrane (Hernandez et al., 2003; Heusinkveld et al., 2019). This defect results in a gain-of-function phenotype, in which mature granulocytes are not capable of moving out from bone marrow niche, causing chronic noncyclic neutropenia. However, also some amino acids localized on the IL3 of hCXCR4 as for other GPCRs were described as important sites with a role in facilitating the arrestin recruitment (Cheng et al., 2000; Gustavsson et al., 2017).

Another disease in which the correct maintenance of hCXCR4 C-tail represents a critical point is Waldenström macroglobulinemia, a type of B cell proliferative disease (lymphoplasmacytoid tumour) with a phenotype that represents a combination of both non-Hodgkin lymphoma (lymphoid tumour) and multiple myeloma (plasmacytoid tumour), in which cancerous WM cells grow in the bone marrow and can push out the hematopoietic stem cells from their niche (Drandi et al., 2022). The origin of this disease is to search in gain-of-function mutations in *myd88* gene, which codes for MYD88 (Myeloid Differentiation primary response 88) adaptor protein involved in regulation of signalling of immune cells. Additionally, together with additional nonsense mutations located 20 amino acids upstream of the stop codon of hCXCR4 and some frameshift mutations contained in a region up to 40 amino acids upstream of the receptor (Treon et al., 2014). These genetic mutations result in anaemia, immune deficiency, thrombocytopenia and causes symptoms such as weakness and fatigue in patients, which also show a high sensitivity to bruising, excessively bleeding and severe symptoms in presence of infections. Indeed, these immune cells are fundamental in the regulation of production of IgM antibodies (macroglobulins). Because of their malfunctioning, these cells can make blood thicker and produce symptoms such as alteration in vision, headaches, vertigo, and changes in the mental status (Castillo et al., 2015; Naderi and Yang, 2013).

Another disease for which hCXCR4 has been intensively studied for is T-tropic HIV-1 infection of T cells. HIV-1 gp120 (glycoprotein 120) is a protein of the virus envelop binding with high affinity to CD4 (cluster differentiation 4), a 55 kDa membrane protein found predominantly on a subset of T cells named T helper (T_h cells), which plays a major role in mediating immune response through the secretion of specific cytokines and activating cells of the innate immune system, but expressed also on the surface of monocytes/macrophages and dendritic/Langerhans cells (Zaitseva et al., 1997; Luckheeram et al., 2012).

When gp120 glycoprotein interacts with CD4, it is subject to a structural change that favours the binding of gp120-CD4 complex either to CCR5 receptor (in case of M-tropic HIV-1 strain, so monocyte/macrophage tropic or R5 virus) or to hCXCR4 (in case of T-tropic HIV-1 strain, or X4 virus). However, this scheme of the infection method adopter by HIV-1 can be simplistic, since there are many strains in nature that are defined “dual tropic” because of their ability to use both hCXCR4 and hCCR5 receptors to start the infection process (Bleul et al., 1997).

Therefore, it is commonly accepted that both hCXCR4, together with hCCR5, are co-receptors of HIV-1 entry into $CD4^+$ T cells, since usage of hCXCR4 by HIV-1 isolates often evolves during disease progression associated with the emergence of syncytium-inducing (SI) variants, while usage of this receptor by primary HIV-1 strains appears to be critical in the establishment of persistent infection (Ostrowski et al., 1998). After the chemokine receptor is bound by gp120, the process called "fusion" starts because of the exposure of gp41, another envelope protein helping the anchoring of the viral cells and the penetration of the target cells membrane. This action leads, as consequence to the release of virions within the infected cells. In this process, hCXCR4 shows a crucial role to help the fusion, and this feature granted the name of "fusin" when referring to hCXCR4. (Endres et al., 1996).

Alterations in hCXCR4 expression has been found in several cancer cell lines, and this leads to more aggressive phenotypes and lower survival rates, due to the enhanced ability of these types of cancer cells to invade other tissues and form metastasis (Liu et al., 2009; Iwasa et al., 2009). This aggressive phenotype is the result of the action of cancer cells with overexpressed hCXCR4 and this overexpression has the aim of hijacking the hCXCR4/CXCL12 axis to establish distant organ metastasis. Indeed, this hypothesis finds support when CXCL12 expression levels are analysed in different areas of body and levels of this chemokine are higher in common sites of metastasis such as brain, bone marrow, lungs, and live (Chatterjee et al., 2014). This overexpression of hCXCR4 has been investigated and encountered in around 23 different human cancer types, such as blood, lung, brain, kidney, ovary, pancreas, and skin neoplastic diseases (Balkwill, 2004*a*).

In past years, it became clear that hCXCR4 has a prominent role in cancer biology field. In fact, stimulation with basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF), and activation of transcription factors such as nuclear respiratory factor-1 (NRF-1) are all mediated by hCXCR4 upregulation (Phillips et al., 2005; Zhuo et al., 2012). Also, hypoxia-induced factor-1α (HIF-1α) accumulation and activation is mediated by nuclear hCXCR4, which, in turn, induces *cxcr4* transcription, leading to hCXCR4 upregulation (Bradley, 2018; Bao et al., 2019).

hCXCR4 has been described to be an essential receptor in regulating different physiological processes, and one these is the embryo development. This role of

hCXCR4 was shown in a number of works from many different groups, in which hCXCR4 knockout (KO) mice showed aberrant vascular formation in the intestine (Tachibana et al., 1998), cardiac ventricular malformations (Ivins et al., 2015), an abnormal migration of the cerebellar neurons and a severe impairment of haemopoiesis, affecting especially the myeloid progenitors and the B lymphoid lineage (Qing et al., 1999). Because of all these problems found in mice development, generally hCXCR4-KO mice die perinatally.

An important regulatory function of hCXCR4 has recently been described as a suppressor of type-I interferons production by directly suppressing the activation of TLRs (Toll-like receptors) signalling pathways with a role in many auto-immune diseases, that include interferonopathies such as systemic lupus erythematosus (SLE), or rheumatoid arthritis or multiple sclerosis (Smith et al., 2017; Smith et al., 2019, McHugh, 2019; Caspar et al., 2022). These diseases show a direct connection to elevated interferon levels due to hCXCR4 activity, making this receptor a promising target (De Ceuninck et al., 2021) to treat these pathological conditions. Additionally, overexpression of hCXCR4 receptor has been reported on circulating B cells in patients with SLE and, therefore, the establishment of non-physiological interactions between B and T cells represents an important biomarker of these diseases (Hanaoka et al., 2015; Wang et al., 2009). Indeed, the expansion of T follicular helper cells and of some subsets of T peripheral helper cells is one the typical trait of patients displaying SLE disease (Caspar et al., 2022)

1.7.2.1 CXCR4 ligands

The endogenous agonist of hCXCR4 is the ELR$^-$ CXC chemokine ligand CXCL12 (CXC motif chemokine ligand 12), in the past also known with the name stromal-derived factor-1 (SDF-1) (Xu et al., 2015). CXCL12 is expressed ubiquitously in many tissues and cells. Even though it is the endogenous agonist of hCXCR4, it also can bind to the atypical chemokine receptor hACKR3, for which this receptor has been proposed to act as a scavenger receptor (see more detail in the section relative to hACKR3, in paragraph 1.7.4).

In humans, six different splice variants of CXCL12 were found (named from α to φ), which derive from alternative splicing events occurring between the fourth and final exon of the gene codifying for this ligand and which differ mainly for

their specific tissue distribution and activity (Janssens et al., 2018). CXCL12α, 89 residues long, is the shortest and most expressed isoform of this ligand (Yu et al., 2006) and it can exist both as in monomeric and dimeric forms, which can have different implications in binding hCXCR4 and exerting its effect on this receptor. In fact, the monomeric form of CXCL12 has a higher effectiveness in signalling through hCXCR4 to block the production of intracellular cAMP and stimulate AKT, as secondary effects mediated by G protein activation. Instead, dimeric forms of CXCL12 ligand show more efficiency in recruiting β-arrestin 2 to hCXCR4 and act as more potent chemoattractants to facilitate the migration of breast cancer cells expressing hCXCR4, also affecting the chemotaxis process via β-arrestin 2 (Fong et al., 2002; Ray et al., 2012).

CXCL12 KO mice display similar pathological phenotypes as seen for hCXCR4 KO, showing that an impairment of this axis results in serious pathological conditions, which are characterized by severe alterations in neural progenitor proliferation, migration and differentiation (Trousse et al., 2019; Ma et al., 1998), problems in vascularization of the gastrointestinal tract (Tachibana et al., 1998), dysregulation of cardiac functions (LaRocca et al., 2019).

The list of ligands exerting an effect after binding hCXCR4 receptor is vast and all these molecules vary in terms of function and chemical nature (Bianchi, and Mezzapelle, 2020). hCXCR4 represents the receptor of another ligand called macrophage migration inhibitory factor (MIF), an inflammatory cytokine with chemokine-like activity involved in the regulation of innate immunity. MIF promotes leukocyte recruitment, which mediate the role of MIF itself in atherosclerosis and contribute to the wealth of other MIF-related biological activities (Rajasekaran et al., 2016). This ligand interacts with the amino terminal sequence (1-27 region) of hCXCR4 receptor and with the external surface area of transmembranes helices (at the level of EL1, EL2, EL3) but it does not bind inside the TM pocket (Rajasekaran et al., 2016; Lacy et al, 2018). MIF also acts as an agonist for other receptors, including hCXCR2, CD74/CD44, and hACKR3 (Jankauskas et al., 2019).

Another ligand for hCXCR4 is extracellular ubiquitin (eUb), included in the category of a damage-associated molecular patterns (DAMPs), which are molecules released from dead or severely stressed cells to alert about any damage or infection to cellular microenvironment and the innate immune system. Extracellular ubiquitin can exert an agonist effect on hCXCR4, without

competing with CXCL12 in promoting the agonist-mediated signalling of the receptor (Saini et al., 2010). Indeed, different groups adopting molecular modelling and mutagenesis analysis to determine the binding site of eUb suggested the possibility that this molecule may bind to hCXCR4 inside the cavity delimited by TMs domains (EL2 and EL3) (Tripathi et al., 2013; Saini et al., 2011), but the interaction occurs through hCXCR4 residues which do not contribute to CXCL12 binding (Scofield et al., 2018), since eUb lacks of the interaction with the N-terminal region of hCXCR4 that is a crucial step in CXCL12 binding (Saini et al., 2011).

In the past, another molecule has been related to CXCR4 for its ability to bind this receptor and this is called β-defensin-3 (HBD3), which can compete with CXCL12 for CXCR4 binding. It is involved in promoting receptor internalization without inducing Ca^{2+} flux, ERK phosphorylation, and chemotaxis (Feng et al., 2006), therefore it can be considered a novel antagonist at CXCL12-promoted signalling of hCXCR4 receptor. Additionally, it has been shown that β-defensin-3 cysteine residues, specific surface-distributed cationic residues, the electrostatic properties, and availability of both β-defensin-3 termini are very important in allowing hCXCR4 binding (Feng et al., 2013).

The list of diseases which are developed upon dysfunctions of hCXCR4 also includes Kaposi's sarcoma, which is caused by Kaposi's sarcoma Herpes virus, or Human Herpesvirus 8 (KSHV or HHV8). This γ herpesvirus which, after it has infected the cells, initiates the production of the "master KEYmokine" named v-MIP II or v-CCL2 (viral Macrophage Inflammatory protein II, or viral CC motif chemokine ligand 2), which acts as an antagonist on hCXCR4 (Szpakowska and Chevigne', 2016). Together with vMIP I/v-CCL1 and vMIP III/v-CCL3, they block the T_{h1}-mediated response upon viral infection: vCCL2 facilitates immune system evasion by blocking the action of chemokines towards the infection (Nicholas, 2010; Szpakowska et al. 2018).

Another ligand for hCXCR4 reported in literature is the high mobility group box 1 protein (HMGB1), member of DAMPs molecules (Ge et al., 2021). This molecule can form heterocomplexes with CXCL12 (HMGB1·CXL12) and it has been observed that the structural changes of hCXCR4 occurring upon ligand binding show differences depending on whether CXCL12 alone or HMGB1·CXCL12 complex binds the receptor. Additionally, the complex is over one order of magnitude more potent than CXCL12 alone in inducing cell

migration (Schiraldi et al, 2012; Bianchi and Mezzapelle, 2020). However, HMGB1 can bind CXCL12 and interact with CXCR4 only when HMGB1 is found in its reduced form, which presents the pair of cysteine residues localized in the HMG-box domain A that in this case are not forming any disulphide bonds (Venereau et al., 2012). Another study using a 3S-HMGB1, where serine residues replaced all three cysteines involved in CXCL12 binding, demonstrated that this ligand acquired the ability to bind CXCR4 directly even without intercting with CXCL12 and showed the same efficacy as HMGB1-CXCL12 complex to induce migration of immune cells and muscle regeneration (Tirone et al., 2018).

In the past years, because of the physiological relevance of this receptor, many synthetic ligands, such as plerixafor (or AMD3100) and isothiourea-1t (IT-1t), were developed, initially as T-tropic HIV-1 blockers. (Hendrix et al., 2004). AMD3100 is a bicyclam molecule, in which an aromatic bridge tether the two cyclam rings, that reversibly antagonizes hCXCR4 (De Clerq, 2005). This small molecule ligand showed efficacy also promoting the mobilization the $CD34^+$ stem cells from the bone marrow into the peripheral blood stream by interacting with CXCL12 (Bachelerie et al., 2015; Eby et al., 2017; De Clerq, 2019). Later, it has been successfully approved by US FDA (United States Food and Drugs Administration) as treatment drug for autologous transplantation in patients with non-Hodgkin's lymphoma or multiple myeloma, with possible beneficial effects even in other malignant diseases as well as hereditary immunological disorders such as WHIM syndrome.

The isothiourea secondary amine IT1t, is the most known and characterized ligand showing an immunomodulating function at hCXCR4 (Thoma et al. 2008), with the most potent and concentration-dependent blockade of interactions between CXCL12 and hCXCR4 (Van Hout et al., 2017; Caspar et al. 2022). It has also demonstrated the effect of this small antagonist ligand in disrupting the oligomerization state of CXCR4, and even more efficiently than AMD3100, with important consequences on the receptor signalling and function (Ward et al. 2021). Additionally, IT1t is structurally similar to clobenpropit and it has been shown to inhibit type I interferon production by pDCs in vitro (Smith et al., 2019). Smith et al. (2019) also showed that in ex vivo tonsil mononuclear cell suspensions, IT1t inhibited TLR7-mediated production of IFNα. Furthermore, IT1t treatment reduced systemic inflammation and autoantibody production, and prevented glomerulonephritis, in a TLR7-dependent mouse model of SLE

(pristane-induced lupus) (McHugh, 2019). These findings are important because of the ability of CXCR4 to interact with TLRs as explained in more depth in the next paragraphs (1.7.3.3).

Other molecules directed against hCXCR4 have been or currently are under clinical trials assessments (US clinicaltrialsgov.com), even though low efficacy, high toxicity, or poor pharmacokinetic properties represented the most common problems of these drugs (Van Hout et a., 2018). The more the knowledge about hCXCR4 pharmacology, signalling and functioning acquired grows, the more it becomes helpful to realize new synthetic molecules targeting hCXCR4 in many ways and that could be, in the future, new therapeutic agents to treat all diseases related to hCXCR4/CXCL12 axis.

1.7.2.2 CXCR4 binding pockets

Based on several studies using crystal structure of the receptor fused to the T4 lysozyme as a starting point, it has been possible to investigate and characterize the binding positions of chemokines and small molecules at hCXCR4 (Wu et al., 2010). Other works in which mutational studies were carried out, instead, helped in describing the signalling transmission for different ligands interacting with hCXCR4 through the transmembrane helices but also through the N-terminal region of the receptor. (Wescott et al., 2016; Adlere et al., 2019).

The extracellular interface of hCXCR4 is formed by an amino terminal region, the ECL1 linking helices II and III, ECL2 linking helices IV and V and ECL3 linking helices VI and VII, with two disulphide bonds critical for ligand binding (Caspar et al., 2022). Differently from other GPCRs, hCXCR4 helix II is rotated which contrasts with predictions generated about the receptor binding pocket by using homology models. Additionally, helices V and VII are projected into the extracellular space, while helices I, IV and VI are shifted and there is also an additional disulphide bond in the extracellular space which has the role of connecting the N-terminal region and helix VII of hCXCR4 (Wu et al., 2010, Caspar et al., 2022). The ECL2 and amino-terminal domains of the receptor are connected by two disulphide bonds that cooperate in order to shape the entrance required to the ligand to allocate in its binding pocket (Wu et al., 2010).

Once it binds to hCXCR4, CXCL12 occupies the full binding pocket and induces conformational changes at the level of N-terminal and extracellular domains of the receptor. This effect is facilitated thanks to an abundance of non polar and polar intermolecular interactions, including salt bridges formed between positively charged CXCL12 residues and negatively charged hCXCR4 residues (Tamamis and Floudas, 2014).

The binding interface between CXCL12 and hCXCR4 predicted by molecular docking studies shows a good degree of overlap with the binding positions of IT1t antagonist (Xu et al., 2013; Busillo and Benovich, 2007), which is then classified as orthosteric negative modulator for hCXCR4, since it acts by antagonizing CXCL12-mediated effect by competing with CXCL12 for the same binding pocket on the receptor.

All chemokines have a flexible N-terminus which has a key role in promoting signalling through their cognate receptors. In fact, the area comprised between residues 12–17 of CXCL12 N-loop domain, usually referred to as RFFESH from the letters of the amino acid residues which form this sequence, interact with residues of the TM bundle of hCXCR4, while the core region of CXCL12 composed by 24–50 residues interact with hCXCR4 N-terminus. These interactions represent the basis for the initial contact between ligand and receptor (Crump et al., 1997; Tamamis and Floudas, 2014). Upon interactions between CXCL12 N-terminal residues and hCXCR4 TM region, the basic Lys1 residue located in the CXCL12 N-terminus sequence, and which is fundamental for activating the receptor, interacts with Glu32 residue by establishing a strong polar interaction and this bond takes place in the hCXCR4 extracellular surface. This bond eases the movement of TM5 and TM6 in a transitional conformation associated with G protein signalling pathway (Xu et al., 2013).

When different ligands are compared to each other in terms of predicted binding positions, CXCL12, AMD3100 and IT1t bound to hCXCR4 show clear differences, with a larger, more open binding cavity for IT1t, and placed closer to the extracellular surface (Kufareva et al., 2015). Generally, for chemokine receptors, the extracellular loop 2 partially covers their large binding pocket. The binding pocket of hCXCR4, as similarly for other chemokine receptors, is larger also because CXCL12, endogenous agonist of this receptor, is a relatively large molecule (89 amino acid, 10 kDa) and the region of CXCR4 forming the binding pocket needs to be large enough to allocate this chemokine. Additionally, other

differences are due to the presence of negatively charged residues which have the aim of promoting ligand binding once this is situated in the binding pockets of the receptor. Therefore, these residues may affect ligands binding according to their chemical structure and physical properties (Arimont et al., 2017).

The orthosteric antagonist IT1t, differently from CXCL12, binds the receptor in the minor pocket, which is located in the middle of the receptor transmembrane region, formed by side chains belonging to helices I, II, III and VII, with no contacts with the other helices. The nitrogens of the symmetrical isothiourea group are both protonated with a net positive resonance charge, helping the stabilization of the interactions between IT1t and hCXCR4, which make effective the inhibition of hCXCR4 and the blockade of CXCL12 activity in presence of IT1t (Caspar et al., 2022). Instead, differently from IT1t, the antagonist AMD3100, formed by two cyclam units linked by a heteroaromatic phenylenebis(-methylene) linker, has been suggested to bind CXCR4 at the level of the major binding pocket with one cyclam ring, whereas the other ring is sandwiched between helix VI and VII (Rosenkilde et al., 2007).

A full list of the key amino acid residues involved in interactions between hCXCR4 and its ligands CXCL12, IT1t and AMD3100 is reported in **Table 1.2**. Moreover, a graphical representation of the regions of hCXCR4 forming the major (coloured in blue) and minor (coloured in red) subpocket for IT1t and AMD3100 binding is represented in **Fig.1.4** (adapted from Caspar et al., 2022). Since IT1t is an orthosteric antagonist for hCXCR4, its binding site mostly overlaps with CXCL12 binding site, with some differences in residues listed in **Table 1.2**.

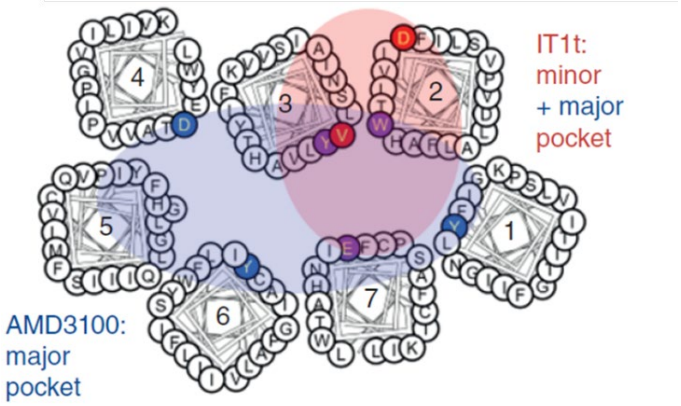


Fig.1.4: Comparison of binding sites of hCXCR4. Top view of the receptor. Key interaction partners of IT1t are highlighted in red, key residues interacting with AMD3100 are highlighted in blue, and shared residues in purple (adapted from Caspar et al., 2022).

	Residues (CXCR4)	CXCL12	IT1t	AMD3100
N-terminus	E2	X		
	E14	X		
	E15	X		
	D20	X		
	Y21	X		
	E26	X		
Helix I	E32	X		
	Y45			X
Helix II	F87	X		
	W94	X	X	X
	D97	X	X	
ECL1	W102		X	
Helix III	V112		X	
	Y116		X	X
Helix IV	D171			X
	A175			X
ECL2	R183		X	
	I185		X	
	C186	X	X	
	D187	X	X	X
	R188	X		
	F189	X		X
	Y190			X
Helix VI	E255			X
	D262	X		X
	D268	X		
ECL3	K271	X		
Helix VII	E277	X		
	V280	X		
	H281	X		
	E288	X	X	X
	F292	X		

Table 1.2: Key residues involved in the interactions between hCXCR4 and its ligands CXCL12, IT1t and AMD3100. A full list of residues is reported in this table, where "x" indicates the role of that specific residue in the binding of the respective ligand.

1.7.2.3 CXCR4 oligomerization

In the past, several studies demonstrated the hCXCR4 tendency to homo- and heterodimerize, either constitutively or upon ligand binding (Babcock et al., 2003; Percherancier et al., 2005; Vila-Coro et al., 1999; Levoye et al., 2009; Salanga et al., 2009). The oligomerization of GPCRs is a key event which affects many aspects of their life cycle, so it does not surprise that also hCXCR4 can form homo- and heterodimers with other GPCRs. Although the functional importance of hCXCR4 dimerization is still not fully understood, the pharmacological implications of this phenomenon are commonly accepted. For example, in WHIM syndrome, hCXCR4 dimerization has been suggested to be the most likely

mechanism to justify the observed dominance of truncated form of hCXCR4 over the wild-type receptor (Balabanian et al., 2005; Lagane et al., 2008).

Different ligands can affect in many ways the oligomerization of hCXCR4, so they should be considered individually. It has been reported that the homodimerization of hCXCR4 increases in number with an increase in expression level and it is highly promoted by CXCL12, while AMD3100 has no effect and IT1t has been demonstrated to be able to selectively disrupt the oligomerization of hCXCR4 (Ward et al., 2021; Işbilir et al., 2020), even though mutants in specific amino acid residues that are considered important for hCXCR4 dimerization did not show any difference or reduction in their signalling ability (Ward et al., 2021). Additionally, an important consideration to take into account is that dimerization can also cause changes in basal activity of hCXCR4 and, nevertheless, it has been demonstrated that hCXCR4 homodimers tend to be internalized more easily than monomers (Ge et al., 2017).

Using a single-molecule microscopy approach, it has been shown that hCXCR4 has the tendency to be expressed mostly in its monomeric form. However, increasing the expression levels of the receptor, as it happens frequently in several cancer phenotypes, where hCXCR4 tends instead to exist in its dimeric form (Işbilir et al., 2020), hCXCR4 starts to form high-order oligomers with a potential affection on receptor pharmacology and signalling (Heuninck et al., 2019).

It is relevant to say, also, that mutants generated considering the structure of hCXCR4 bound to IT1t showed no significant differences in signalling by using a bioluminescence resonance energy transfer (BRET) approach, even though there is evidence that hCXCR4 signalling is affected by the receptor oligomerization by using different approaches looking at diverse signalling pathways. This could be explained by the idea that many different interfaces in hCXCR4 homodimers can be generated, and hCXCR4 homodimers formation is a highly dynamic mechanism of hCXCR4 regulation. Therefore, these aspects may determine eventual changes in receptor's signalling (Hamatake et al., 2009).

hCXCR4 is known to be able to form heterodimers with hACKR3, because of the connection that these two receptors have, since CXCL12 acts as an agonist on both receptors. However, several studies using transiently co-transfected cells showed that hCXCR4 can form heteromers and/or crosstalk not only with hACKR3, which is the receptor mostly investigated in this thesis among the other partners involved in hCXCR4 hetero-dimerization, but also with chemokine

receptors hCCR2, hCCR5, hCCR7, and hCXCR3 in the family of chemokine receptors and with adrenergic receptors, opioid receptors, cannabinoid receptors, BILF1 receptor and acquiring different roles in influencing several cellular and physiological processes (Heuninck et al., 2019; Sohy et al., 2007; Coke at al., 2016).

One of the crucial points of GPCR heterodimerization is represented by the effects that heterodimerization has on GPCR complexes in terms of signalling and pharmacological profile. For example, in the case of heterodimerization with hACKR3, unlike when hCXCR4 is alone in cells, there is an increased CXCL12-mediated activation of both receptors, which leads to an increased β-arrestin signalling and a sustained activation of ERK1/2 and p38 MAPK (mitogen-activated protein kinase) signalling pathways Decaillot et al., 2011; Levoye et al., 2009). At a physiological level, hCXCR4-hACKR3 heterodimeric complexes have proven to be critical in valve formation in the heart, and integrin activation in T-cells (Yu et al., 2011; Hartmann et al., 2008).

hCXCR4-hCCR2 heterodimerization has also been studied for a long time and the functional effects of this heterodimerization have been observed, such as trans-inhibition of chemotaxis and calcium response and antagonist trans-inhibition of ligand binding (Sohy et al., 2007). This proved that allosterism is a key process in regulating the effects ligands may exert on GPCRs also in presence of heterodimers, since switching off a member of the heterocomplex may lead to a consequential inhibition of the second member by using one single ligand specific for a single receptor.

Also hCCR5 can form heterodimers with hCXCR4, with important consequences in HIV-1 infection, for which both receptors act as co-receptors to allow the entry of the virus via gp120. The effects of the formation of complex by these two receptors have been investigated as well and the modulation of T lymphocytes responses represents the most important physiological process for which these two receptors are required (Contento et al., 2008). However, hCCR5 and hCXCR4 are protagonists of a “paradoxical finding”, since it has been demonstrated that CCR5 ligands protect neurons from HIV/gp120 and CXCL12 toxicity (Kaul et al., 2007), because hCCR5 ligands might cross-compete with hCXCR4 ligands and prevent their neurotoxic effects (Cocchi et al., 1995; Springael et al., 2005).

A link between hCXCR4 and hCCR7 has been shown in metastatic breast cancer, where both CXCL12 and CCL19, cooperates in a synergistic way to activate signalling responses in aggressive types of breast cancer (Poltavets et al., 2021). Furthermore, hCXCR4-hCCR7 complexes were found in advanced primary mammary tumours where number of hCXCR4-hCCR7 heretodimers directly correlate with the severity of the disease, leading to a more invasive and aggressive phenotype for this type of cancer (Poltavets et al., 2021).

hCXCR4 possesses a high tendency to form heterodimers also with hCXCR3. Both receptors are over-expressed in several types of cancer, and this made them interesting targets for new therapeutic agents. As for hCCR2 and hCCR5, examples of negative binding cooperativity have been showed to occur between hCXCR3–hCXCR4 complexes, since CXCL10 and CXCL12 binding to the complex is mutually exclusive, since the binding of an agonist causes conformational changes which affect the binding of the other agonist to the other member of hCXCR3–hCXCR4 heteromers (Watts et al., 2013).

The variety by which GPCRs can oligomerize still require further analysis due to the huge variability of mechanisms by which this phenomenon happens. About hCXCR4 specifically, a new study published by Paradis et al. (2022) hypothesised the existence of an open- and a closed-dimer conformation of hCXCR4, observed also for other different GPCRs. This difference in receptor's conformations may lead to a preference towards a specific signalling pathway or to another. In the case hCXCR4, it has been shown that while the open-dimer conformation could form active state complexes with both $G_{\alpha i}$ and β-arrestin, the closed-dimer conformation was demonstrating a preference for $G_{\alpha i}$ but not for β-arrestin in forming active state complexes. Moreover, the closed-dimer conformation tends to be more stable in the active state, maybe due to major conformational changes to which GPCRs are subjected. This hypothesis might explain differences in signalling pathways preferences when GPCRs are oligomerized rather than in their monomeric forms, but also how ligands may influence the choice of a signalling pathway rather than another.

Therefore, in pharmacology and drug discovery it is important to take into account the oligomerization state of the target receptors studied, and this is why, although a lot of information has been acquired recently, more investigation is required to fully understand the mechanisms behind GPCRs oligomerization and how to use this aspect in generating new therapeutic agents.

1.7.2.4 CXCR4 phosphorylation

Phosphorylation of GPCRs represents a key chemical modification that regulates GPCRs signalling. hCXCR4 is rapidly phosphorylated upon ligand binding within its serine/threonine amino acidic residues located in its C-terminal tail. Many of these sites, phosphorylated by CXCL12 or PKC (protein kinase C) are important for regulating the internalization and the degradation of the receptor (Busillo et al., 2010; Orsini et al., 1999). In addition, GRK2, GRK3 and GRK6 have been shown to be important to regulate the phosphorylation of this receptor (Orsini et al., 1999; Marchese and Benovich, 2001; Fong et al., 2002) and they might represent an important component in defining hCXCR4 phosphorylation "barcoding".

A study by Busillo et al. (2010), showed the importance of seven serine residues phosphorylated in response to CXCL12 stimulation, but this phosphorylation occurs with distinct kinetics and kinase specificity. In more details, phosphorylation of Ser324 and Ser325 residues is rapid, transient, and mediated by PKC, GRK2, GRK3 and GRK6 (Zhuo et al., 2022), Ser330 phosphorylation has a slower kinetic and is mediated by GRK6, while Ser339 is phosphorylated rapidly by GRK6. Other sites of phosphorylation currently know in hCXCR4 C-tail are Ser338, Ser346 and Ser347, even if the characterization of the phosphorylation patterns of these residues is still not fully complete because of the high degree of phosphorylation present in the region where these residues are placed.

The phosphorylation of hCXCR4 dependent on GRKs has diverse effects on arrestin recruitment affecting other signalling pathways such as the Ca^{2+} mobilization. In this case, negative regulation is the result of the action of GRK2, GRK6, and arrestin3. Another signalling pathway that can be affected by GRK action is ERK1/2 activation, positively regulated by GRK3, GRK6, and arrestin2 (Busillo et al., 2009). Furthermore, a work from Zhuo et al. (2022) provided evidence that distal C-tail, but not proximal, phosphorylation of hCXCR4 specifies β-arrestin 1-dependent signalling and specify the GRK–β-arrestin-mediated signalling by hCXCR4, where a preference for GRK2, GRK3 and GRK6 was observed. In fact, specific phosphorylation sites localized in the distal C-tail are crucial players to recruit an adaptor protein called STAM1 (signal-transducing adaptor molecule 1) to β-arrestin 1 and to promote focal adhesion kinase-mediated phosphorylation but not the phosphorylation mediated by

extracellular signal-regulated kinase 1/2. However, these sites, that have been found similarly in the sequence of other GPCRs, might not be sufficient alone as specific mechanism to recruit STAM1 to β-arrestin 1 for other receptors.

The intricate world of GPCR phosphorylation makes clear that the importance of this chemical modification in GPCR regulation, signalling and functionality is still not fully characterized and understood. Thus, it is important to consider the presence of phosphorylation on GPCRs sites to identify the specific GPCR barcode to investigate their signalling and pharmacology to develop new therapeutics and having a better understanding of diseases in which these receptors are involved.

1.7.3 CXCR7/ACKR3 and its pathophysiological role

Human Atypical Chemokine Receptor 3 (hACKR3), in the past also known as CXC-motif chemokine receptor 7 (CXCR7), is a chemokine receptor that has been discovered more recently than hCXCR4 and might have a possible role in the regulation of the hCXCR4/CXCL12 axis. In fact, because of the effects observed on hCXCL12, which acts as an agonist for hACKR3, it has been proposed to be a "scavenger receptor" for CXCL12, preventing the binding of this ligand to its cognate chemokine receptor hCXCR4 and bringing CXCL12 to degradation (Naumann et al., 2010; Balabanian et al., 2005).

The binding of CXCL12 to hACKR3 is favoured by the higher affinity that the monomeric form of CXCL12 possesses for hACKR3 compared to hCXCR4, for which CXCL12 is the endogenous ligand (Ray et al., 2012). The binding of this ligand involves a two-step mechanism in which the N-terminus of the chemokine initially forms interactions with the extracellular loops (EL2 and EL3) and transmembrane pocket of the receptor, followed by the receptor N-terminus wrapping around the core of the chemokine to prolong its residence time (Gustavsson et al., 2019; Gustavsson et al., 2017; Hanes et al., 2015; Costantini et al., 2013; Gustavsson et al., 2019). The activation of hACKR3 mediated by agonists has been shown to be unable to elicit a G protein-dependent signal, and this means that this receptor can only signal via *β*-arrestin recruitment, resulting in MAP kinases activation (Rajagopal et al., 2010).

Because of this reason, hACKR3 is often referred to as a "biased" receptor, and this feature that makes it "atypical" compared to canonical chemokine receptors

is probably due to an altered DRYLAIV motif commonly present in many chemokine receptors and localized in the region between TM3 and IL2 (Lokeshwar et al., 2020). This motif is instead replaced by a DRYLSIT sequence which might explain the unusual behaviour of this receptor. However, the replacement of a DRYLSIT motif with a DRYLAIV motif in a chimeric version of ACKR3 did not result in G protein activation upon CXCL12 stimulation, did not cause intracellular calcium mobilisation, did not promote ERK phosphorylation or chemotaxis (Hoffmann et al., 2012; Naumann et al., 2010). Even though several experimental approaches have been used to characterize the signalling pathways that hACKR3 can use, the possibility of G protein coupling for this receptor has not been completely ruled out. Indeed, in a precedent work from Levoye et al. (2009) ACKR3-eYFP and $G_{\alpha i2}$ generated a BRET signal, with a decrease observed upon treatment with $^{[35S]}$GTPγS, suggesting that hACKR3 could recruit $G_{\alpha i/0}$ proteins in its resting mode, but it probably fails to activate these G proteins (Levoye et al., 2009).

The peculiarity of this receptor made it an interesting topic to investigate to understand the role that this receptor could have in human body physiology. Until now, hACKR3 has always been considered as a decoy receptor for CXCL12, exerting a strong influence on CXCL12/hCXCR4-dependent guidance of migratory cells.

Differently from the other canonical chemokine receptors, hACKR3 activation does not drive cell chemotaxis, but its expression by non-migratory cells helps to shape chemokine gradients and prevents migration to undesired locations; moreover, hACKR3 can be also expressed by migratory cells, and in this case, it has the role of helping to sustain responsiveness of the hCXCR4 pathway (Sánchez-Alcañiz et al., 2011).

Another interesting feature of this receptor is that, even without CXCL12 binding, hACKR3 undergoes ligand-independent internalization, even though it is not fully understood yet how ligand-induced (upon CXCL12 stimulation) and ligand-independent internalization affects hACKR3 degradation (Naumann et al., 2010). However, the binding of CXCL12 to this receptor activates the recruitment of β-arrestin, which leads to the activation of other signalling pathways, such as Akt and ERK activation and phosphorylation, together with JAK2/STAT3 (Janus Kinase 2/Signal Transducer and Activator of Transcription 3) activation

(Decaillot et al., 2011; Hattermann et al., 2010; Odemis et al., 2012; Rajagopal et al., 2010).

In terms of cellular localization, hACKR3 tends to be localized more intracellularly when expressed in HEK cells (Naumann et al., 2010; Szpakowska et al., 2018), although it has been described how shortening or removing the carboxy-terminal tail of hACKR3-GFP resulted in an increased plasma membrane localisation of the receptor (Ray et al., 2012). The receptor can also undergo constitutive rapid recycling and moving back to the cell surface, and this process is fundamental for allowing a continued membrane localization of hACKR3, since it has been showed that the receptor can signal only when expressed on the membrane (Ray et al., 2012; Canals et al., 2012).

In the recent years research on hACKR3 has brought chemokine receptors experts to hypothesize and discover alternative roles for this receptor in human physiology, including contributions to cardiovascular and lymphatic vessel growth, embryonic development, and central nervous system function (Quinn et al., 2018). During embryonal development, the distribution of chemokine ligands in the right place and at the correct time represents a fundamental aspect in determining important processes such as lineage commitment, organogenesis, and chemotaxis. One of the first studies carried out *in vivo* to define the role of hACKR3 used zebrafish embryo as a model. In this study, generation of knockdown of hACKR3 by using specific morpholinos resulted in an impairment of the migration of primordial germ cells (PGCs), which caused defective gonad development (Boldajipour et al., 2008).

In another study from Sierro et al. (2007), ACKR3-KO (knockout) mice in which the receptor was not expressed were created and phenotypically characterised. This showed that over 95% of knockout mice died within 24 hours after birth, due to an abnormal heart valve development and an impaired expression of the angiogenic factor Hbegf (Heparin-Binding EGF-like growth factor) and of adrenomedullin in cardiac valves (Sierro et al., 2007). Another study showed that among hACKR3 knockout mice, only few animals survived to adulthood, but they showed as phenotype a compromised heart function, that ended up anyway to cause an earlier death as well for these survivors (Gerrits et al., 2000). Therefore, the evidence of hACKR3 expression in brain, kidney, and trophoblast cells of the placenta suggests the possibility that there may be other alternative functions that

are still not fully clarified for hACKR3 beside its role in regulating cardiac physiology (Gerrits et al., 2000; Quinn et al., 2018).

In addition to all these studies, other works proved other roles of hACKR3. For example, the inhibition of hACKR3 in human umbilical and aorta endothelial cells resulted in a significant decrease of the angiogenesis (formation of new blood vessels), and this led to consider hACKR3 as a key player for regulation of vascular functions (Hao et al., 2017), and in affecting physiological mechanisms which were connected to cardiac failure by regulating CXCL12 and adrenomedullin (ADM) signalling. In fact, Hao et al., (2017) showed how mice with endothelial deletion of *ackr3* gene and which were experimentally induced to develop myocardial infarction were characterized by elevated levels of CXCL12 and weakening of heart functions. On the other hand, it has been reported that genetic overexpression of ADM ligand in $Adm^{hi/hi}$ mice resulted in consistent cardiac enlargement caused by cardiac hyperplasia originated during embryogenesis which closely resembles the phenotype of the dysmorphic cardiac hyperplasia found in $ack3^{-/-}$ mice (Wetzel-Strong et al., 2013). To support the connection between ADM and hACKR3, a study from Klein et al. (2015) showed that in the presence of haploinsufficiency of ADM in $ackr3^{-/-}$ animals, cardiac and lymphatic hyperproliferation was effectively reversed, demonstrating that hACKR3 is required as a molecular rheostat for controlling ADM ligand availability during development. These findings helped in considering the possibility of important roles of CXCL12 and ADM ligands in regulating functionality and signalling of hACKR3 *in vivo* (Quinn et al., 2018).

To further corroborate the hypothesis of a link between ADM and hACKR3, some studies *in vitro* were carried out and it has been demonstrated that hACKR3-transfected HEK 293 cells were able to internalize biotinylated adrenomedullin, demonstrating adrenomedullin being a ligand for hACKR3 (Klein et al., 2014).

Studies on $ackr3^{-/-}$ mutants displayed the essential role of hACKR3 for positioning and regulating migration of cortical neurons. In fact, conditional genetic deletion of *ackr3* gene in interneurons can lead to insensitivity towards CXCL12 and an increase in concentrations of this ligand, which can then drive degradation of hCXCR4 in the cell (Sánchez-Alcañiz et al., 2011); moreover, since a co-expression of hACKR3 and hCXCR4 is observed in migrating medial ganglionic eminence progenitors, and migrating cells in hACKR3-null mice do not produce hCXCR4 protein (Sánchez-Alcañiz et al., 2011), it is reasonable to

consider that a relationship between hACKR3 and hCXCR4 may be crucial for proper neuronal development. Additionally, hACKR3 also modulates CXCL12 signalling in astrocytes and Schwann cells (Odemis et al., 2012), where the receptor can signal through pertussis toxin sensitive $G_{i/o}$ proteins by binding to CXCL12 and activating Akt and ERK signalling, suggesting the role of hACKR3 as potential therapeutic target for central nervous system-related diseases.

hACKR3 can be overexpressed in lung, brain, pancreatic, prostate, and renal cancer (Liu et al., 2013; Heinrich et al., 2012; Kallifatidis et al., 2016; Wang et al., 2012). At the level of arteries, an upregulation of hACKR3 receptor expression after injury has been reported, and after myocardial infarction, this can provoke angiogenesis induced by ischemia and proliferation of endothelial cells (Hao et al., 2017).

Furthermore, in atherosclerotic plaques resident macrophages express hACKR3 which stimulates their phagocytic activity through MAPK-mediated signalling (Chatterjee, Borst et al., 2014). Finally, it has also been reported that even HIV-1 and -2 gp120 proteins may use hACKR3 as a co-receptor, as it happens for hCXCR4 (Shimizu et al., 2000) and hCCR5, for viral envelope fusion with the plasma membrane, showing a role in viral infection for hACKR3 as well as described for hCXCR4 and hCCR5.

1.7.3.1 ACKR3 ligands

hACKR3 has been reported to be able to bind different types of ligands which differ not only for their physiological role but also for their chemical nature and structure. The most important and well characterized, as described previously, is CXCL12. This interaction is often overlapping and interconnected physiologically with CXCL12-hCXCR4 interactions. Therefore, nowadays it became more common considering hCXCR4-CXCL12-hACKR3 axis as an intricate complex, influenced by other players. These could be either different GPCRs able to heterodimerize with hACKR3 or hCXCR4 or both, or other ligands that can bind these receptors with different degrees of affinity.

Indeed, other ligands that have been reported to be able to bind and exert an effect on hACKR3 can be either chemokines or non-chemokine ligands. For example, the recognized and characterized ligands are CXCL11, the macrophage inhibitory factor (MIF), adrenomedullin, opioid peptides, and the viral chemokine

vCCL2/viral macrophage inflammatory protein-II (Wang et al., 2018; Meyrath et al., 2020; Perpiñá-Viciano et al., 2020; Koch and Engele, 2020), although not always the mechanisms by which these interactions occur are totally clear. For example, CXCL11, previously known as interferon-inducible T-cell alpha chemoattractant (I-TAC), is a member of ELR^- CXC chemokines, which is found in high levels after interferon-β and -γ stimulation. CXCL11 is the endogenous agonist for CXCR3, and its chemoattractant role is important for activation of memory T cells during the adaptive immune response. However, CXCL11-hACKR3 interactions have been characterised only pharmacologically and our knowledge about this interaction is still poor (Quinn et al., 2018; Burns et al., 2006).

The macrophage migration-inhibitory factor (MIF) is a pleiotropic cytokine exerting chemokine-like functions of mediator of inflammation process (Alampour-Rajabi et al., 2015). hACKR3 interacts with hCXCR4 and CD74 in binding MIF, and a blockade of hACKR3 inhibited MIF-mediated ERK- and zeta-chain-associated protein kinase (ZAP)-70 activation and the primary murine B-cell chemotaxis triggered by MIF, but not promoted by CXCL12, confirming the importance of ACKR3 in MIF-promoted B-cell migration (Alampour-Rajabi et al., 2015).

Viral chemokine v-MIP II/v-CCL2, together with hCXCR4, can also interact with hACKR3 (Szpakowska et al., 2016). V-CCL2 is related to Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman disease and while it acts as an antagonist at hCXCR4, it shows agonist activity towards hACKR3 (Szpakowska, and Chevigné, 2016). This ligand recruits β-arrestin 2 to hACKR3 and modifies surface levels of receptor in a concentration-dependent manner; thus, it has been thought that hACKR3 could maybe exert the role of scavenger for vCCL2 ligand by affecting the binding of ligands interacting with hACKR3 and modifying signalling activity of this receptor (Szpakowska et al., 2016; Quinn et al., 2018).

Another interesting point about the scavenging function of hACKR3 is that this receptor showed a scavenger behaviour towards a series of opioid peptides, especially enkephalins and dynorphins, reducing their availability for the classical opioid receptors (Meyrath, 2020). hACKR3 has a good level of selectivity to bind the proper opioid peptides, since several of them, such as endorphins, short endomorphins and leu- or met-enkephalin did not trigger β-arrestin recruitment to

this receptor when HEK cells expressing hACKR3 were stimulated with these ligands (Meyrath, 2020).

Other non-chemokine ligands have been reported as able to interact with and exert an effect on hACKR3, and these include adrenomedullin and BAM22 peptide. Adrenomedullin is a vasodilator peptide 52 residues long, which belongs to the family of calcitonin gene-related polypeptides (CGRP), which also includes ligands for calcitonin gene-related receptor, adrenomedullin receptor and amylin receptor. Its ring structure consists of six residues and a C-terminal amide structure (Kitamura et al. 1993) and this peptide is widely expressed throughout the blood vessels, heart, lungs, kidneys, and gastrointestinal tract and is highly concentrated in the adrenal medulla, where it was firstly found and from where it gets its name (Ashizuka et al., 2021). Adrenomedullin is considered the endogenous ligand for human adrenomedullin (hAMR) receptor, which is the result of the formation of a complex between human calcitonin receptor-like receptor (hCLRL) and a human receptor activity-modifying protein (hRAMP). There are three subtypes of hRAMP receptors, referred to as hRAMP1, hRAMP2, and hRAMP3. hCRLR/hRAMP1 complex has a high affinity for CGRP, while hCRLR/hRAMP2 and hCRLR/hRAMP3 preferentially bind adrenomedullin. The hCRLR/RAMP2 complex comprises the human adrenomedullin-1 (hAM1R) receptor, and the hCRLR/hRAMP3 complex constitutes the adrenomedullin-2 (hAMR2) receptor (Ashizuka et al., 2021; Russell et al., 2014).

In many works it has been shown the huge variety of physiological effects that adrenomedullin has. Indeed, it acts as a paracrine factor to stimulate heart development, vasodilation and angiogenesis, and this is why it can lead to development of pathological conditions such as hypertension, heart failure, and renal failure (Eto and Kitamura, 2001). Besides, anti-inflammatory effects of adrenomedullin have been observed, since it was able to inhibit the secretion of TNF-α and IL-6 after LPS treatment of immune cells such as monocytes and macrophages (Kubo et al, 1998). Other physiological effects of adrenomedullin include the suppression of gastric acid secretion via somatostatin in the stomach, enhancement of electrolyte secretion in the colon, suppression of gastrointestinal motility, and influence on microcirculation flux, as well as antibacterial effects in contributing to the functioning of the mucosal defence system by regulating the oral and intestinal microbiome (Ashizuka et al., 2021; Marutsuka et al., 2003; Martinez-Herrero and Martinez, 2016).

Because of the variability of the physiological processes in which this peptide is involved, it is not so easy to characterize and, in fact, only recently it has been related to hACKR3. It is generated through the proteolysis of its precursor pro-adrenomedullin (proADM), that represents a key step in the generation of the pro-adrenomedullin N-terminal 20 peptide (PAMP), very similar structurally to adrenomedullin but characterized by the inability to activate ADM receptors (Meyrath et al., 2021). Additionally, it was demonstrated that PAMP has a shorter duration of antihypertensive activity when compared to adrenomedullin and it can cooperatively regulate blood circulation in combination with adrenomedullin ligand (Tsuruda et al., 2019). However, even though ADM and hACKR3 expression has been linked genetically and physiologically, mechanism used by hACKR3 to regulate ADM signalling, as well as the pharmacological properties of ADM toward hACKR3 have not been comprehensively understood (Sierro et al., 2007; Klein et al., 2014). Meyrath et al. (2021) demonstrated that ADM was the only ligand among the members of CGRP family to show moderate activity towards hACKR3, while PAMP and PAMP12 displayed a stronger potency towards hACKR3 than ADM, extending the variety of agonists acting and regulating hACKR3 functioning.

Lastly, BAM22, peptide I and peptide E can activate β-arrestin recruitment through hACKR3 and modulate the circadian glucocorticoid oscillation, linking hACKR3 to the regulation of human emotional behaviour (Ikeda et al., 2013). Several efforts in designing synthetic antagonist ligands against failed, since molecules functioned instead as receptor agonists (Wijtmans et al., 2012).

1.7.3.2 ACKR3 binding pocket

Differently from what reported earlier for hCXCR4, structural data about hACKR3 are more recent, with a work from Yen et al., (2022), where a first cryo-EM model for hACKR3-CXCL12 interaction was proposed (Yen et al., 2022). The hardship of obtaining this model were due to hACKR3 inherent flexibility and low stability, but also because of the limited surface area required for forming crystal contacts and additionally even the need for slow off-rate ligands to allow the crystallization process (Terrillon and Bouvier, 2004). The most characterized interaction of hACKR3 with a ligand is its binding with CXCL12. The proposed model is strongly based on the mechanism described for most receptor-chemokine

complexes, with a CRS1 that works to make the N-terminus of the receptor to interact with the N-loop of the chemokine and a CRS2 where the N-terminus of chemokine interacts with ECL2 and the TM domain pocket of the receptor (Gustavsson et al., 2017).

By contrast, a study proposed by Szpakowska et al. (2018) suggested a different mechanism of ligand recognition and activation of hACKR3, which could depart from the two-step model. Indeed, in this work it has been demonstrated by using binding assays that truncation applied in the N-loop dramatically influenced the interactions of CXCL12 and peptides which were structurally derived from this chemokine to hCXCR4, whereas it partially reduced peptide ability to bind and activate hACKR3. The truncation of the N-terminus is not the only difference it is possible to observe with hCXCR4. For example, specific N-loop contacts are not required for hACKR3 binding or activation by CXCL11 and CXCL12, that is instead an important aspect of binding process of these chemokines to hCXCR3 and hCXCR4 respectively, showing a high degree of insensitivity of hACKR3 to N-loop composition of chemokine ligands and in general to their proximal residues (Szpakowska et al., 2018).

Even if these data showed a high degree of flexibility in hACKR3 activation and binding to CXCL11 and CXCL12, compared to other chemokine receptors and considering the importance of the N-loop in the general mechanism of binding for chemokine receptors, hACKR3 N-terminus possesses a critical role that is not only dependent on the specific “CRS1” interactions, but more interestingly on the total sum of interactions that the chemokine makes with other regions of the receptor (Gustavsson et al., 2019). So, truncations and mutations of the receptor or chemokine N-terminus may affect the potency and efficacy of activation only if the truncated or mutated region contributes a large enough fraction of the total binding energy. In the case of CXCL11 and CXCL12, the latter showed less affection in its binding to hACKR3 in case of mutations and truncations, due to a slower off-rate, thus dissociation rate, of CXCL12 to hACKR3, especially in comparison with hCXCR4 (Gustavsson et al., 2019).

Indeed, Crump et al. (1997) showed that even single-site mutations of CXCL12 (such as $CXCL12_{P2G}$) convert this chemokine into an antagonist for hCXCR4 for which normally this ligand would be an agonist, but also orthosteric pocket mutations such as $Y116^{3.32}A$ and $D187^{ECL2}A$ or conservative substitutions like $D97^{2.63}N$ and $E288^{7.39}Q$ effectively abolish arrestin recruitment to hCXCR4. On

the other hand, this sensitivity in ligand binding and generation of effects promoted as result of this binding was not observed in hACKR3 in presence of single mutations in key residues (Yen et al., 2022). In fact, some mutations (hACKR3-Q301$^{7.39}$ and hACRK3-Y268$^{6.51}$) results in increased constitutive activity of the receptor with increased arrestin recruitment, suggesting that the distortion of the binding pocket by steric bulk may be sufficient for hACKR3 activation (Yen et al., 2022).

Moreover, the structural diversity of some side chains of the receptor, a segment of TM5 at the base of the orthosteric pocket and the absence of disulphide bonds between the receptor N terminus and ECL3 could facilitate binding of diverse ligands and allow for distortion-driven activation and helping the deformability of the orthosteric pocket of hACKR3 upon ligand binding (Yen et al., 2022).

Another important point is represented by the biased behaviour of this receptor, which cannot signal via G proteins but only via arrestin recruitment. In their study, Yen et al. also concluded that ICL region properties may play a key role in dictating bias behaviour of hACKR3, in particular the lack of a kink at the cytoplasmic end of TM4 in ICL2, and this element directly interacts with heterotrimeric G proteins in many of the active class A receptor structures (Ahn et al. 2021). This means that in the future, further studies are required to understand the binding of ligands to hACKR3, especially other chemokines such as CXCL11, to facilitate our understanding on the mechanism of binding and to determine whether the CXCL12-hACKR3 interaction may be unique or shared among other similar ligands.

1.7.3.3 ACKR3 oligomerization

As the most of GPCRs and as described before for hCXCR4, also hACKR3 may be found in a co-existence of monomers and dimers/oligomers, with a certain percentage of constitutively preformed oligomers (Albee et al., 2017; Salazar et al., 2014). Even though little information is known about hACKR3 homodimerization, it has been reported the ability of hACKR3 to heterodimerize with hCXCR4 and this is mostly due to its role of regulation of hCXCR4 functions (Levoye et al., 2009; Luker et al., 2009). Both works from Levoye et al. and Luker et al., provided evidence that in presence of both receptors transiently co-transfected into HEK 293T cells, hACKR3 tends to form homodimers with the

same tendency and kinetics in forming heterodimers with hCXCR4. As immediate effect of the heterodimerization with hCXCR4, hACKR3 causes an attenuation of hCXCR4-induced increases in intracellular Ca^{2+}, even if this mechanism is still not wholly understood (Levoye et al., 2009).

Another regulatory effect of hACKR3 on hCXCR4 is detectable in cancer cells, where both receptors normally would be overexpressed, but silencing of gene codifying for hACKR3 expression led to an increase of CXCL12 with a more rapid and slightly higher Ca^{2+} response when compared with wild-type cells (Qiao et al., 2016). Other effects where hACKR3 has been related to hCXCR4 functioning regulation include cell migration and cell proliferation of immune cells, especially human monocytes, and human hematopoietic stem cells (Chatterjee et al., 2015; Hartmann et al., 2008).

All these findings enforce the idea of hACKR3 acting as a scavenger receptor for CXCL12, with the important function of regulating the binding of this chemokine to hCXCR4 to mediate the effects originated from this interaction.

Moreover, hACKR3 can interact and form heterodimers also with other GPCRs, and especially with non-chemokine receptors. For example, hACKR3 can interact with the human α1 adrenergic receptor (α1-hAR), in a complex composed by α1-hAR:hACKR3:hCXCR4 hetero-oligomers, that have been found in vascular smooth cells, and the activation of hACKR3 can inhibit the α1-hAR activity (Albee et al., 2017).

hACKR3 can work in combination with another non-chemokine receptor, the human epidermal growth factor receptor (hEGFR) in a β-arrestin-2–dependent fashion and hACKR3 also mediates hEGFR phosphorylation. Together, they are involved in mitosis of breast cancer cells fostering cancer progression (Salazar et al., 2014; Neves et al. 2022).

Considering the role of hACKR3 in modulating the activity of different receptors and how poorly known is still the dynamic by which hACKR3 interacts with other GPCRs and also exert different functions by itself, it is clear the importance of the characterization of this receptor in terms of signalling and structure for helping a drug discovery approach.

1.7.3.4 ACKR3 phosphorylation

As for other GPCRs and as seen also for hCXCR4, several kinases can phosphorylate different residues present in the C-tail of hACKR3, regulating the phosphorylation pattern and the behaviour of this receptor in terms of β-arrestin recruitment, internalization, trafficking and recycling. However, the mechanism by which this regulation is carried out is still not fully understood.

The most common kinases that phosphorylate hACKR3 are members of GRK family and, in particular, GRK2 and GRK3 have been reported to be able to phosphorylate hACKR3 at its C-terminus, at the level of a cluster of phosphorylation formed by the amino acidic residues Ser^{350}, Thr^{352} and Ser^{355}, with a crucial role especially for the threonine residue in this process (Zarca et al., 2021). From the same study of Zarca et al. (2021), it has been demonstrated that the phosphorylation of these sites is CXCL12-dependent, and it affects even the internalization rate of the receptor, that decreases in presence of mutated residues in this region. Although these changes were observed, the internalization still occurred, suggesting a non-total dependence of the internalization process from β-arrestin in the case of hACKR3.

GRK5 has been reported to be involved in phosphorylation of the receptor. This in interesting because this GRK is generally bound to the membrane and its strong interaction with hACKR3 may be due to a co-internalization of both receptor and GRK5 in early endosomes, with a regulatory function that requires the presence of hACKR3 to the membrane.

The full mechanism of phosphorylation of hACKR3 still requires further investigation, starting from the C-tail region of the receptor, to have a better understanding of how this process works in case of hACKR3 and how it is interconnected with hACKR3 functions, to determine even for hACKR3 its "barcode" to highlight the signalling mechanism by which this receptor works.

1.8 Aim of the project

Because of their important involvement in several physiological role and their participation in development of several pathological conditions, both hCXCR4 and hACKR3 are considered interesting therapeutic targets for different types of human cancers, for tackling HIV-1 infection, to treat (auto)immune diseases such as multiple sclerosis or systemic lupus erythematosus and many other diseases

that could be less common in the world population, but not less relevant. However, many aspects about the mechanisms by which these receptors signal and start signalling cascades leading to related biological effects is still to characterize and investigate. Moreover, also the outcome of the combined action of both hCXCR4 and hACKR3 needs to be highlighted because of the tight connection that these two receptors show.

Additionally, in recent years many ligands have been classified as agonists for both receptors, although they were not belonging to the group of chemokine ligands, such as adrenomedullin. This peptide has been studied because of the variety of functions in which it may be involved, such as the regulation of the angiogenesis. This physiological process, indeed, in case of pathological situations such as cancer, it may favour the progression the disease towards a more aggressive phenotype. Moreover, this small peptide has been recently correlated with hACKR3, for which this ligand could act as an agonist or the receptor might act scavenging this ligand with regulatory function similar to what hypothesized for CXCL12. This hypothesis opens to the possibility to include this peptide in the intricated network represented by hCXCR4-CXCL12-hACKR3.

Therefore, the first aim of this project aimed to characterize the role of the possible interactions between adrenomedullin and its putative receptor hACKR3, to determine whether this ligand could play a role in shaping the signalling of hACKR3.

Because of the hypothesized interaction between hACKR3 and hCXCR4, especially in pathophysiological conditions, the second aim of the project was to understand the role of the possible interactions between adrenomedullin and hCXCR4. This should clarify whether this small peptide might influence the hCXCR4-hACKR3-CXCL12 axis by regulating the CXCL12-mediated signalling on hCXCR4.

Since a recent trend topic in pharmacology consists of the “barcode theory”, the third and last aim of this project is the characterization of the phosphorylation of both hCXCR4 and hACKR3 by investigating the role of GRK enzymes in this process. By using both CXCL12 and adrenomedullin, in combination with small GRK inhibitors, it was possible to highlight whether and how these kinases are able to determine a “barcode” for these receptors, with the phosphorylation of different sites. This could further strengthen the current knowledge on how the

phosphorylation might affect the signalling and functionality of these receptors, helping the development of new therapeutic strategies against different diseases.

CHAPTER 2 – Materials and Methods

2.1 Pharmacological and biochemical reagents

- CXCL12: purchased from Peprotech and dissolved in water at the desired concentration

- Adrenomedullin: purchased from Alfa Aesar and dissolved in 1% acetic acid at the desired concentration or, alternatively, from Genscript and dissolved in water at the desired concentration

- IT1t: purchased from Tocris Biosciences and dissolved in water at the desired concentration

- VUF16840: kindly provided by Prof. Rob Leurs, Vrije University of Amsterdam

- Barbadin: purchased from Sigma Aldrich and dissolved in DMSO (2mg/ml)

- Compound 101: purchased from Tocris Biosciences and dissolved in water

- Compound 19: kind gift of Dr. David E. Uehling, Ontario Institute for Cancer Research, Canada, and Dr. Rodger E. Tiedemann, Princess Margaret Cancer Centre, Canada

- Primary antibodies: anti-CXCR4 no phospho antiserum, anti-CXCR4 $pSer^{324}/pSer^{325}$ antiserum, anti-CXCR4 $pSer^{330}$ were purchased from 7TM antibodies, anti-CXCR4 $pSer^{338}/pSer^{339}$ antiserum was purchased from ThermoFisher and anti-FLAG antibody was purchased from Proteintech

- Secondary antibody: IRDye 800CW Donkey anti-Rabbit IgG, purchased from LICOR

- Pertussis toxin from Bordetella pertussis: purchased from Invitrogen

- Λ protein phosphatase enzyme kit: purchased from New England Biolabs

2.2 Buffers and solutions

- Solution 1, used to prepare competent bacteria cells (for a total volume of 100 ml buffer solution) was prepared by adding: 1 M potassium acetate (3.00 ml), 1 M $RbCl_2$ 1.00 ml, 1 M $CaCl_2$ (1.00 ml), 1 M $MnCl_2$ (5.00 ml) 80% (w/v) glycerol (18.75 ml), and at the end pH was adjusted to 5.0 with acetic acid

- Solution 2, used in the preparation of competent bacteria cells (total volume of buffer solution prepared: 40 ml), was prepared by adding: 100 mM MOPS pH 6.5 (4.00 ml), 1 M $CaCl_2$ (3.00 ml), 1 M $RbCl_2$ (0.40 ml), 80% (w/v) glycerol (7.50 ml)

- Hank's balanced salt solution (HBSS): without Ca^{2+} and Mg^{2+}, purchased from ThermoFisher Scientific

- DMEM: purchased from Gibco, it contains 1x Dulbecco's modified Eagle's medium with high glucose, L-glutamine and phenol red, without sodium pyruvate and without HEPES

- DNA loading buffer (6x): purchased from BioLabs

- Laemmli buffer for SDS-PAGE experiments either 5x or 2x according to different experimental conditions used: 60 mM Tris, 80 mM sodium dodecyl sulphate (SDS), 50 mM dithiothreitol, 10% (v/v) glycerol, 0.25% (w/v) bromophenol blue

- Luria-Bertani (LB) agar: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, pH 7 (Marsango et al., 2015)

- Luria-Bertani broth: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7 (Marsango et al., 2015)

- Ampicillin: powder was dissolved into distilled water to obtain a final concentration of 100 mg/mL (1000x) and it was therefore filtered to increment the sterilization level. Working concentration used was 100 µg/mL.

- Kanamycin: powder was dissolved into distilled water to obtain a final concentration of 50 mg/mL (1000x) and it was then filtered in a similar way as seen for ampicillin. Working concentration was 50 µg/mL.

- Doxycycline 10000x stock: doxycycline powder was dissolved into distilled water (1 mg/mL), and it was filter-sterilised. Working concentration was 100 ng/mL

- Lysis buffer 7TM: 150 mM NaCl, 50 mM Tris-HCl, 5mM EDTA, 1% Igepal (Nonidet P-40), 0.5% Sodium deoxycholate, 0.1% SDS according to 7TM protocol (https://uk.7tmantibodies.com/)

- Lysis Buffer DDM: 150 mM NaCl, 2 mM EDTA, 0.01 mM Na_2HPO_4, 0.5% (w/v) DDM (n-dodecyl β-D-maltoside), 5% glycerol (Ward et al., 2021)

- Running buffer western SDS-PAGE: NuPAGE MOPS 1x (Invitrogen)

- Transfer buffer: 200 mM glycine, 25 mM Tris, 20% (v/v) methanol (Ward et al., 2021)

- 1x Native PAGE buffer: diluted from 20x stock and purchased from ThermoFisher

- Dark Blue Native PAGE buffer: 10 ml 20x Native PAGE buffer, 10 ml Native PAGE Cathode buffer purchased from ThermoFisher and 180 ml distilled water

- Light Blue Native PAGE Buffer: 10 ml 20x Native PAGE buffer, 1 ml Native PAGE Cathode buffer purchased from ThermoFisher and 189 ml distilled water

- G250: purchased from ThermoFisher

- Tris-acetate-EDTA (TAE) buffer: 40 mM Tris, 1 mM EDTA (pH 8), 20 mM acetic acid (Viskaitis et al., 2017)

- Tris-buffered saline (TBS): 20 mM Tris, 150 mM NaCl, 3 mM EDTA, pH adjusted to 7.4 (Barki et al., 203)

- TBST: TBS with 0.1% Tween 20

- Tris-EDTA (TE) buffer: 10 mM Tris, 0.1 mM EDTA, pH 7.4 (Mahmud et al., 2017)

- Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4 (Divorty, 2017)

- GTPγS binding buffer used for $^{[35S]}$GTPγS incorporation assay: 20 mM Hepes, 5 mM $MgCl_2$, 160 mM NaCl, 0.05% BSA (Sergeev et al., 2017)

- [^{35}S]GTPγS solution: purchased from Perkin Elmer and diluted in 10 mM tricine, 10 mM DTT (dithiothreitol), pH 7.6

- Blocking buffer: TBST with 5% BSA

- Primary antibody solution: TBST with 5% BSA, 1:2000 antibody

- Secondary antibody solution: TBST with 5% BSA, 1:10000 antibody

- HMW Native Marker Kit: purchased from Cytiva

- Chameleon® Duo Pre-stained Protein Ladder: purchased from LICOR

- Poly-Ethylene-Imide (PEI): purchased as powder from Polysciences Inc.

- Coelenterazine H: purchased from Nanolight technology and dissolved adding 0.1 M HCl in methanol

2.3 Molecular biology and cloning

2.3.1 Preparation of competent bacteria

XL1-Blue competent bacteria were striped on LB agar plates, and they were incubated overnight at 37°C. An individual colony was picked up and inoculated into 10 mL LB broth and incubated overnight at 37°C to allow bacterial growth in this solution. On the following day, this solution was therefore sub-cultured into 100 mL LB broth and grown at 37°C for a few hours (time variable according to the colony picked, although few hours were usually enough) until they reached an OD value (where OD means optical density) at 550 nm of 0.48. At this point, the culture obtained was left to chill on ice for 5 minutes and then the solution was divided into two 50 mL aliquots, which were centrifuged at 135 rcf for 10 mins at 4°C. Each pellet obtained was therefore resuspended in 20 mL solution 1 prepared as described earlier, then chilled on ice for 5 minutes and subjected to a second step of centrifugation in the same conditions as before. The new pellets were resuspended each in 2 mL solution 2 prepared as written earlier and kept on ice for 15 minutes. The resulting XL1-Blue competent bacteria were aliquoted and quickly stored at -80°C (Divorty, 2017; Capoferri, 2020).

2.3.2 Chemical transformation

This method was used to transform XL1-Blue competent bacteria with DNA plasmid encoding the construct of interest. 1 μg in total (or as much as closer to this when less amount of stock was left) of plasmid containing the construct of interest was added to 50 μL competent bacteria and left on ice for around 20 minutes. A heat shock method was used for these cells to allow the transformation, heating up the samples for 2 minutes at 42°C, and then bacteria cells were incubated on ice for 2 minutes. After that, LB broth without antibiotics was added to bring up the volume up to 1 mL and cells were incubated and let them to shake for 45-60 minutes at 37°C. After this step, 100 μL bacteria cells solution were plated on LB agar plates containing the specific antibiotic required for the selection (either ampicillin or kanamycin in this case). Plates were then incubated overnight at 37°C.

2.3.3 Miniprep purification

DNA plasmids were purified from bacteria colonies collected from plates and incubated overnight. For the purification, the QIAprep® Spin Miniprep Kit from Qiagen was used. Following the instructions provided in the protocol, 5-10 ml bacteria previously cultured overnight were pelleted by centrifugating them at 1503 rcf for 10 mins at room temperature. After removing the supernatant, pellets were resuspended in resuspension buffer (Buffer P1) containing RNAse A, and transferred to microcentrifuge tubes, where the same amount of lysis buffer (Buffer P2) was added. As soon as the colour of the samples changed to blue, after they have been mixed, a neutralization buffer (Buffer N3) was added to the samples which became white.

The next step was a centrifugation for 10 minutes at 13000 rpm and the supernatant from this step was applied to the QIAprep spin columns to be centrifuged for 60 seconds. After discarding the flow-through, columns were washed with a first wash buffer (Buffer PB) and centrifuged again to discard the flow-through. After this step, columns were washed with a second wash buffer to remove salts (Buffer PE) and the flow-through obtained by centrifugation was discarded. After transferring the columns to the collection tubes, a centrifugation for 1 min was done to remove residual wash buffer and ethanol residues and then columns were placed in clean microcentrifuge tubes. To elute DNA, water was added for 1 minute before a quick centrifugation 1 minute long.

2.3.4 Determination of DNA concentration

To determine the plasmid DNA concentration, absorbance values at 260 nm were measured. Therefore, a 1:100 dilution of the stock or sample to measure was prepared in suitable microcuvettes, and the concentration was measured by using a spectrophotometer. An important parameter that was checked was the DNA purity, which was assessed checking whether the value expressing the A_{260}/A_{280} ratio was in a range between 1.7 and 2.0, in order to be considered pure (Capoferri, 2020).

2.3.5 Polymerase chain reaction (PCR) (Divorty, 2017)

A classical PCR technique was used when there was a need of introducing convenient restriction sites and to amplify determined DNA sequences. All reactions were carried out in sterile PCR tubes containing 50 µL of mix, composed as described to follow:

- 5 µL of 10x Pfu DNA Polymerase Buffer with 20mM $MgSO_4$ (Promega)
- 1.25 µL of 10 mM deoxyribonucleotides (dNTPs) (Promega)
- 1.25 µL each forward and reverse primers, 25 pmol/µL
- 100 ng template DNA
- 1 µL Pfu DNA Polymerase (final concentration: 1.25 units/50 µL) (Promega)

Reaction mixtures were subjected to thermal cycling using the following conditions:

1. Preheating: 95°C, 2 mins
2. Denaturing: 95°C, 1 min
3. Annealing: 55°C, 1 min
4. Extension: 72°C, 3 mins
5. Repeat steps 2–4 (x 29)
6. Final extension: 72°C, 10 mins
7. Hold 4°C, ∞

* The annealing temperature may vary according to the melting temperature of the primers used for PCR reactions. However, this is the general protocol used for this method.

2.3.6 PCR Purification

Products obtained from PCR reactions were purified with QIAquick® PCR Purification Kit (QIAGEN), by diluting the samples in five volumes of binding buffer (Buffer PB). Diluted samples were put on QIAquick spin column and centrifuged at very high speed (18407 rcf) for 1 min. After removing the flow-through, columns were then washed by using Buffer PE, and they were again centrifuged at the same conditions as described before. After the removal of new flow-through obtained, an additional centrifugation step was used to remove residual traces of ethanol in the columns. The last step consists of eluting DNA in 35 µL sterile water, which was added for a minute at room temperature before running a new centrifugation round.

2.3.7 Restriction endonuclease digestion

Restriction endonuclease enzymes were used to carry out a digestion when needed. This step was helpful for the generation of sticky-end DNA fragments, which would have made the following ligation reaction easier, allowing a more successful integration of the sequence of interest. The two most used vectors were pcDNA3 or pcDNA5/FRT/TO/. Insert DNA sequences were digested overnight at 37°C by adding them to a 50 µL mix which was formed by the following ingredients:

- 2 µL 10x CutSmart® Buffer (New England Biolabs)
- Up to 50 µg vector DNA or the full amount of PCR product
- 1 µL each of both restriction endonucleases enzymes (usually purchased from New England Biolabs)

2.3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to assess the right size of the construct generated and/or to separate the DNA insert and vector fragments. 1% agarose and 1x SYBR® Safe DNA stain (Life Technologies) was dissolved in TAE buffer and heated, before allowing the solution to chill at room temperature to let it become jelly. Before starting the reaction, solidified

gels were soaked in a variable volume of TAE buffer to cover the gels in the apparatus. 5x DNA loading buffer was added to each sample before loading them on the gel. Then, electrophoresis started by applying a voltage of 125 V for 20–30 min. As a control to compare size and concentration of samples ran, an additional lane alongside the samples was prepared containing 10 µL Hyperladder^{TM} 1kb (Bioline).

2.3.9 Gel extraction

The following step after a gel electrophoresis was to extract the DNA insert and vector and this was achieved with QIAquick Gel Extraction Kit from QIAGEN. By using a UV light to visualize them correctly, bands were cut out from the gel. Three gel volumes of solubilisation buffer (Buffer QG) were added to one volume of gel and samples were incubated at the temperature of 50°C for 10 minutes to dissolve the pieces of gel collected.

One gel volume of isopropanol was added, and the mix was transferred on QIAquick spin columns and centrifuged at high speed (18407 rcf) for 1 minute. After discarding the flow-through, columns containing the samples were washed and, after removing the new flow-through, residual traces of ethanol were removed by a further step of centrifugation. DNA was eluted by adding sterile water to the columns (50 µL) placed on new microcentrifuge tubes and centrifuging them an additional time.

2.3.10 DNA ligation

To reconnect the sticky ends of plasmid DNA to make this circular again, a ligation reaction was set up by preparing a mix with insert and vector fragments in a ration 3:1 in a final volume of 20 µL mix containing:

- 1 µL 10x T4 DNA Ligase Reaction Buffer (New England Biolabs)
- 100 ng vector DNA
- 300 ng insert DNA
- 1 µL (400 units/µL) T4 DNA Ligase (New England Biolabs)

Incubation of these samples occurred overnight on ice at 4 ̊C to allow a gradient of temperature and the following day, 2 μL of ligated products were transformed into competent cells (for details, see paragraph 2.3.2).

2.3.11 Site-directed mutagenesis

Specific point mutations were put in place into DNA sequences of interest when needed for specific purposes by using primers containing the sequence encoding for an alanine residue in place of the amino acid to substitute. In this way, the side chain of the newly added alanine residue is not going to sterically affect the structure of the receptor because it is small and neutral For the amplification of this construct, 50 μL reactions were prepared in sterile PCR tubes as it follows:

- 5 μL 10x Pfu Turbo Reaction Buffer (Agilent Technologies)
- 1.25 μL of 10 mM dNTPs (Promega)
- 1.25 μL each forward and reverse primers, 25 pmol/μL
- 50 ng template DNA
- 1 μL Pfu Turbo DNA Polymerase (Agilent Technologies)

Thermal cycling used for these samples was set up with the following conditions:

1. Preheating: 95°C, 30 secs
2. Denaturing: 95°C, 30 secs
3. Annealing: 55°C, 1 min
4. Extension: 72°C, 10 mins
5. Repeat steps 2–4 (x 16)
6. Hold 4°C ∞

Dpn I restriction enzyme (Promega), was added to mutagenesis product and samples were incubated for at least 2 hours at 37 °C to allow the digestion of

template DNA, characterized by a high methylation, which makes it more sensitive to Dpn I-mediated digestion. Hence, 25 µL digested mutagenesis product were transformed into competent bacteria cells as seen before.

2.3.12 DNA sequencing

To assess the quality of the construct and to determine if changes introduced were correct, cloning and mutagenesis products were sequenced by using DNA Sequencing & Services (MRC I PPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk). DNA sequences were assessed using SNAPgene software, Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/), NCBI Basic Local Alignment Search Tool (BLAST) (www.blast.ncbi.nlm.nih.gov) and EMBOSS needle online software (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The primers used were the standards CMV forward and BGH reverse for both receptors, CXCR4 for sequencing (5'-GACCACAGTCATCC-3') and CXCR7-846-forward (5'-GGCTGATCGGCATGG-3').

2.3.13 Generation of FLAG-hCXCR4-mEGFP and FLAG-hACKR3-mEGFP and derived constructs

Human CXCR4 and ACKR3 constructs with an N-terminal FLAG epitope tag (amino acid sequence DYKDDDDK) and a C-terminal monomeric enhanced green fluorescent protein (mEGFP) tags (FLAG-hCXCR4-mEGFP and FLAG-hACKR3-mEGFP) in pcDNA5/FRT/TO were generated. FLAG-hCXCR4-mEGFP was a kind gift from Richard J. Ward (Ward et al., 2021) and it was used as template for the generation of FLAG-hACKR3-mEGFP construct.

As already described in Ward et al. (2021), human CXCR4 was fused in frame at its C-terminal position to monomeric (m, $Ala^{206}Lys$) EGFP by subcloning after PCR amplification (using primers designed to add Xho1 and EcoR1 sites) into the Xho1 and EcoR1 sites of pEGFP-N1, modified to

include the monomeric $Ala^{206}Lys$ mutation. Therefore, hCXCR4mEGFP construct was generated as result of these steps. For human ACKR3, the construct previously described for hCXCR4-mEGFP was used as starting material to build up the FLAG-hACKR3-mEGFP construct. FLAG-hACKR3-mEGFP was digested with XhoI and AgeI restriction enzymes to sub-clone FLAG-hACKR3 into mEGFP-pcDNA3. These restriction sites were added by modifying the DNA sequence of the FLAG-hACKR3-mEGFP construct to obtain the restriction sites required by XhoI and AgeI.

After this step, I digested FLAG-hACKR3- mEGFP-pcDNA3 with NheI and EcoRV to generate blunt ends and pcDNA5/FRT/TO was digested with NotI because of the same reason. As following step, the digestion product of hACKR3 was finally digested with Not I, ligated and sub-cloned into pcDNA5/FRT/TO, to generate the final constructs.

To follow, a full list of all the primers used for site-directed mutagenesis, cloning and/or DNA sequencing reactions is reported for both hCXCR4 and hACKR3 in **Table 2.1**.

Name	**Sequence**
CXCR4 for sequencing	5'-GACCACAGTCATCC-3'
CXCR7-846-forward	5'-GGCTGATCGGCATGG-3'
CXCR7-XhoI-up	5'-GAGTACTCGCTCGAGATGGATTACAAGGATGACGAC -3'
CXCR7-AgeI-dw	5'-GGCCTAGTCACCGGT.CGTTTGGTGCTCTGGCTCTGCTCCAA – 3'
CXCR4 forward stop	5'- GTTTTCACTCCAGCTGAATTCTGCAGTCG – 3'
CXCR4 reverse stop	5'- CGACTGCAGAATTCAGCTGGAGTGAAAAC – 3'
CXCR7 forward stop	5'-CAGAGCACCAAATGAACCAAATGAACCTAGGTGAGC – 3'
CXCR7 reverse stop	5'- GCTCACCTAGGTTCATTTGGTGCTCTG – 3'

Table 2.1. List of primers used to generate the different constructs.

2.4 Mammalian cell culture

2.4.1 HEK293(T) cells

Human embryonic kidney 293 cells transformed with large T-antigen (HEK293T cells) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, from ThermoFisher), 2 mM L-glutamine (from ThermoFisher), 100 units/mL penicillin (from ThermoFisher) and 100 µg/mL streptomycin (from ThermoFisher) at 37°C and 5% CO_2 in a humidified atmosphere (Divorty, 2017). The same conditions were used to maintain HEK293 parental cells and HEK293 GRK-knockout cells, gently provided by Prof. Carsten Hoffmann, University of Jena.

2.4.2 Flp-In T-REx-293 cells

Flp-In T-REx HEK293 cells (Life Technologies) were maintained in Dulbecco's Modified Eagle's Medium High Glucose without sodium pyruvate supplemented with 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin, 100 µg/mL streptomycin and 10 µg/mL blasticidin S (InvivoGen) (at 37°C and 5% CO_2) in a humidified atmosphere (Divorty et al., 2022).

2.4.3 Cryopreservation

Upon generation of a new cell line, cells deriving from this clone were cryopreserved for long-term storage in liquid nitrogen. Cells were detached with trypsin-EDTA when they reached 90% confluence and centrifuged at low speed (135 rcf for 3 min) to allow the formation of pellet and the removal of supernatant containing traces of trypsin. FBS +10% (v/v) DMSO was used to resuspend pellets and 1.5 mL aliquots were kept frozen at -80°C before transferring to liquid nitrogen storage. To recover cryopreserved cells, these were thawed as quick as possible in a 37°C water bath and transferred to 9 mL culture medium and centrifuged. The cells were then resuspended in a flask in

10 mL medium, that was changed the following day to remove any residual trace of DMSO left during the defrosting process.

2.4.4 Transient transfection of cell lines with polyethyleimine (PEI)

PEI is a non-viral nucleic acid delivery system which is made of repetitions of an imine (NH) group spaced by two aliphatic carbons (CH2-CH2) forming a cationic polymeric structure. This feature helps it packing DNA into positively charged particles, which can then bind the cells surface, which is rich in anionic residues. Hence, by endocytosis, DNA is endocytosed into transfected cells. Inside the cells, environment acidification causes the amine protonation of vesicles containing PEI-DNA. This generates an influx of counterions and, consequently, a progressive unbalance of the cellular osmotic equilibrium. This allows a burst of vesicles, helping the DNA-PEI complex to diffuse into the cytoplasm. Following the de-condensation of these complexes, the transfected DNA is now able to diffuse to the nucleus. This method was the standard used whenever a transient transfection was performed.

For a 10 cm^2 culture dish, 5 µg of DNA were diluted in 250 µL of 150 mM NaCl solution and mixed 1:1 with 250 µL of 150 mM NaCl containing 30 µg PEI. PEI was previously prepared by reconstituting the powder into milli-Q water and stirred. After adjusting the pH to 7.0, the PEI solution was filter-sterilized and divided in aliquots kept at -20 °C. The DNA-PEI mixture was briefly vortexed and incubated for 10 min at room temperature before adding the mix dropwise to the dish to transfect. Cells were incubated with the PEI overnight at 37°C, then transfection medium was replaced with fresh culture medium or cells were seeded to carry out the assays (Marsango et al., 2022).

2.4.5 Flp-In T-REx doxycyline-inducible 293 cell lines

FLAG-CXCR4-mEGFP and FLAG-ACKR3-mEGFP were stably transfected into Flp-In T-Rex 293 cells. The pcDNA5/FRT/TO vector containing the relevant cDNA was transfected into Flp-In T-REx 293 parental cells using the FRT stable integration site. Cells were co-transfected with the relevant cDNA/pcDNA5/FRT/TO construct and the pOG44 Flp recombinase vector

in a 1:8 ratio using PEI similarly as described before in paragraph 2.4.4 (Capoferri, 2019). pOG44 is a plasmid encoding for a Flp recombinase, which facilitated the integration of the construct of interest stably in the host genome by recombining the DNA through FRT sites.

The following day the medium was changed to transfected cells to remove the PEI traces which could have cytotoxic effects on cells. After 24 hours more, cells were split into new flasks in a ratio 1:5 and 1:10, and two days later medium was refreshed with new maintenance medium containing additionally 10 µg/mL blasticidin S and 200 µg/mL hygromycin (from InvivoGen) to select for stable transfectants. Medium was changed every three days for around three weeks to allow the generation of colonies visible by eye. When cells were confluent enough, they were then treated with trypsin-EDTA to allow their detachment and pooled to give polyclonal cell lines, which were grown in selection medium. To express the receptor codified by the integrated gene of interest, 100 ng/mL doxycycline (dox) were added to cells for 18-24 h, since the promoter upstream of the integrated construct was repressed by a Tet repressor, expressed in these cells from the pcDNA6/TR regulatory plasmid (Capoferri, 2019). This possesses a single stably integrated FRT (Flp Recombination Target) site with a crucial role for the integration of the construct of interest.

2.5 Biochemistry

2.5.1 Membrane preparation

Membrane preparation consist of a disruption of the cell membrane using a combination of both mechanical methods and differential centrifugation steps, which help to isolate the membranes from soluble proteins, nuclear material, and other internal membranes. 10 or more tissue culture dishes containing confluent Flp-In T-Rex 293 cells were harvested with 10 mL cold sterile PBS after a preparatory wash step with the same buffer. To harvest Flp-In T-Rex 293 cells, a cell scraper was used, and the next step consisted of resuspending the cells in the dish by pipetting. The cells solution was

centrifuged at 135 rcf 4 ˚C for 5 min and the pellet was kept at -80 ˚C after the removal of supernatant for at least 30 minutes.

After this step, 1 mL of TE buffer containing protease inhibitor cocktail was added to resuspend the pellet that was subsequently homogenised in a glass homogeniser (40 strokes). The homogenate was transferred into a 1.5 mL centrifuge tube and centrifuged for 10 minutes at 135 rcf. The supernatant was recovered, moved to glass ultracentrifuge tubes, and subjected to another centrifugation step at 109000 rcf, 4 ˚C for 45 minutes by using Beckman Optima XL-80K Ultracentrifuge. After this step, the supernatant was removed, and the pellet resuspended in 500 µL TE buffer (volume to adjust according to pellet seize) and moved to a new microcentrifuge tube. A 2 mL syringe with a 25G needle was used to disaggregate the homogenate which was filtered through the needle several times.

The membranes were then quantified by BCA (Pierce) assay and aliquoted to be stored at -80 ˚C diluting according to the required concentration, usually 1µg/µL.

2.5.2 BCA (Bicinchoninic acid) assay

The bicinchoninic acid assay was used to determine the amount of protein in a solution and it consisted of a kit which was purchased from ThermoFisher Scientific. The kit contained two solutions to mix: the first, indicated with "A", (pH 11.25) contains bicinchoninic acid, sodium carbonate, sodium bicarbonate and sodium tartrate, while the second, referred to as "B", it is basically copper sulphate. Because of the presence of proteins peptide bonds, Cu^{2+} ions are reduced to Cu^{+} and these reduction is strictly proportional to the amount of protein. Bicinchoninic acid can chelate Cu^{+} ions, and this complex absorbs light at 562 nm. So, proteins concentrations were determined by using a plate reader to determine the absorbance value at 562 nm. The values obtained were mathematically interpolated with the ones derived from the standard curve made with known concentration of a standard protein such as bovine serum albumin (0.1 to 2 µg/µL).

On a clear 96-well plate, in triplicate, 10 µL BSA standard concentrations (0 to 2 mg/mL) were loaded. In the same plate, 10 µL of sample lysates or membranes were loaded in triplicate in different wells. 200 µL of 50:1

Solution A: Solution B mixture were added to each well and the plate was incubated at 37 ˚C for up to 30 minutes in the dark. After reading the plate with Pherastar FS plate reader (BMG labtech), values were normalized by subtracting the vehicle values to other concentrations values. Once the normalized values were obtained, they were correlated through known concentrations of standards and their absorbance values by using GraphPad Prims to interpolate with a known standard curve. Once concentration of the samples was calculated, these were diluted then at the desired concentration according to the assay to be carried out.

2.5.3 Cell lysate preparation

Confluent cells previously cultured in 10 cm^2 dishes were stimulated with the ligand(s) of interest. Pre-treatment with antagonist ligands was usually between 15 and 30 minutes long (according to the specific ligand used and features of assays carried out), the agonist treatment was between 5 and 15 minutes long (conditions variable according to ligands and assay features).

To lyse cells, they were washed with ice-cold PBS, harvested with a cell scraper as previously seen for membrane preparation, and centrifuged at 1200 rpm at 4°C for 5 minutes to allow the formation of pellet. The supernatant was removed, and the pellet was resuspended in 500 µL (volume to adjust according to pellet size) of the proper lysis buffer (7TM buffer, prepared for SDS-PAGE experiments according to the manufacturer's protocol, or DDM buffer, prepared for blue native PAGE as described in 2.2). The lysis buffer, in both cases, contained cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) (with the addition also of PhosSTOP™ Phosphatase Inhibitor Cocktail (Roche) when lysates were prepared for immunoblots experiments for detection of phosphorylation in proteins).

Lysates were rotated at 4 °C for 30 min in a rotating wheel and then subjected to centrifugation at 18407 rcf for 10 min at 4 °C to remove cell waste. The supernatant was then recovered and moved to a clean 1.5 mL microcentrifuge tube to be quantified by using BCA assay in the same as described before at 2.5.2. The lysates were then diluted according to the desired concentration depending on the assay they would have been used for.

2.5.4 Immunoprecipitation

To enrich the yield of the samples detected by immunoblots in SDS-PAGE, samples were immunoprecipitated by using GFP-Trap® Agarose or DYKDDDDK Fab-Trap™ Agarose kit (for receptors tagged with FLAG), purchased from Chromotek, according to manufacturer's protocol. GFP-Trap® Agarose is an affinity resin which has been developed for immunoprecipitating proteins fused to GFP. It is formed by a GFP nanobody/VHH which is coupled to beads coated with agarose, and it was used in case of SDS-PAGE experiments involving lysates derived from Flp-In T-Rex 293 cells expressing FLAG-hCXCR4-mEGFP. In the case of DYKDDDDK Fab-Trap™ Agarose, it consists of an anti-DYKDDDDK-tag (also known as FLAG tag) Fab-fragment, covalently bound to agarose beads, and it was used in case of SDS-PAGE experiments involving lysates derived from Flp-In T-Rex 293 cells expressing FLAG-hACKR3-mEGFP.

These kits were used following the instructions provided by the company. To sum up, 25 µL beads were equilibrated in 380 µL of 7TM lysis buffer (without protease inhibitor cocktail and phosphatase inhibitor cocktail tablets added) in 1.5 mL microcentrifuge tubes, washed by inverting the tube and centrifuged at 2500 rcf, at 4 °C and for 5 minutes to allow the deposition of the beads. The washing step was repeated for 3 times to allow the beads to be properly cleaned by 7TM lysis buffer. After this step, the buffer from the last washing step was removed and the lysates prepared for SDS-PAGE experiments were transferred into the same microcentrifuge tubes containing the beads. The volume changed according to the quantity of lysates to use for the experiments and to the desired concentration. Therefore, samples with beads were left to rotate overnight in a rotating wheel at 4 °C, to allow receptors to bind to the beads. The following day, samples were centrifuged at 2500 rcf, at 4 °C and for 5 minutes to let the beads to which the receptors bound to deposit at the bottom of the microcentrifuge tube. The supernatant containing unbound proteins was removed, and three steps of washing were done by adding new wash buffer (lysis buffer with no protease inhibitor cocktail and phosphatase inhibitor cocktail) every time and centrifuging three times to remove the supernatant containing contaminating proteins at the same conditions as seen before.

After the third wash, the beads are resuspended in 2x Laemmli buffer and the samples incubated at 65 °C for 10 min to induce the denaturation of the proteins, breaking of disulphide bonds and coating with negative charges. Then, samples are centrifuged at room temperature 2500 rcf for 2 minutes to favour the deposit of beads and facilitating the sample loading into the gels. Then, the samples were loaded onto a gel and an SDS-PAGE experiment was started.

2.5.5 Immunoblot: SDS-PAGE

Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In case of lysates, 20-30 μg protein/well were loaded onto NuPAGE™ 4-12%, Bis-Tris gels (ThermoFisher). Instead, when immunoprecipitated samples were loaded into the gels, 20 μl protein/well were loaded. Gels were run by using NuPAGE® MOPS SDS Running Buffer (Invitrogen) applying a voltage of 180 V for 60-75 mins.

To follow this step, proteins were therefore transferred onto nitrocellulose membranes taking advantage of a wet transfer system. In doing so, proteins were transferred in transfer buffer applying a voltage of 35 V for 90 mins. Once the transfer finished, the membranes were incubated in blocking buffer, which could be either milk powder or TBS (Tris-buffered saline) buffer with 5% BSA (bovine serum albumin) powder, for at least an hour at room temperature shaking the membranes gently. Successively, the blocking buffer was removed, and the primary antibody solution was added, which contained an appropriate antiserum/antibody (**Table 2.2**) in a ratio that was variable and dependent on the specific antiserum/antibody (although, in general, 1:2000 dilution was used). The membranes were left overnight incubating at 4 °C on a shaker, to facilitate recognition and binding of the specific amino acid sequence of the protein of interest.

The day after, the solution containing the primary antibody used was removed and, after three washes with TBS-T (TBS with addition of 0.1% Tween20) for 10 mins each at room temperature, the secondary antibody solution was added. This solution contained an IRDye® fluorescently labelled secondary antibody in a dilution 1:10000, which was chosen according to the host animal in which the primary antibody was produced (**Table 2.3**). The

membranes were incubated at room temperature on a shaker in the dark, protected from the light, for 90-120 minutes.

Membranes were then washed three times for 10 mins each with TBS-T, left them to dry covered in aluminium foil to keep them protected from light and then images were collected by scanning the membrane on a LI-COR Odyssey Imager using the 700 nm and 800 nm channels on Image Studio version 5.2 software.

Table 2.2: List of primary antibodies/ antisera used for immunoblots.

Anti-serum	Company	Dilution
Anti-CXCR4 no phospho	7TM antibodies	1:2000
Anti-CXCR4 $pSer^{324}/pSer^{325}$	7TM antibodies	1:2000
Anti-CXCR4 $pSer^{330}$	7TM antibodies	1:2000
Anti-CXCR4 $pSer^{338}/pSer^{339}$	Invitrogen	1:2000
Anti-CXCR4 $pSer^{346}/pSer^{347}$	7TM antibodies	1:1000
Anti-FLAG	Proteintech	1:2000
Anti-GFP	In-house made	1:10000

Table 2.3: List of secondary antibodies/antisera used for immunoblots.

Anti-serum	Company	Dilution
IRDye® 800CW Donkey anti-Rabbit IgG	LICOR	1:10000
IRDye® 800CW Donkey Anti-Goat IgG	LICOR	1:10000

2.5.6 Immunoblot: Blue Native PAGE

In a similar way as seen for SDS-PAGE version, proteins were separated by Blue Native PAGE (BN-PAGE) to investigate how ligands would affect the homo-oligomerization of the receptors under study. The protocol used was an optimization of the one described in Ward et al. (2021). 16-18 µg protein/well with the addition of G250 blue dye (Thermofisher) were loaded into each lane of Native PAGE Novex 3-12% Bis–Tris gels (ThermoFisher). Gels were run in Native PAGE® Running Buffer (Invitrogen), with Dark blue buffer (prepared as described in paragraph 2.1) in the inner chamber, at 180 V for 25 mins at room temperature. After removing dark blue buffer and adding in the inner chamber light blue buffer (prepared as described in paragraph 2.1), gels were run for additional 30 mins still at room temperature. Finally, the gels were run at 250 V, 4°C (on ice), for an hour.

Proteins were then transferred by using a transfer buffer (90 min at 25 V) on to a polyvinylidene fluoride membrane, which had been prewetted for 30 s in methanol and then soaked for several minutes in transfer buffer prepared during the previous running step. Membranes were then fixed in 8% acetic acid, gently shaking for 15 min at room temperature and stained with Ponceau S (Sigma–Aldrich) (0.2% in 1% acetic acid) to allow the markers to be visualized and labelled. Then, membranes were rinsed to remove the Ponceau S solution, and blocking buffer solution, prepared as described for SDS-PAGE in paragraph 2.5.5, was added to membranes which were incubated for at least an hour shaking gently at room temperature.

Once this step was finished, membranes were incubated letting them to shake overnight at 4 °C with the antisera listed in the previous paragraph (**Table 2.2**) as seen already for SDS-PAGE. On the following day, membranes were washed for 10 mins with TBS-T for 3 times shaking at room temperature and then incubated with secondary antibodies listed in **Table 2.3** for 90-120 mins at room temperature in the dark.

Membranes were then analysed by using Pierce ECL western blotting substrate horseradish peroxidase (HRP) substrate for enhanced chemiluminescence (ECL) (ThermoFisher). The substrate was a mix of detection reagents 1 and 2 combined in ratio 1:1 before applying this solution to the membranes. The development of the medical X-ray films (Kodak) on which the reaction happening was impressed took place in a dark room by

using black boxes to impress the membranes content on the films which were developed by using an X-OMAT developer. Exposure times were different according to how strong the signal given by the bands was, although generally 30 seconds or one minute exposure was enough.

2.6 Functional assays

2.6.1 [^{35}S]GTPγS incorporation assay

[^{35}S]GTPγS incorporation assay was carried out to investigate the activation of the $G_{\alpha i/o}$ protein signalling for hCXCR4 stimulated with CXCL12 and to check whether adrenomedullin could affect this signalling pathway. The protocol represents an optimization of the conditions already described in Sergeev et al. (2017). Therefore, 10 µg of membranes prepared as described in paragraph 2.5.1 from cells expressing FLAG-hCXCR4-mEGFP were incubated either with 20 µL of increasing concentrations of CXCL12 agonist. For antagonist test, 20 µL of a fixed concentration (EC_{80}) of agonist was used to stimulate membranes either in absence or presence of pre-treatment with increasing concentrations of IT1t antagonist or adrenomedullin (ADM) (20 µL, 15 minutes incubation) before adding the agonist. Ligands dilutions were prepared in assay buffer as described in section 2.2.

After addition of the agonist, 100 µL of assay mix, containing 1 µM GDP, 0.1 nM (50 nCi) [^{35}S]GTPγS (specific activity: 539.24 Ci/mol) and assay buffer were added to each well. Plates were incubated for 60 mins in a water bath (at 30 °C). During this time, ice cold 1x PBS was prepared, and the harvester was washed together with the filter plates soaked in PBS. Plates incubated earlier were harvested onto the filter plates by rapid filtration through GF/C glass fiber filter-bottom 96-wells microplates using a UniFilter FilterMate Harvester (PerkinElmer); after labelling the plates, these were left to dry for few hours (or overnight).

Once the plates were completely dry, 100 µL of scintillation liquid (MicroScint-20, PerkinElmer) were added to each well and a liquid

scintillation spectroscopy approach was used to quantify the incorporation of [^{35}S]GTPγS. Data from agonist test were normalized on the average of values recorded for the vehicle, while for the antagonist test, data were normalized on CXCL12 percentage.

2.6.2 Cyclic adenosine monophosphate (cAMP) accumulation assay

Cyclic adenosine monophosphate (cAMP) accumulation assay was carried out to check whether adrenomedullin influenced the accumulation of second messenger intracellular cAMP for hCXCR4 upon $G_{\alpha i/o}$ protein activation. The assay was carried out and optimized according to protocol described by the kit cAMP G_s dynamic kit HTRF®, purchased from CisBio Bioassays SAS. HTRF (Homogeneous Time Resolved Fluorescence) represents a wash-free technology, which combines the principles of FRET approach with time-resolved measurement of fluorescence. These features facilitate the elimination of unspecific background fluorescence. This technology takes advantage of two antibodies, one coupled to a donor and the other to an acceptor, which are able to recognize the analyte of interest. Upon recognition of the analyte by the antibodies, the donor unit emits a fluorescent signal after being excited and the energy generated is transferred to the nearby coupled acceptor, which can therefore provide a fluorescent signal specific for this interacting couple. To quantify this signal, fluorescence generated from the donor is measured and a ratio of this value over the acceptor fluorescence is calculated. This method represented an adaptation of the one described by Sergeev et al. (2017).

Therefore, in the specific case of this project, all the ligands' dilutions have been prepared in assay buffer. Five thousand Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP resuspended in assay buffer (Cisbio) were plated onto a 384-well plate and incubated with 2 µl of varying concentrations of antagonist ligand in order to generate a concentration-response curve, for a time of 15 minutes at room temperature (only in case of IT1t and ADM test).

Following this incubation, 2 µl of either a fixed concentration of CXCL12 agonist (EC_{80}, used for the antagonist test), or increasing concentrations of

agonist were added and incubated at room temperature for 15 minutes. After this step, the appropriate concentration of forskolin is added to stimulate the production of cAMP. This event has the purpose of lowering cAMP levels because of negative regulation of adenylate cyclase activity mechanisms adopted by cells. Indeed, native intracellular cAMP or unlabelled cAMP enter in competition with d2-labeled cAMP to bind to monoclonal anti-cAMP Eu^{3+} cryptate.

The incubation is done for an hour at room temperature, before adding, at last, 2 µl each of Terbium cryptate donor and d2 acceptor (prepared diluting the stock in lysis buffer provided with the kit) to the mix, to allow the generation of fluorescent signal. This last incubation is carried out in the dark at room temperature and it can last up to 24 hours.

The plate was read taking measures at two different wavelengths, 620nm (donor) and 665nm (acceptor) by using a PheraStar FS plate reader (BMG labtech). The data collected provide a concentration-response curve of HTRF signal, and this specific signal recorded is inversely proportional to the concentration of cAMP levels measured in the standard curve or in samples tested. Thus, for data analysis, data are plotted with a standard curve of cAMP known concentrations. As a result of this plot, because hCXCR4 signal via $G\alpha_{i/0}$, higher concentrations of agonist correlate to lower levels of cAMP accumulated, while an antagonist will show higher levels of cAMP accumulation.

2.6.3 Bystander Bioluminescence Resonance Energy Transfer (Bystander BRET)

Bystander BRET was used to assess the β-arrestin recruitment to hCXCR4 and hACKR3 with different ligands, including CXCL12 and adrenomedullin for both hCXCR4 and hACKR3, IT1t for hCXCR4 only and VUF16840 for hACKR3 only. This approach represented an adapted version of the method explained in Namkung et al. (2016).

Bystander BRET signal originates from random collisions between energy donors and acceptors, differently from classical BRET approaches which rely on the unnatural combination of luciferase from *Renilla reniformis* (Rluc) and

GFP variants from *Aequorea victoria*, which do not interact spontaneously, hence, limiting non-specific signals that originate from random interactions (Namkung et al., 2016).

Plasmids were generated by using human GPR35 untagged receptor as backbone and example. This was a kind gift of Dr. Brian Hudson (University of Glasgow), as well as the pIRES plasmid. FLAG-hCXCR4 or FLAG-hACKR3 untagged receptors constructs were generated, starting from the original constructs sub-cloned in pcDNA5/FTR/TO described in 2.3.12, where stop codons were added at the end of the receptors sequence to remove the fluorescent protein tag at the C-terminal side of receptors.

HEK293T were transfected transiently by using PEI in a ratio 1:6 with either FLAG-hCXCR4 or FLAG-hACKR3 untagged receptor and an internal ribosome entry site (pIRES) plasmid that allows expression of two proteins mammalian cells from the same bicistronic transcript in a ratio 1:2 (2.5 μg each component). These were β-arrestin-2-nanoluciferase and mNeonGreen. When the mNeonGreen is expressed, it becomes linked to the plasma membrane because its sequence has been modified to incorporate the fatty acylation motif of the Lyn non-receptor tyrosine kinase.

Cells were therefore cultured overnight after changing the medium to refresh cells. On the following day, 60000 cells per well were seeded on a 96-well plate and cultured overnight with poly-D-lysine (1:20 dilution) to help the attachment of the cells to the wells. The following day, medium was changed to the cells with HBSS (without calcium chloride and sodium magnesium) after washing a couple of time with HBSS and cells were then incubated for at least 30 minutes at 37 °C.

Dilutions of the ligands of interest were prepared in HBSS and, after the incubation step ended, they were added similarly to how described already for other assays before. Cells were firstly pre-treated with 10 μL of increasing concentrations of antagonist for 15 minutes (in case of antagonist test), then 10 μL of fluorescent substrate NanoGlo (Promega) were added and after 5 minutes of incubation, 10 μL of agonist were added (EC_{80} of agonist in case of antagonist test or increasing concentrations of agonist in absence of antagonist). Dual 535 and 475 nm luminescent emission measurements were then taken at 90 seconds intervals using a PheraStar FS plate reader (BMG labtech) before adding the agonist, to assess the signal background of the

assay and after agonist addition and up to 30-45 mins following the addition of the indicated test compounds (Scarpa et al., 2021).

Net BRET responses were calculated as the 535/475 ratio after correcting values obtained for both the wells baseline and test compound vehicle response (Scarpa et al., 2021). BRET data were then reported as the concentration-response curve showing net BRET signal at the time point before the plateau started (usually 20 mins since agonist addition).

2.6.4 Schild regression analysis

The Schild plot is a pharmacological method of receptor classification, which helps in studying the influence of specific ligands on the response generated in response to ligand-receptor complex formation or to the receptor itself. In fact, with Schild analysis it is possible to assess the rightward shift of ligands concentration–effect curves in presence of different concentrations of the respective receptor antagonist and present their relationship in a double log plot, referred to as Schild plot. The pA_2 value determined from this experiment gives a measure of affinity of the antagonist for its receptor and, thus, the equilibrium dissociation constant.

The Schild analysis was carried out by using a cAMP accumulation assay, with a protocol already described for this assay in relative paragraph 2.6.2. Differences with the previously described cAMP accumulation assay reside in the fact that, to determine the effects of adrenomedullin on hCXCR4, six different concentrations of ADM were used to pre-stimulate Flp-In T-REx 293 cells stably expressing FLAG-hCXCR4-mEGFP (no ADM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM), before adding increasing concentrations of CXCL12 agonist. Data generated relative to HTRF signal were analysed to determine whether ADM was able to induce a shift either in potency or efficacy of CXCL12, which would give insights about the nature of this ligand either as orthosteric antagonist or an allosteric antagonist.

2.6.5 NanoLuc® Binary Technology (NanoBiT) assay

The NanoBiT assay was carried out to investigate the role of different ligands, especially ADM, in GPCR-GRK interactions taking advantage of the nature of this complementation assay. For both hCXCR4 and hACKR3, the assay conditions were optimized accordingly. HEK 293 cells in which expression of all GRKs was eliminated via CRISPR-Cas9 were used in this assay and they were kindly provided by Prof. Carsten Hoffmann, University of Jena. The plasmids containing GRKs-LgBiT and hCXCR4/hACKR3-SmBiT were a kind gift of Dr Andy Chevigné, Luxembourg Institute of Health.

These cells were transfected transiently with 500 ng of receptor and 50 ng of GRK of interest and with empty vector pcDNA3 to fill up the total amount of DNA to 5 μg by using the PEI transfection method as described in the related paragraph 2.4.4. Cells were cultured overnight, then the following day 100000 cells/well were seeded on 96-well plates coated with poly-D-lysine (1:20 ratio) and cultured overnight.

The day of the assay, dilutions of increasing concentrations of drugs to use were prepared in HBSS without calcium chloride and sodium magnesium and a dilution 1:20 of coelenterazine H was used as luminescent substrate preparation. Coelenterazine substrate was added to the plate, and this was incubated for 5 minutes before reading the plate a first time to establish the baseline values to use for data analysis. After these 5 minutes, agonist dilutions were added to the plate and the reading was carried out by using a CLARIOstar (BMG LabTech).

In case of antagonist test, the antagonist was added by pre-treating cells for 15 minutes before adding coelenterazine H substrate to the plates, and after that a fixed concentration of agonist (EC_{80}) was added to cells. Data were collected and analysed as ratio between the absorbance value after ligand administration over the absorbance value after coelenterazine addition. The values obtained were then plotted on a graph by using GraphPad Prism software, where the X axis represented the concentrations of ligands and the Y axis the percentage of GKR recruitment detected.

2.7 Cell biology assays

2.7.1 Fluorescence microscopy

Fluorescent images of different cell lines generated were acquired using a 40x Plan-Apochromat objectives of the Zeiss 880 Axio Observer Z1 Laser Scanning Confocal Microscope (Zeiss).

This visualization approach was used to assess the correct expression of the constructs encoding for the inducible expression of FLAG-hCXCR4-mEGFP and FLAG-hACKR3-mEGFP and their respective cellular localization in Flp-In T-REx 293 cells transfected to express stably these constructs by detecting the emitted green mEGFP signal.

2.7.2 Label-free impedance assay via xCELLigence Real Time Cell Analysis (RTCA)

xCELLigence RTCA (Real Time Cell Analysis) (Agilent) approach was used to investigate the role of different ligands, especially ADM, in affecting the cell's adhesion properties once these ligands were added to the cells. In a cellular impedance assay such as the xCELLigence RTCA, the functional unit are golden microelectrodes placed at the bottom surface of a microtiter well plate (E-plates, purchased from Agilent). When submersed in an electrically conductive solution, which in this specific case was tissue culture medium for Flp-In T-REx 293 cells (see the relevant paragraph for more information), an electric potential was applied through the microelectrodes and this induced the electrons to exit the negative terminal, pass through the bulk solution, and then deposit onto the positive terminal.

This event depends on interactions between microelectrodes with bulk solution used for the assay. Therefore, since adherent cells are placed at the microelectrode-bulk solution interface, the flow of electron finds an impedance. The magnitude of this impedance depends on the number, size and shape of the cells, but also on the quality of the interaction between cells

and substrate. Instead, cells health or behaviour is not affected at all during the process.

E-plates used for this assay are covered by gold microelectrode biosensors, which are linked into “strands” disposed in a way to form an interconnected array, and this settlement helps the simultaneous monitoring of cells under study and furnishes a high level of sensitivity to several parameters providing information of cells plated in the wells.

For the assay, conditions used represented the optimization of the method previously described in Lin et al. (2021). Therefore, Flp-In T-REx 293 cells stably expressing either hCXCR4-mEGFP or hACKR3-mEGFP upon doxycycline addition were seeded onto xCELLigence E-plates (4×10^4 cells/well) after passing them through a syringe to allow a better separation of cells plated. Cells were then left at room temperature for 30 minutes to allow their attachment to the plate. Before plating the cells, 100 µL of medium was added to the plate and a first read of the plate was executed to determine the baseline. Cells were added to each well topping up the volume to a total of 200 µL per well. Cells were then incubated overnight at 37 °C.

The following day, medium was changed to cells and doxycycline was added to the usual concentration described previously (100 ng/mL) to allow the expression of the receptor. The following day, dilutions of the ligands of interest were prepared as required after warming them up at 37 °C for 10 minutes before adding them to the cells. In case of antagonist test, cells were pre-treated for 20 minutes with the antagonist before adding the agonist.

Upon ligand or vehicle control addition, the impedance (represented as “cell index”) was recorded every 30 seconds over an hour period using xCELLigence RTCA recorder. The Cell Index (CI) value is described with the following mathematical equation, as already described in Lin et al. (2021):

CI = (impedance at time point n – impedance in the absence of cells)/nominal impedance value

2.8 Small molecule inhibitors and enzymes treatments

2.8.1 Compound 101 and compound 19 treatment

Compounds 101 (Tocris Biosciences) and compound 19 (kind gift of Dr. David E. Uehling, Ontario Institute for Cancer Research, Canada, and Dr. Rodger E. Tiedemann, Princess Margaret Cancer Centre, Canada) were used to block, respectively, the recruitment of GRK2/GRK3 and GRK5/GRK6 in SDS-PAGE and NanoBiT assay. In all the assays where they were used to characterize the role and the effects of GRK2/3/5/6, cells were pre-treated with one of these small molecules for 30 minutes with a concentration of 10 μM (diluted in DMSO) at 37°C before adding the agonist and compared with vehicle controls in absence of agonist.

2.8.2 Barbadin treatment

Barbadin (Sigma Aldrich) treatment was used because this small molecule can prevent β-arrestin and β2-adaptin subunit of the clathrin adaptor protein AP-2 from interacting among each other. Anyway, this does not affect the formation of functional complexes formed between receptor and β-arrestin upon GPCRs activation, and this leads, as a result, to block the agonist-promoted endocytosis of the receptor (Beautrait et al., 2017). In impedance assay via xCELLigence RTCA the concentration used was 5 μM administered to cells incubated for 30 minutes at 37°C before adding the agonist.

2.8.3 Pertussis toxin from Bordetella pertussis treatment

$G\alpha_i$ protein activation is sensitive to pertussis toxin (PTX), an exotoxin which is normally secreted by a gram-negative bacterium called *Bordetella pertussis*. The first time this exotoxin was discovered and purified, showing important pharmacological properties was in a work published by Katada and Ui (1979).

It was observed that, when this exotoxin crosses the plasma membrane, its subunit A catalyses the adenosine diphosphate (ADP) ribosylation of $G\alpha_i$ subunit, locking the G proteins in their inactive state (in which a molecule of GDP is bound) and preventing its activation (Holz et al., 1998; Capoferri, 2020). This treatment was used to characterize the potential involvement of G proteins in CXCL12 signalling, in particular in xCELLigence RTCA, to rule out the possibility of CXCL12-mediated hACKR3 signalling.

For impedance assay via xCELLigence RTCA Flp-In T-Rex 293 cells were pre-treated for 16 hours with PTX (1:8000 dilution) before starting the assay to determine a possible role of $G_{\alpha i/o}$ proteins involvement in affecting cell adhesion properties upon treatment with CXCL12.

2.8.4 λ protein phosphatase enzyme (λPP) treatment

In SDS-PAGE experiments, to remove phosphate groups, samples were treated with λ-PP (lambda protein phosphatase) enzyme at a final concentration of 10 units/µl for 90 mins at 30 °C before elution with 2×SDS-PAGE sample buffer as explained previously for SDS-PAGE in the relative paragraph (Marsango et al., 2022).

2.9 Data analysis

2.9.1 GraphPad Prism

All data generated from different functional assays were analysed by using GraphPad Prism 9.5.1 version software, according to the suitable statistical analysis required and where possible, a one-way ANOVA test was used for statistical analysis.

3. CHAPTER 3 (Results part 1) - Characterisation of hACKR3 adrenomedullin interactions

Klein et al. (2014) showed that loss of ACKR3 results in postnatal lethality in mice due to aberrant cardiac development and myocytes hyperplasia. They also demonstrated how the dosage and signalling of the mitogenic peptide hormone adrenomedullin, normally required for normal cardiovascular development, is controlled by ACKR3 in mice. This observation was interpreted as a result of a possible scavenging activity for hACKR3 towards adrenomedullin *in vitro*. Indeed, it was observed an upregulation of *cxcr7* gene expression in $ADM^{hi/hi}$ mice cardiac tissue when compared to that of wildtype littermates. Conversely, loss of *adm* gene expression in isolated endothelial cells resulted in a consistent reduction in ACKR3 expression. Moreover, haploinsufficiency of adrenomedullin in $ACKR3^{(-/-)}$ mice led to a gain-of-function cardiac and lymphatic vascular phenotype reverting the severeness of these pathological conditions. These outcomes demonstrated that hACKR3 is required as a molecular rheostat for controlling AM ligand availability during development. By contrast, Szpakowska et al. (2018), using glioma U87 cells, showed no interaction between adrenomedullin and ACKR3 in the same concentration range for which all other known ligands for this decoy receptor were observed. This information still leaves an open interpretation about the possible interactions between adrenomedullin and ACKR3 and, if so, the eventual mechanism of action by which this interaction occurs. To study potential interactions between ACKR3 and adrenomedullin, therefore, hACKR3-expressing stable cell lines were generated and characterized.

3.1 Generation of a FLAG-hACKR3-mEGFP construct and production of Flp-In T-REx 293 stable cell lines

Sequence details of vector constructions have been described previously in chapter 2 “Materials and methods”, section 2.3.12. Briefly, a hACKR3 construct with an N-terminal FLAG epitope tag and C-terminal monomeric enhanced green fluorescent

protein (mEGFP) in pcDNA5/FRT/TO was generated using as a backbone construct FLAG-hCXCR4-mEGFP, by replacing the sequence of hCXCR4 with hACKR3. After confirmation of the correct sequence, the final construct was then transfected stably into Flp-In TREx 293 cells to generate a stable cell line allowing expression of the receptor construct in an inducible fashion. Induction was performed routinely by adding doxycycline overnight.

3.2 Assessment of receptor expression and localisation

Once obtained, cells of this clone were validated for expression and membrane localisation of the receptor. Cells were treated overnight with increasing concentrations of doxycycline to establish how the expression of the receptor varied with such treatment. Cell lysates obtained were immunoprecipitated with a FLAG tag Fab-Trap kit, resolved by SDS-PAGE, and then immunoblotted with anti-FLAG primary antibody that identified the FLAG tag sequence DYKDDDDK. Such immunoblots were then exposed to an infrared dye-labelled donkey anti-rabbit secondary antibody (**Fig. 3.1**). Although several structural hACKR3 antisera from different companies were tested, none of them successfully detected the receptor, leading to the choice of the anti-FLAG antibody. The possible explanation behind the unsuccessful outcome of testing these antisera can be due to the epitope used for the generation of these antisera. In this case, the antigen-binding site maybe does not recognize the antibody, making harder the proper detection of the hACKR3 polypeptide. Also experimental conditions such as pH of the buffer used, temperature and concentration used might have affect the outcome of this method. The experimental condition used were an optimization of 7TM antibody company protocol, since the majority of the antisera tested were provided from this company.

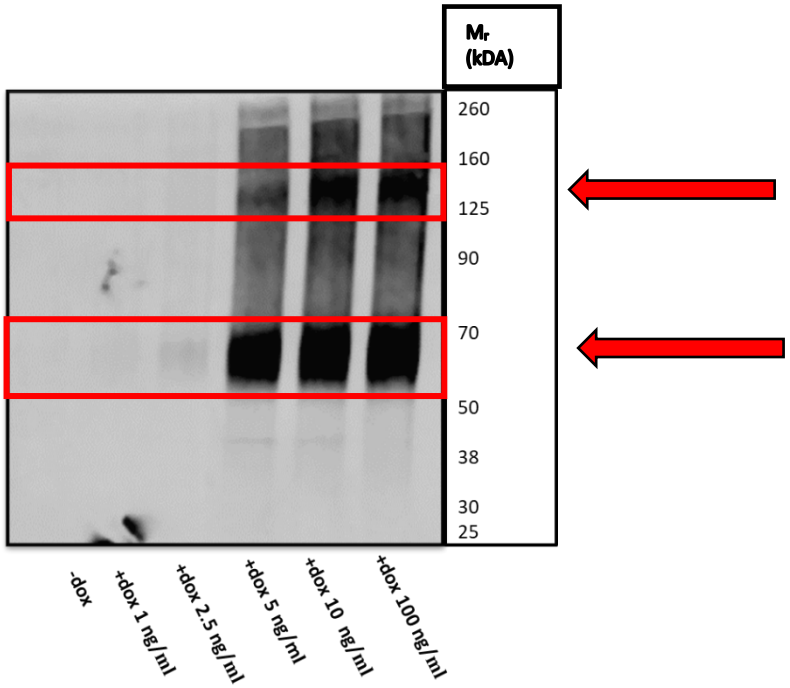


Fig. 3.1: Detection of the expression of FLAG-hACKR3-mEGFP in Flp-In T-REx stable cells by immunoblot. Stable Flp-In T-REx 293 cells developed to be able to express FLAG-hACKR3-mEGFP were treated with the indicated concentrations of doxycycline overnight and immunoprecipitated with a FLAG Trap kit. After this step, samples were resolved on by SDS-PAGE and immunoblotted by using an anti-DYKDDDDK (FLAG) antibody. The anticipated molecular mass of the receptor construct is 69 kDa (41 kDa hACKR3, 27 kDa mEGFP and 1 kDa FLAG tag). Red arrows (lower and upper) represent immunoreactivity corresponding to the receptor construct upon doxycycline treatment.

As shown in **Fig. 3.1**, the predominant polypeptide detected was generated in a doxycycline-induced fashion and migrated with M_r (relative molecular mass) in the region of 65-70 kDa, as anticipated. Higher molecular mass polypeptides were also detected with M_r corresponding to some 125 kDa-160 kDa, which may correspond to oligomeric forms of hACKR3, due to the nature of their molecular mass. A similar behaviour it has been observed for other GPCRs and it might depend on experimental conditions used. Indeed, some groups (Blum et al., 2024) observed that decreasing the temperature used for heating the samples from 65 to 55 ℃ and increasing the incubation time up to 20 mins, it improves the quality of the immunoblots. In fact, the detection of higher molecular mass polypeptides is strongly reduced, allowing a more specific detection of the correct mass of the polypeptide of interest, maybe

because of a major breaking of chemical bonds between different domains of the receptor of interest.

It is evident that concentrations of doxycycline as low as 5 ng/ml were able to induce detectable expression of the receptor. However, as this was not reduced by treatment with higher concentrations of doxycycline, therefore, to achieve the highest levels of expression of the receptor, 100 ng/ml doxycycline was chosen the standard concentration to use in subsequent experiments. This immunoblot shows some smeared bands, which can be due to overloaded samples (higher amount of lysate was used to help the detection of the receptor and to allow a more clear visualization of the polypeptide of interest). Because these samples were immunoprecipitated with a FLAG-Trap Kit, it is impossible to have a control represented by an housekeeping protein-targeting antibody, because the housekeeping protein would not be recognized because of the absence of the FLAG tag. An alternative could have been keeping a fraction of the lysates before immunoprecipitating them and resolving them with an anti-actin antibody, to have an additional control.

To determine whether the receptor expressed by these cells was localized either to the plasma membrane or intracellularly, cells from this clone were grown on coverslips according to the protocol described in chapter 2, section 2.7.1, and induced with 100 ng/ml doxycycline treatment overnight, before examining the intensity of the fluorescent signal. The fluorescence of mEGFP was detected by fluorescence microscopy using a Zeiss LSM 880 confocal microscope (**Fig. 3.2**).

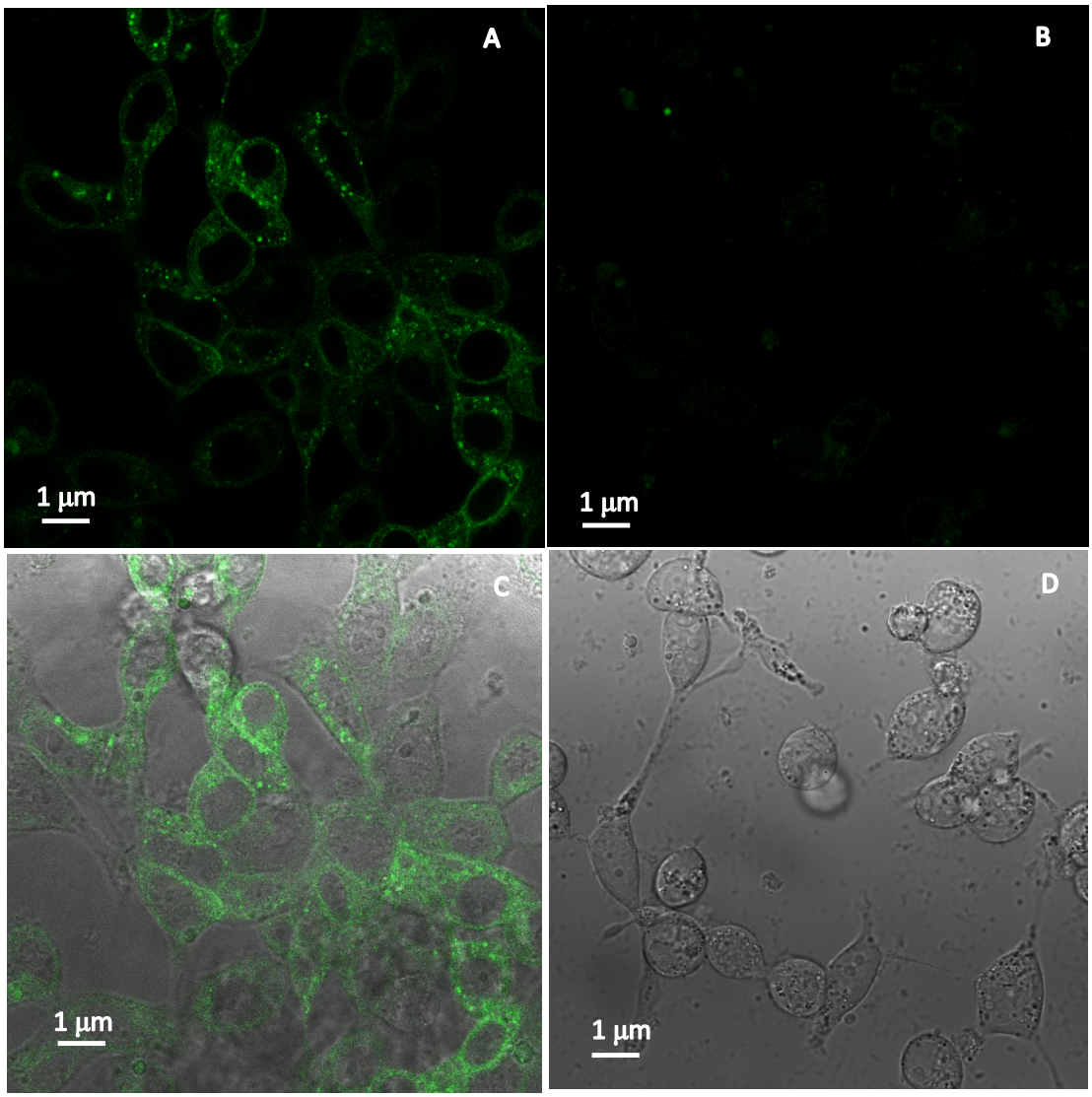


Fig. 3.2. Assessment of ACKR3 localization in FLAG-hACKR3-mEGFP-expressing Flp-In T-REx 293 cells by fluorescence microscopy. Flp-In T-REx 293 cells able to express FLAG-hACKR3-mEGFP were examined by fluorescence microscopy either after (**panel A**) and without (**panel B**) overnight doxycycline treatment (100 ng/ml). Green fluorescent signal corresponds to FLAG-hACKR3-mEGFP**.** The same cells are also shown by using a bright-field filter (**panel C**, cells induced with doxycycline, and **panel D**, cells without any doxycycline addition).

This analysis showed the localization of the hACKR3-mEGFP construct, that appears constitutively internalized **(Fig. 3.2A)** and grouped into intracellularly located clusters, with a clear cytoplasmic localization. The signal observed was clearly receptor-specific, since this was absent without treatment with doxycycline **(Fig. 3.2B).** As a comparison, same cells are shown after applying a bright-field (**Fig.**

3.2C, cells treated with doxycycline, and **Fig. 3.2D**, cells without doxycycline addition). As both immunoblots and confocal microscopy confirmed doxycycline-induced receptor expression I proceeded to perform assays to assess functionality of the receptor construct.

3.3 xCELLigence RTCA approach to assess the role of CXCL12 and ADM at hACKR3

To determine whether ligands such as CXCL12 and adrenomedullin (ADM) would have measurable effects at hACKR3 xCELLigence RTCA (Real Time Cell Analysis) studies were carried out. This is an elegant and sensitive approach, by which it is possible to monitor, in real-time, variations in the adherent properties of cells in a non-invasive and label-free manner. To do so cells are grown as a monolayer on a support able to record alterations in electrical conductance (referred as cellular impedance) and they respond over time as when they are challenged with ligands of interest. CXCL12 was used as a reference agonist as it is well established to be an endogenous ligand of hACKR3. Each experimental condition was tested in 3 separate wells of an E-plate, to have a biological replicate. This setting was kept in n = 4 independent experiments.

When added to cells (at 100 nM), CXCL12 produced a time-dependent increase in the signal that reached a maximum within 20 mins (**Fig 3.3A**). However, after this time, the signal decreased until it reached a plateau phase. This could be due to the desensitization of hACKR3, which could lead to a decreased response to the agonist over time. In such assays parallel vehicle controls are required as cells may respond to a wide range of stimuli. Although effects of the vehicle were observed (**Fig 3.3A**) clear separation of the extent of the cell response to vehicle and to CXCL12 were observed (**Fig 3.3A**). The response generated by the vehicle could be explained by changes in cells environment. Indeed, together with the change of cell medium before the assay, water was added to the wells of the vehicle simultaneously with the addition of the ligands to other wells. This was done to keep similar experimental conditions between agonist-treated cells and vehicle. However, this could cause a response-like situation, although not really significant. Additionally, normalization of the values recorded were done over the values collected on the wells containing medium before seeding the cells. Therefore, the values of the vehicle do not reach 0

because cells might induce a background response, due to their attachment to the wells and their differentiation. Another explanation about the high baseline recorded could depend on the number of cells, that could have been to high, or to the presence of clumps of cells, which could impact the electrons flux and then the cell index values recorded. (Hamidi et al., 2017).

Since adrenomedullin (ADM) has been reported in certain settings to be an agonist for hACKR3, this ligand was also tested, and added to cells at 1 µM. As observed for CXCL12, ADM generated a time-dependent increase in the signal with a plateau reached within 20-30 mins that was substantially greater that with treatment with vehicle (**Fig 3.3B**).

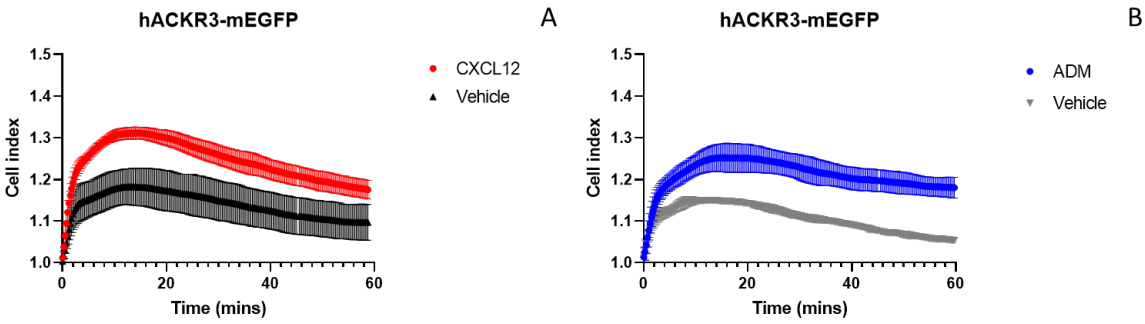


Fig. 3.3. Effects of CXCL12 and ADM on hACKR3-mEGFP-expressing Flp-In T-REx 293 cells measured using xCELLigence RTCA. Representative traces are shown from n=4 independent experiments, in which 3 wells of a single E-plate have been used for each condition to have a biological replicate. Kinetic curves were recorded after treatment either with CXCL12 (100 nM, **panel A**) or ADM (1 µM, **panel B**), compared to vehicle (water for CXCL12 and 0.001% acetic acid in water for ADM). The intensity of the response was measured as variation of cell index over a 60 min time period.

The choice of the concentration depends on literature describing these ligands and their effects on this receptor. Indeed, various work reported the potency of CXCL12 towards hACKR3, with a working concentration used in several assays between 10 nM and 100 nM. In case of adrenomedullin, little is known about its effects on hACKR3, and in vitro these effects are observed at a concentration >1 µM. This explain the reason behind the choice of these concentrations, rather than carrying out

a concentration-response curve, which would have been too expensive, especially for adrenomedullin.

Data derived from n=4 different independent experiments were combined (**Fig. 3.4**). Both CXCL12 and ADM increased response in a statistically significant manner compared to the vehicle-generated response. As an additional control, Flp-In T-REx 293 cells in absence of doxycycline treatment were used, to assess whether the signal observed in this assay were specific and determined by hACKR3 expression (**Fig. 3.5**). In this case, the ligand of choice used to determine the robustness of this approach was CXCL12, already established as an agonist for hACKR3. In **Fig. 3.5**, cells induced with doxycycline (+dox) to express hACKR3 generated a higher response compared to cells in absence of doxycycline (-dox). However, this response was higher in presence of CXCL12 stimulation, as expected from previous experiments. The presence of an effect on +dox cells might be due to a basal activity of the receptor combined with the addition of water to the vehicle, which can cause a small change in the cell environment which the plate reader records as a partial positive signal.

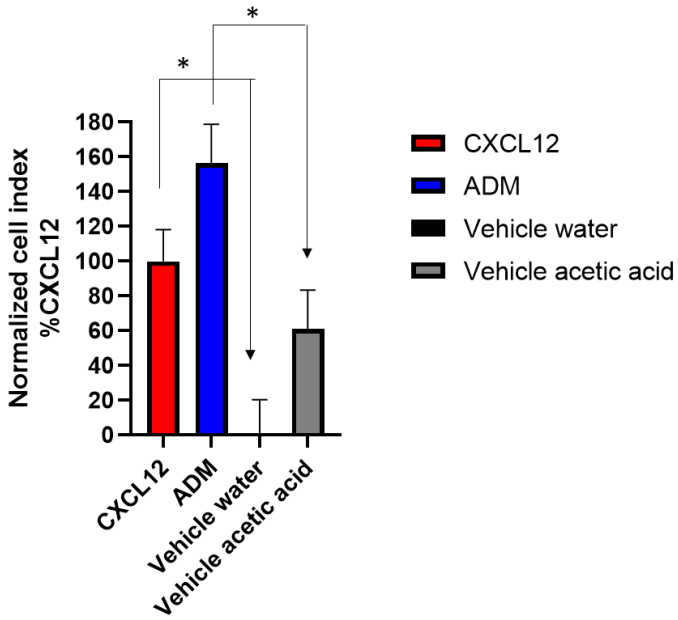


Fig. 3.4. Both CXCL12 and ADM increase xCELLigence cell index in hACKR3-mEGFP-expressing Flp-In T-REx 293 cells. The graph is the analysis of the combination of n=4 independent experiments. Both CXCL12 (100 nM) and ADM (1 μM) induce a statistically significant increase in response compared to vehicle (*, $p < 0.5$, one-way ANOVA).

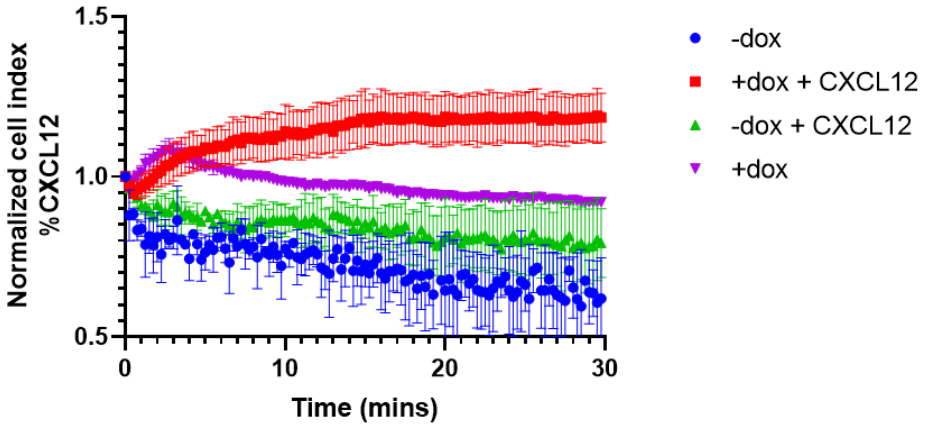


Fig. 3.5. CXCL12 and doxycycline treatment induce a major response in Flp-In T-REx 293 cells compared to untreated cells belonging to the same clone. The figure represents a n=1 experiment. It shows the effects observed when inducing hACKR3 expression via doxycycline treatment (+dox and +dox/+CXCL12 in legend, respectively purple and green) when compared to cells where doxycycline treatment was not performed (-dox and -dox/ +CXCL12, respectively blue and red). The intensity of the response was measured as variation of cell index over a 30 min time period.

As hACKR3 is considered to produce signals only via β-arrestin recruitment and fails to signal via G proteins of the inhibitory G_i family, I then used xCELLigence RTCA assays on hACKR3-mEGFP expressing Flp/In T-REx 293 cells after treatment with either pertussis toxin or barbadin (**Fig. 3.6**). Pertussis toxin (PTX) is secreted by the gram-negative bacterium *Bordetella pertussis*. When this toxin crosses the plasma membrane, the subunit A catalyses the ADP-ribosylation of $G_{\alpha i}$, locking the G protein in its inactive (GDP bound) state and preventing its activation by GPCRs. By contrast barbadin is reported to be a β-arrestin/β2-adaptin interaction inhibitor, hence potentially blocking the agonist-promoted endocytosis of the receptor if this route is engaged.

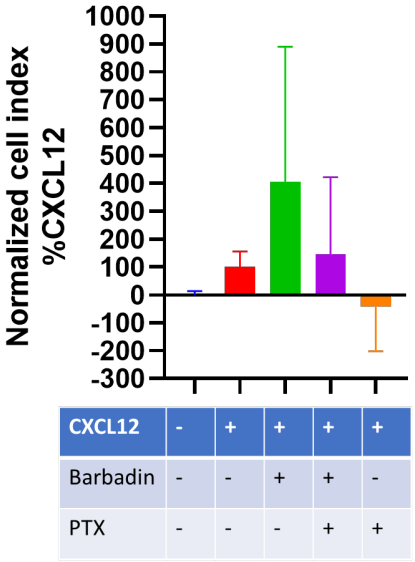


Fig. 3.6. Barbadin and pertussis toxin treatment affect ACKR3 functioning with diverse outcomes. The figure represents a combination of n=2 independent experiments, in which each condition have been tested in triplicate on a 96-well E-plate. The figure shows Flp-In T-REx 293 cells expressing hACKR3-mEGFP with CXCL12 or vehicle and barbadin, pertussis toxin (PTX) separately and/or in combination. The intensity of the response was measured as variation of cell index over a 30 min time period.

Fig. 3.6 shows a trend about how barbadin and PTX affect hACKR3 activation. As shown in previous xCELLigence experiments in this chapter, CXCL12 promoted the activation of hACKR3 (**Fig. 3.6**, red bar) compared to vehicle (**Fig. 3.6**, blue bar). This activation was hugely increased by administering barbadin (5 µM) to cells and incubating them for 30 mins before adding CXCL12 (**Fig. 3.6**, green bar). Even though it is known from literature the ability of hACKR3 of recruiting G proteins, it has been demonstrated that this receptor fails to generate a signalling response through them. Therefore, a possible role of G proteins in affecting hACKR3 activation was investigated by adding PTX (200 ng/ml) to cells overnight before barbadin (30 mins before adding agonist) and CXCL12 on the day of the assay (**Fig. 3.6**, purple bar). This re-established activation levels of hACKR3 almost to CXCL12-only condition, evidencing a role of PTX in affecting CXCL12-promoted hACKR3-activation. As an additional control, cells were pre-treated with PTX before adding CXCL12 and in absence of barbadin (**Fig. 3.6**, orange bar). This treatment completely abolished the activation of hACKR3 initially observed after CXCL12-treament, and this strengthens the hypothesis considered previously about

the possibility of G proteins (and in particular of $G\alpha_{i/0}$) in regulating CXCL12-mediated activation of hACKR3 receptor.

However, because of the high statical error of these last experiments, it is hard to say whether the explanation is strong enough, and further experiments or different approaches should be used.

3.4 Characterization of adrenomedullin effects on ß-arrestin recruitment to hACKR3 using a bystander Bioluminescence Resonance Energy Transfer assay

hACKR3 is believed to signal only via β-arrestin recruitment. I therefore explored interactions between hACKR3 and β-arrestin-2 by using a bystander Bioluminescence Resonance Energy Transfer (BRET) approach. Unlike the standard BRET approach, where a GPCR of interest is tagged either with a nanoluciferase or with a fluorescent protein, in the 'bystander' assay an untagged receptor, in this case hACKR3 is used. hACKR3 was co-transfected with an internal ribosome entry site plasmid (pIRES) that allows for expressing two genes in mammalian cells from the same bicistronic transcript. These were β-arrestin-2-nanoluciferase and mNeonGreen which when expressed becomes linked to the plasma membrane via incorporation of the fatty acylation motif of the Lyn non-receptor tyrosine kinase. Initially I assessed the effect of varying concentrations of CXCL12 in a set of kinetic assays (**Fig. 3.7A**). Concentrations are expressed as nano/micromolar in case of kinetics experiments, while they are expressed in logarithmic scale in case of concentration-response curve. pEC_{50}/pIC_{50} values are the negative logarithm of EC_{50}/IC_{50} values.

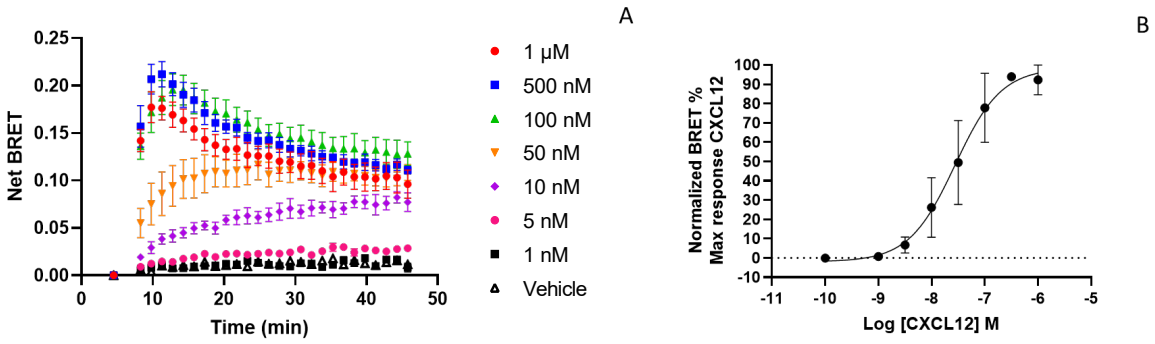


Fig. 3.7. CXCL12-promotes β-arrestin-2 recruitment to hACKR3. A) Increasing concentrations of CXCL12 were used to treat HEK293T cells transiently co-transfected to express hACKR3 and with a pIRES vector able to express both β-arrestin-2-nanoluciferase and Lyn11-mNeonGreen. Samples were measured every 90 seconds for 45 minutes after agonist addition. The association kinetics of hACKR3 and β-arrestin-2 increased with CXCL12 concentration. B) A concentration-response curve was generated by selective values obtained from the kinetic curves (20 mins after agonist addition). The graph shows a concentration-dependent effect of CXCL12 in recruiting β-arrestin-2 to the receptor. pEC_{50} for CXCL12 in this assay was 7.5 ± 0.4 (n =3)

These showed that CXCL12 promoted robust β-arrestin-2 recruitment into proximity with hACKR3 in a concentration-dependent manner. They also showed that the kinetics of this measurement varied with different concentrations of CXCL12 tested. Indeed, a peak is observed at 10 mins upon CXCL12 addition at concentration ≥ 100 nM. This peak is not observed in concentrations tested up to 50 nM instead, although the receptor is still activated. This could be explained by the possibility that hACKR3 could be active but not internalized yet, while at higher concentrations, a quick and transient overactivation of the receptor may lead to internalization of a portion of hACKR3 expressed. Therefore, the BRET signal generated is still positive, but it lowers progressively across the time. Also, this peak could result from a concentration-dependent conformation of hACKR3-β-arrestin 2 complex, which can be quickly de-associated upon agonist physiological depletion. By taking the net BRET values after exposure to CXCL12 for 20 minutes it was possible to generate a concentration-response curve to CXCL12 with pEC_{50} 7.5±0.4. (**Fig. 3.7B**).

To further characterize the robustness and sensitivity of the assay and its ability to explore the pharmacological profile of hACKR3, a small synthetic ligand that has been described as an inverse agonist at this receptor was tested. This ligand (VUF16840) was a generous gift from Prof. Rob Leurs, from Vrije University of Amsterdam. Increasing concentrations of VUF16840 decreased the effect of a fixed concentration of CXCL12 (EC_{80} = 80 nM) in a concentration-dependent fashion (**Fig. 3.8**).

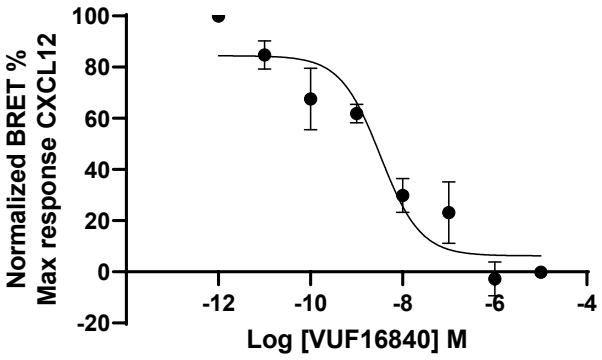


Fig. 3.8. VUF16840 blocks CXCL12-promoted β-arrestin-2 recruitment to hACKR3. HEK293T cells were transiently co-transfected to express hACKR3 and with a pIRES vector able to express both β-arrestin-2-nanoluciferase and Lyn11-mNeonGreen. Cells were pre-treated for 15 mins with varying concentrations of VUF16840 before adding a fixed concentration (80 nM) of CXCL12 (EC_{80} as assessed from **Fig 3.6B**) and reading the plate for 45 minutes. The concentration-response curve for this small ligand was generated choosing a single time point in the plateau phase of the kinetic curve (20 mins). The graph is representative of n=3 independent experiments. The calculated pIC50 for VUF16840 in this assay was 8.4 ± 0.3.

These experiments showed good suitability of bystander BRET assay to detect β-arrestin-2 recruitment to hACKR3. Because the experimental conditions required for the quality of the assay were assessed with the previous experiments, the same conditions and protocol were used to determine whether ADM had an effect in promoting β-arrestin-2 recruitment to hACKR3. However, although a substantial effect was observed when using an exceptionally high concentration of ADM, no significant effect was observed at concentrations at or below 1 μM (**Fig 3.9A** and **Fig. 3.9B**). Additionally, even considering the possibility of comparing earlier time points between CXCL12 and adrenomedullin, the current data did not differ substantially (data not shown), showing that the time point chosen (20 mins) can be

indicative in the same way as an early one. At an earlier time point there could be the risk of a higher window for CXCL12, due to the nature of the ligand effect on kinetics of hACKR3 (as reported in **Fig.3.7A**).

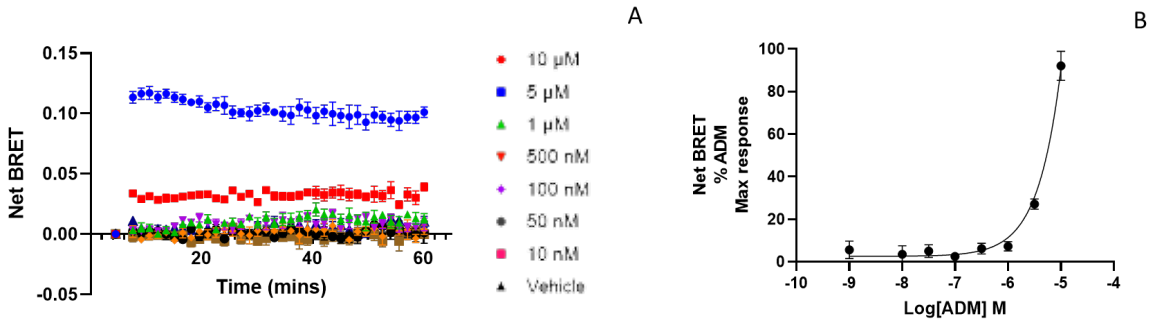


Fig. 3.9. Adrenomedullin is able to promote recruitment of β-arrestin-2 to hACKR3 only at very high concentrations. The figure is a representative of n=2 independent experiments. A) Kinetics studies performed using increasing concentrations of ADM to treat HEK293T cells co-transfected transiently with hACKR3 and the pIRES vector as described in **Figures 3.6** and **3.7** before reading the plate for 60 minutes. B) The concentration-response curve was generated choosing a single time point in the plateau phase of the kinetic curve (20 mins).

Adrenomedullin was not able to efficiently promote β-arrestin-2 recruitment to hACKR3 except at the highest concentrations tested (> 1 µM). This means it was impractical to assess a pEC_{50} value.

Although several assays were performed to determine whether interactions between adrenomedullin and hACKR3 could exist, this possibility cannot be ruled out yet, because only higher concentrations of adrenomedullin seem to exert an effect on hACKR3, with important consideration about the physiological relevance of this outcome. Therefore, many further analyses would be required to (dis)prove this hypothesis.

3.5 Discussion

As discussed earlier in this chapter, the aim of this sub-project was determining a possible role of adrenomedullin ligand at hACKR3. hACKR3 is considered a scavenger receptor for many ligands, such as CXCL12, opioids and adrenomedullin. The scavenging activity of this receptor consists in regulating the clearance of designated ligands and, by this way, influencing the signalling of other GPCRs. The first scavenger receptor was described in the late 1970s by Brown and Goldstein and was defined by its ability to bind and subsequently internalize low density lipoproteins (LDLs) (Patten and Shetty, 2018). This process can be particularly important in both homeostatic and disease conditions, as they detect and remove, or scavenge, unsolicited ligands self-antigens from general circulation.

Adrenomedullin (ADM) is an endogenous agonist for calcitonin-like receptor (CLR), in complex with one of three receptor activity-modifying proteins (RAMPs) and, specifically, for CLR complexed with RAMP2 or RAMP3, which create the adrenomedullin-1 (AM1) or adrenomedullin-2 (AM2) receptors (Weston et al., 2016). This hypotensive peptide was originally discovered in human pheochromocytoma by monitoring the elevating activity of platelet cAMP. Because this peptide was found to be abundant in normal adrenal medulla as well as in pheochromocytoma tissue originating from adrenal medulla, it was designated as "adrenomedullin" (Kitamura et al., 1993). However, in the past years, different groups showed a possible role of this ligand at hACKR3 as an agonist, with the hypothesis that hACKR3 could act as a scavenger receptor for this ligand. Adrenomedullin has been shown to be involved in regulation of several physiological process, such as the promotion of cardiac development, vasodilation and formation of blood and lymph vessels, with important clinical implications. As a first way to screen whether adrenomedullin could be a ligand for hACKR3, an xCELLigence RTCA assay was used, as firstly reported by Stallaert and colleagues (2012). This is based on the principle that the adhesion of cultured cells onto an array microelectrodes embedded at the bottom of wells of a microtitre plate induces changes in the local ionic environment at the electrode/solution interface, conferring an increase in electrode impedance. The result of this is that any changes in cell physiological properties modulating the physical contact between cell and electrode (i.e. morphology, adhesion), will be reflected by changes in the measured impedance between the measurement taken before seeding the cells (background), which is subtracted from the cell index values generated following cell attachment.

This approach has become popular recently in pharmacology and drug discovery because of the possibility of understanding the possible effects of a drug on cells and on particular receptor expressed. This approach was successfully used by Xing and colleagues in measuring the effects of drugs in cardiomyocytes (Xing et al., 2005) or by Boitano and colleagues, which showed the effects of novel compounds acting as agonists for the Protease-Activated Receptor 2 (PAR2) (Boitano et al., 2011).

Data from the xCELLigence RTCA assay showed in this chapter, in which Flp-In T-REx 293 cells stably expressing hACKR3-mEGFP were treated with adrenomedullin, showed an increase response after treatment with this ligand, demonstrating an activation of hACKR3 promoted by adrenomedullin at the minimum concentration of 1 μM. This strengthened the hypothesis already reported in literature of adrenomedullin as an agonist for this receptor.

Looking at xCELLigence experiments, where cells derived from same cell line were tested with CXCL12 after pertussis toxin treatment (PTX) and barbadin addition, two small ligands that block, respectively, $G_{\alpha i/o}$ proteins and a β-arrestin/β2-adaptin interaction, interesting data were generated. According to outcomes derived from these experiments, the blockade of $G_{\alpha i/o}$ proteins affected the signal increase generated as response after CXCL12 addition. This does not surprise, since hACKR3 has been reported to be able to interact and recruit G proteins but fail at activating a signalling pathway through them (Rajagopal et al., 2010). This may suggest that, even though the G proteins do not activate a proper signalling cascade for this receptor, they may have a regulatory role in facilitating CXCL12 effect on hACKR3 and, in a way, may affect also β-arrestin-2 recruitment pathway, since this is the signalling cascade of choice for this receptor (Schafer et al., 2023). On the other side, treatment with barbadin also showed an important aspect to take in consideration. In fact, by blocking the β-arrestin/β2-adaptin interaction, the CXCL12-promoted endocytosis was inhibited. This also affected the response generated after adding CXCL12 to cells, translating a visible increased signal observed when compared to CXCL12 without barbadin. This may find an explanation in a possible major localization of the receptor on the plasma membrane. This results in a disruption of the physiological role of hACKR3 as scavenger receptor, also supported by fluorescence microscopy images reported earlier in this chapter, where receptor seems to be constitutively internalized and grouped in clusters placed intracellularly.

Despite the known localization of the receptor in the cells, it was hard to better characterize the phosphorylation and/or the functionality of the receptor expressed by Flp-In T-REx stable cells via immunoblots, since no valid antibodies specific for hACKR3 are commercially available. Some of them (mostly provided from 7TM antibodies company) were tested as a small side-project before making them commercially available, but unsuccessfully. This led to choose an anti-FLAG (DYKDDDDK) antibody to detect the receptor via immunoblots, since the construct encoding for hACKR3 showed a FLAG tag at the N-terminal side of the receptor. However, when β-arrestin recruitment was assessed for hACKR3, few considerations have been done. While CXCL12 promoted β-arrestin recruitment in a dose-dependent manner, adrenomedullin, even though it has been proposed to be an agonist at hACKR3, showed an effect in stimulating β-arrestin recruitment at this receptor almost only at the highest concentration used (10 μM), in agreement with what has been shown by Meyrath et al. (2021). In their paper, this group reported a partial activity of adrenomedullin in triggering, at the highest concentration tested (9 μM), about 50% of the maximum response observed with the full agonist CXCL12. Because only 10 μM were able to induce an evident positive BRET signal, it may be possible to consider this as an off-target effect, since the concentration levels of adrenomedullin required to promote β-arrestin recruitment to hACKR3 are hugely over the range of adrenomedullin plasma levels in human body in physiological state (range of picomolar/liter, rather than micromolar as observed in vitro). Nishikimi et al. (1995) reported a physiological level of adrenomedullin of 2.5 pmol/liter in control group tested in parallel with heart failure-affected group, where this value was increased according to different severity of this pathological condition. Also, in rats platelet, this peptide was shown to elevate cAMP levels at the maximal concentration of 1 μM. This concentration is still out of the range observed by the few works reported in literature where *in vitro* approaches were used (Meyrath et al., 2021). Indeed, another group from Marcus Thelen reassessed the adrenomedullin scavenging activity of hACKR3 but they did not observe any significant effect, although adrenomedullin seemed to induce proliferation of lymphatic endothelial cells (Sigmund et al., 2023).

Additionally, in plasma, adrenomedullin is specifically bound to adrenomedullin binding protein-1 (AMBP-1), which was later identified as complement factor H (Garayoa et al., 2000). Adrenomedullin bound to complement factor H cannot be easily detected in plasma, so it has been theorized that the total plasma

adrenomedullin could be higher than reported in most studies. Also, the kinetic of this peptide is fast, since the circulating adrenomedullin is rapidly degraded with a half-life of 16-20 minutes (Dupuis et al. 2005, Larrayoz et al., 2014).

To conclude, while adrenomedullin has a significant positive effect in stimulating the receptor by interacting with hACKR3 at a more general cellular level that remains not fully understood, as discussed before, it is hard to confirm solidly whether this ligand may exert an agonist activity in promoting a signalling cascade for this receptor at concentrations that are compatible with general human physiology. Because of the physiological role that adrenomedullin has in human body and the big potential in drug discovery and clinical relevance, it is important to further characterize the interaction adrenomedullin-hACKR3 and the concrete effect of this interaction. These considerations still leave open questions on how to achieve this goal, to further understand the pharmacological profile of this receptor with the aim of developing new drugs based on adrenomedullin interacting with hACKR3, to translate into new therapeutic strategies for pathological conditions in which this receptor is involved.

4. CHAPTER 4 (Results part 2) - Characterization of the effects of hCXCR4-adrenomedullin interactions

The characterization of adrenomedullin-hCXCR4 interactions finds its rationale in the importance of hACKR3 for hCXCR4 physiology and pharmacology, due to its reported role in regulation of hCXCR4 functions (Levoye et al., 2009; Luker et al., 2009), with one of the main results of this interaction consisting of attenuation of hCXCR4-induced rise in intracellular Ca^{2+}. Moreover, an enhanced expression of both receptors has been detected in a wide range of tumours and cells residing within the tumour microenvironment.

hCXCR4 and hACKR3 share CXCL12 as a ligand, with CXCL12 considered to be an endogenous agonist for hCXCR4, but hACKR3 has been proposed to bind several other ligands of various natures, including adrenomedullin. Another important feature of the connection between these two receptors is their localization in the body. While hCXCR4 is ubiquitously expressed in non-hematopoietic cells and in all leukocytes, the expression pattern of hACKR3 is still poorly characterized and a matter of debate (Murphy and Heusinkveld, 2018; Infantino et al., 2006).

Because of their localization, the CXCL12/hCXCR4/hACKR3 signalling pathways have been primarily described in immune cells or in model systems. Because these two receptors can heterodimerize (Martínez-Muñoz et al., 2018), a new hypothesis about a possible interaction between adrenomedullin and hCXCR4 was considered, with the possibility that this ligand may regulate the CXCL12/hCXCR4/hACKR3 axis as a key player in several pathophysiological conditions.

In this chapter, I aimed to characterize the possible interactions between adrenomedullin and hCXCR4, to enlighten the molecular basis of the mechanism of action of this interaction.

4.1 Generation of FLAG-hCXCR4-mEGFP and of a Flp-In T-REx stable cell line able to express this construct

Details of vector constructions have been described in Chapter 2 "Materials and methods", section 2.3.12. Briefly, a hCXCR4 construct with an N-terminal FLAG epitope tag and C-terminal monomeric enhanced green fluorescent protein (mEGFP) in pcDNA5/FRT/TO was a kind gift from Dr. Richard J. Ward (University of Glasgow). After confirmation of the correct sequence, this construct was successively transfected into Flp-In T-REx 293 cells to generate a cell line able to allow expression of the receptor construct in an inducible fashion. Induction was performed routinely by adding doxycycline overnight.

4.2 Assessment of receptor expression and localization

Once obtained, cells of this clone were validated for expression and membrane localisation of the receptor. Cells were treated with increasing concentrations of doxycycline to establish how the expression of the receptor construct varied with such treatment. Cell lysates obtained were resolved by SDS-PAGE and then immunoblotted with an anti-CXCR4 primary antiserum (7TM Antibodies, Jena, Germany), which identified the C-terminal sequence of hCXCR4. Such immunoblots were then exposed to an infrared dye-labelled donkey anti-rabbit secondary antibody (**Fig. 4.1**).

As shown in **Fig. 4.1**, the predominant polypeptide detected was generated in a doxycycline-induced fashion and migrated with Mr (relative molecular mass) in the region of 65-70 kDa, as anticipated. Higher molecular mass polypeptides were also detected with Mr corresponding to some 125 kDa-160 kDa. As already explained for hACKR3 in the previous chapter relatively to **Fig.3.1**, the higher molecular mass polypeptide detected is probably represented by oligomeric form of hCXCR4. The presence of oligomeric forms of the receptor can depend on using specific experimental conditions, such as the temperature. Indeed, decreasing the temperature chosen for heating the sample before loading them on a gel, and simultaneously increasing the incubation time improves the quality of the bands detected in SDS-PAGE, especially for GPCRs (Drube et al., 2022). It is evident that concentrations of doxycycline as low as 1 ng/ml were able to induce detectable expression of the receptor. However, as this was not reduced by treatment with higher concentrations of doxycycline then to achieve the highest levels of expression of the receptor, 100

ng/ml doxycycline was chosen as the standard concentration to use in subsequent experiments. However, no additional controls were used, such as immunoblotting with an antibody for a specific housekeeping protein (i.e. actin), since the purpose of this experiment was to test the right concentration of doxycycline to use to induce hCXCR4 expression.

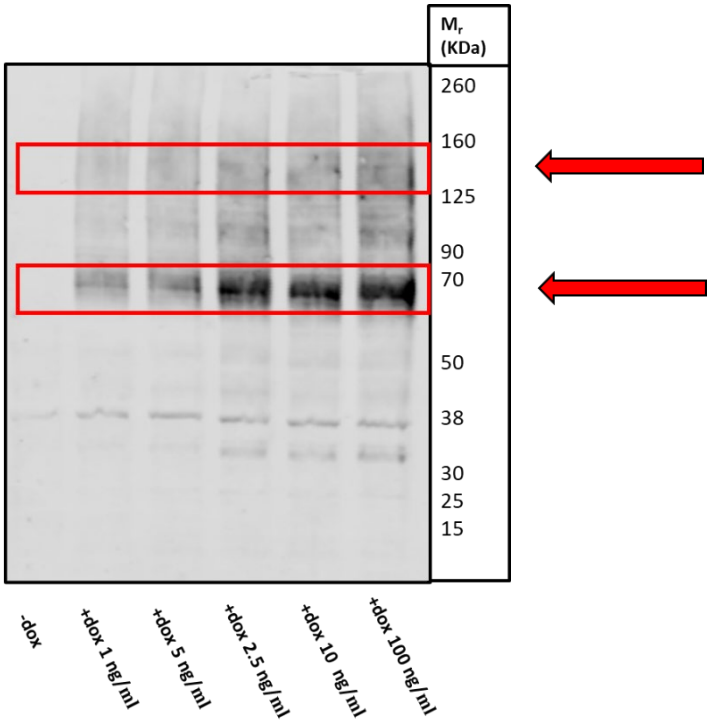


Fig.4.1. Detection of the expression of FLAG-hCXCR4-mEGFP in Flp-In T-REx 293 stable cells by immunoblot. Flp-In T-REx 293 cells able to express FLAG-hCXCR4-mEGFP in a doxycycline-inducible manner were treated with the indicated concentrations of doxycycline overnight, resolved by SDS-PAGE and immunoblotted using an anti-CXCR4 antiserum. The anticipated molecular mass of the receptor construct is 68 kDa (40 kDa hCXCR4, 27 kDa mEGFP and 1 kDa FLAG tag). The red arrows indicate the correct band of the receptor hCXCR4.

To determine whether the receptor expressed by these cells was localized either to the plasma membrane or intracellularly, cells from this clone were grown on coverslips according to the protocol described in chapter 2, section 2.7.1, and induced with 100 ng/ml doxycycline treatment overnight, before examining the intensity of the fluorescent signal. The fluorescence of mEGFP was detected by fluorescence microscopy using a Zeiss LSM 880 confocal microscope (**Fig. 4.2**).

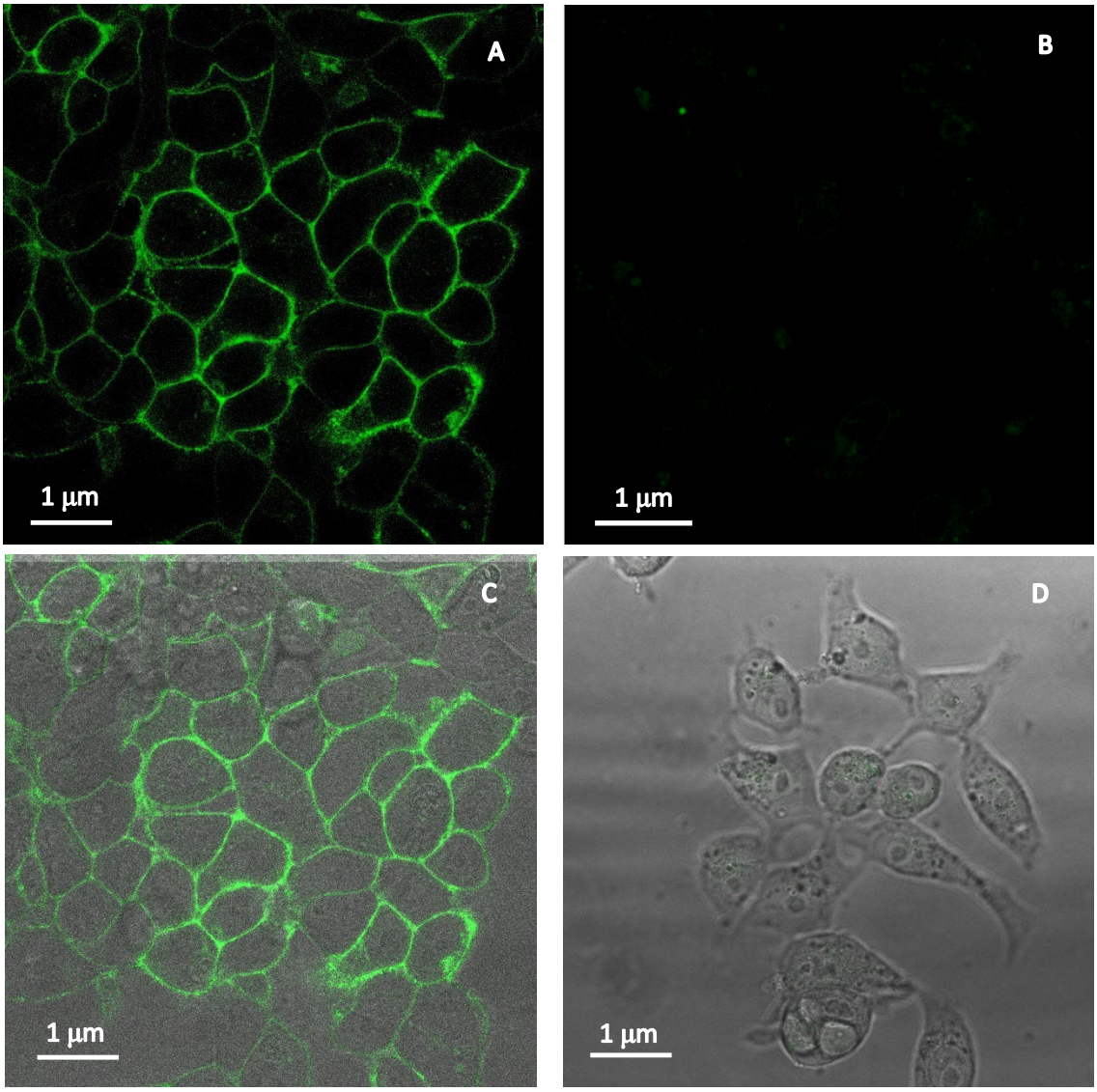


Fig. 4.2. Assessment of CXCR4 localization in FLAG-hCXCR4-mEGFP-expressing Flp-In T-REx 293 cells by fluorescence microscopy. Flp-In T-REx 293 cells able to express FLAG-hCXCR4-mEGFP were examined by fluorescence microscopy either after (**panel A**) and without (**panel B**) overnight doxycycline treatment (100 ng/ml). **Panel C** and **panel D** show the bright field filter applied to cells respectively treated and untreated with doxycycline for comparison.

This analysis showed the localization of the hCXCR4 construct, that appears mainly localized homogeneously along the cell membrane. The signal observed was clearly receptor-specific since this was absent without treatment with doxycycline. As both immunoblots and confocal microscopy confirmed doxycycline-induced receptor expression I proceeded to perform assays to assess functionality of the receptor construct.

4.3 xCELLigence RTCA approach to assess the role of CXCL12 and ADM at hCXCR4

To determine whether CXCL12 and adrenomedullin (ADM) would have measurable effects at hCXCR4, xCELLigence RTCA (Real Time Cell Analysis) studies were carried out (see Chapter 2 "Material and Methods" and Chapter 3 "Characterization of hACKR3-adrenomedullin interactions"). As for hACKR3, CXCL12 was used as a reference agonist as it is well established to be an endogenous ligand of hCXCR4. Since no interaction between ADM and hCXCR4 is currently known from literature, I tested two different concentrations (100 nM and 1 μM) of this ligand to determine the most suitable required to observe any potential regulation of CXCR4, either in the presence or in the absence of CXCL12. As for hACKR3, the concentration chosen for CXCL12 (100 nM) resulted from previous literature where the potency of this agonist was assessed in functional assays. Additionally, this value was confirmed in the experiments I carried out during this project. However, because the main purpose of xCELLigence in this case was to determine an effect of the ligand towards hCXCR4, no concentration-response curve was carried out because too expensive, in particular for adrenomedullin.

When added to cells, CXCL12 (100 nM) added to Flp-In TREx 293 cells stably expressing hCXCR4-mEGFP generated a clear time-dependent increase in signal that reached a maximum within 20 mins (**Fig 4.3A, Fig. 4.3B**) and a plateau reached in 30 mins. Although effects of the vehicle were observed (**Fig 4.3A**), a clear separation of the extent of the cell response to vehicle and to CXCL12 were observed (**Fig 4.3A, Fig. 4.3B**). By comparison ADM alone generated a more limited time-dependent increase in the signal with a plateau reached within 15 mins. However, this was not statistically significant compared to the response generated by the vehicle (**Fig. 4.3B**). Although vehicle seems to generate a response, this does not represent an activation of the receptor and could be due to cell environment. For example, too many cells could have been plated or cells seeded were in clumps, with the results of lowering the window generated during the assay (Hamidi et al, 2017).

By contrast, when pre-stimulating cells with ADM (either 100 nM or 1 μM) for 15 mins before adding 100 nM CXCL12, the response initially observed in CXCL12-only treated cells dropped significantly (especially in presence of 1 μM ADM) (**Fig. 4.3B**). Indeed, this reduction resulted, respectively, in 59.0 ± 11.0% of positive response generated by CXCL12-stimulation (in presence of 100 nM ADM pre-

treatment), and 32.0 ± 4.0% of positive response generated by CXCL12-stimulation (in presence of 1 μM ADM pre-treatment), as shown in **Fig. 4.3.B**.

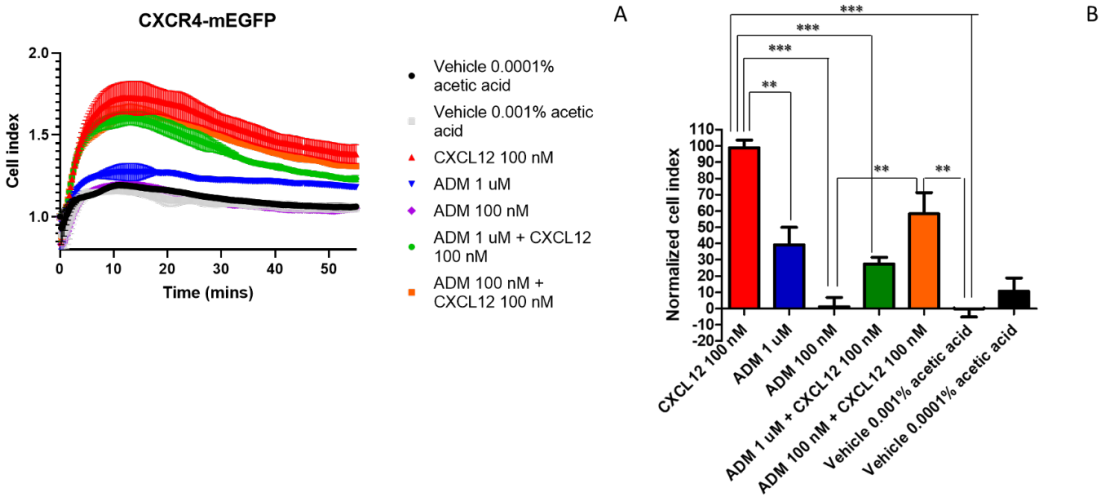


Fig. 4.3. Effects of CXCL12 and ADM on hCXCR4-mEGFP-expressing Flp-In T-REx 293 cells measured using xCELLigence RTCA. Representative traces are shown from n=3 independent experiments. In each case, kinetic curves recorded after treatment either with CXCL12 (100 nM) or ADM (1 μM or 100 nM), or pre-treating cells with ADM for 15 mins before adding CXCL12 demonstrated effects of these ligands compared to vehicle (water for CXCL12 and 0.001% acetic acid in water for ADM). The intensity of the response was measured as variation of cell index over a 60 min time period (**panel A**). CXCL12 (100 nM, red bar) and ADM (1 μM, blue bar) induce a statistically significant increase in response compared to vehicle (*, $p < 0.5$, one-way ANOVA) (panel B), while 100 nM ADM failed to induce a significant increase in response compared to vehicle (**panel B**). When cells from the same clone were pre-stimulated with ADM before adding CXCL12 the intensity of the response measured was lower than cells in presence of CXCL12 only (**panel B**, 1 μM ADM, green bar, 32.0 ± 4% of full response generated by CXCL12; 100 nM ADM, orange bar, 59.0 ± 11% of full response generated by CXCL12). The reduction observed was statistically significant compared to vehicle and CXCL12 (*, $p < 0.5$, one-way ANOVA).

As an additional control, also Flp-In T-REx 293 cells in absence of doxycycline treatment were used to assess whether the responses generated by ligands used in this assay were specific and determined by CXCR4 expression (**Fig. 4.4**). In **Fig. 4.4**, cells induced with doxycycline (+dox) to express hCXCR4 generated a higher response compared to cells in absence of doxycycline (-dox). However, this response was higher in presence of CXCL12 stimulation, as expected from previous

experiments. The presence of an effect on +dox cells might be due to a basal activity of the receptor combined with the addition of water to the vehicle, which can cause a small change in the cell environment which the plate reader records as a partial positive signal. Also, the quality and the number of the cells seeded could affect this response, such as the number of cells plated per well or the presence of clumps, which may elevate the cells index and reduce the window between cells treated with the agonist and untreated cells. Another point is that CXCL12 seems to induce a partial response when added to cells not induced with doxycycline. Flp-In T-REx 293 cells are HEK cells derivatives and hCXCR4 is relatively low endogenously expressed in HEK cells (Atwood et al., 2011). Then, this partial response could be due to a partial presence of hCXCR4 expressed by these cells as a response to CXCL12 treatment.

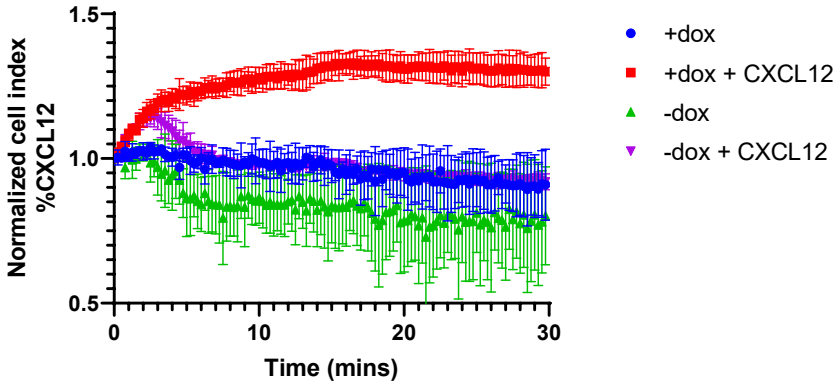


Fig. 4.4. CXCL12 and doxycycline treatment induce response in Flp-In T-REx 293 cells able to express hCXCR4-mEGFP compared to untreated cells. The figure shows the effects of inducing hCXCR4 expression via doxycycline treatment (+dox) and activating this with CXCL12 (+dox/+CXCL12) compared to cells where doxycycline treatment was not performed (-dox and -dox/+CXCL12). The intensity of the response was measured as variation of cell index over a 30 min time period.

4.4 Characterization of ADM effects on $G_{\alpha i/o}$ protein signalling at hCXCR4 using a [^{35}S]GTPγS incorporation assay.

Once the possibility of an adrenomedullin-mediated effect on CXCR4 had been established, different functional assays were carried out to assess the impact of this ligand on CXCR4-signalling pathways. Since the ability of this receptor to signal through $G\alpha_{i/o}$ proteins was known from the literature, the first assay of choice for studying this signalling pathway was a [^{35}S]GTPγS incorporation assay, in which it is

possible to follow the activation of the receptor by assessing the binding of this GTP-analogue to downstream activated G protein(s). This substrate is not hydrolysed during the physiological G protein signalling pathway steps initiated as the consequence of agonist-mediated GPCR activation. Therefore, the response observed is related to the activation of $G\alpha_{i/0}$ proteins.

In **Fig. 4.5**, membranes derived from Flp-In T-REx 293 cells expressing hCXCR4-mEGFP after addition of doxycycline were pre-exposed to ADM for 15 mins before adding a fixed concentration of CXCL12 (EC_{80} = 20 nM, previously calculated from agonist test) or, membranes were stimulated with different concentrations of CXCL12 agonist in the absence of adrenomedullin. In the presence of agonist only, there was an evident concentration-dependent effect in the activation of $G_{\alpha i/o}$ protein mediated by CXCL12 (pEC_{50}: 8.3 ± 0.5, red). This CXCL12-promoted $G\alpha_{i/0}$ signalling of hCXCR4 was limited by pre-exposure of membranes expressing the receptor to ADM, again in a concentration-dependent fashion (pIC_{50} = 7.8 ± 0.7, blue). Concentrations are expressed in logarithmic scale in case of concentration-response curve. pEC50/pIC50 values are the negative logarithm of EC50/IC50 values.

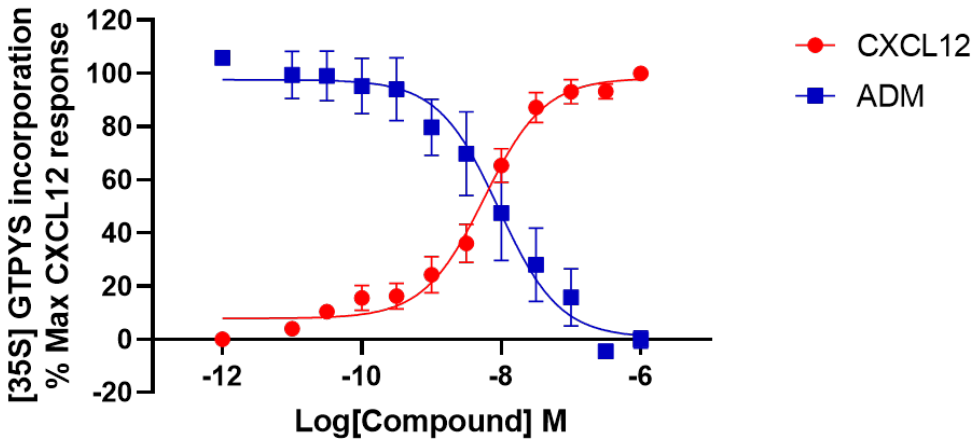


Fig. 4.5. [^{35}S]GTPγS incorporation assay using membranes of hCXCR4-mEGFP expressing cells. The graph is the analysis of the combination of n=6 (CXCL12) and n=4 (ADM) independent experiments. CXCL12 promotes $G\alpha_{i/o}$ protein activation in a concentration-dependent manner (pEC_{50}: 8.3 ± 0.5). When membranes were pre-treated with ADM before addition of CXCL12 (EC_{80}), ADM prevented this effect (pIC_{50}: 7.8 ± 0.7).

These data show a possible antagonistic effect of ADM at hCXCR4, with higher concentrations of this ligand able to inhibit more efficiently the activation of $G\alpha_{i/0}$ proteins. Further experiments were performed as controls by comparing effects of these ligands in cells expressing hCXCR4-mEGFP with cells not induced with doxycycline. In this case, in membranes derived from cells which were treated with increasing concentrations of CXCL12 or pre-treated with ADM before adding a fixed concentration of CXCL12 (EC_{80}) but not induced with doxycycline, no significant activation or inhibition of $G\alpha_{i/o}$ activation were observed (**Fig. 4.6)**.

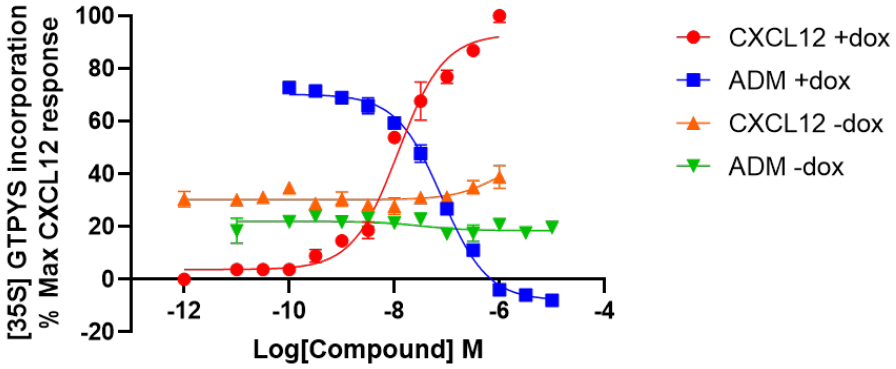


Fig. 4.6. The effect of ADM and CXCL12 on [^{35}S]GTPγS incorporation requires the expression of CXCR4. The graph is the analysis of the combination of n=2 independent experiments. In cells induced to express hCXCR4-mEGFP, CXCL12 promotes $G\alpha_{i/o}$ protein activation in a concentration-dependent manner (pEC_{50}: 8.3 ± 0.2). When membranes prepared from cells deriving from the same clone were pre-treated with ADM before addition of CXCL12 (EC_{80}), ADM prevented this effect (pIC_{50}: 7.8 ± 0.3). In membranes derived from cells treated with the same ligands but not induced with doxycycline, no significant activation or inhibition of $G\alpha_{i/o}$ activation was observed.

The effect of a known CXCR4 antagonist called IT1t (Ward et al., 2021) was also assessed in the same way in cells expressing hCXCR4-mEGFP. This is a drug-like isothiourea derivative that has been used previously as a reference antagonist for this receptor. **Fig. 4.7** shows that IT1t potently antagonised CXCL12-mediated $G\alpha_{i/o}$ protein activation in a concentration-dependent fashion. The figure is a combination of n = 2 independent experiments only, since this ligands has been previously characterized by Ward and colleaugues (2021), where similar data were reported.

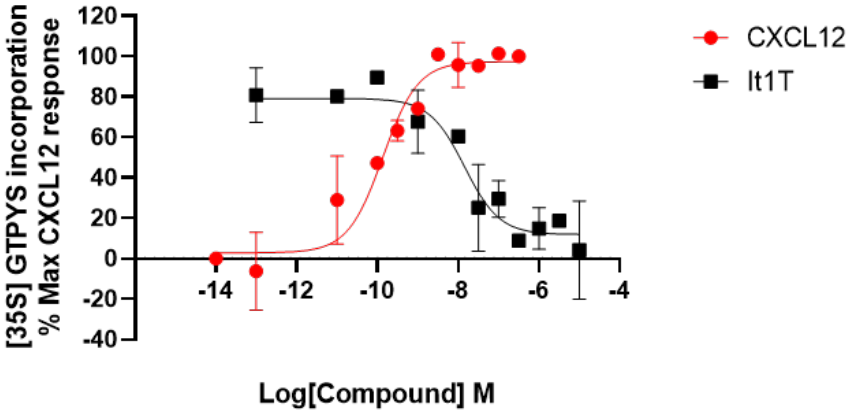


Fig. 4.7 [^{35}S]GTPγS incorporation assay in CXCR4-mEGFP membranes shows an antagonist effect of IT1t on CXCL12-mediated G protein activation. The figure shows analysis of n=2 independent experiments. $G\alpha_{i/o}$ protein activation was promoted by CXCL12 was concentration-dependent (red, pEC_{50}: 8.8 ± 0.5). Inhibition of an EC_{80} concentration of CXCL12 was observed with pre-treatment with IT1t (black, pIC_{50}: 7.4 ± 0.2).

This experiment demonstrated that IT1t displayed a potent inhibitory effect on G protein activation similar to the effect observed with adrenomedullin, providing certainty about the robustness of the assay and the effects caused by adrenomedullin.

4.5 Characterization of adrenomedullin effects on G protein signalling at hCXCR4 via a cAMP accumulation assay

A cAMP accumulation assay was used as an additional approach to characterize the G protein signalling of hCXCR4. This functional assay helps in investigating $G\alpha_{i/0}$ or $G\alpha_s$ activity by measuring the production of the intracellular second messenger cAMP, regulated via G proteins. In the specific case of receptors signalling via $G\alpha_{i/0}$, which is the category to which hCXCR4 belongs, once $G\alpha_{i/0}$ protein is activated upon agonist stimulation, one of the consequences is the inhibition of adenylyl cyclases, leading to reduced production of intracellular cAMP. The role of the cAMP is physiologically important, because it regulates the activity of cAMP-dependent protein kinase A (PKA), which plays an important role in a variety of downstream cellular processes, and other cellular effectors, such as exchange proteins directly activated by cAMP (EPAC), popeye domain containing proteins (Popdc) and cyclic nucleotide–gated ion channels (CNG channels).

Also in this case, adrenomedullin was tested to further confirm the results obtained with the [^{35S}]GTPγS incorporation assay. Additionally, IT1t was used as a reference antagonist for hCXCR4. In this assay CXCL12 reduced cAMP levels in a concentration-dependent manner with pEC_{50}: 8.8 ± 0.6 (**Fig. 4.8),** whilst adrenomedullin (pIC_{50}: 8.0 ± 0.7) prevented the effect of an EC_{80} concentration of CXCL12, causing an increase of intracellular cAMP. Concentrations are expressed in logarithmic scale in case of concentration-response curve. pEC_{50}/pIC_{50} values are the negative logarithm of EC_{50}/IC_{50} values.

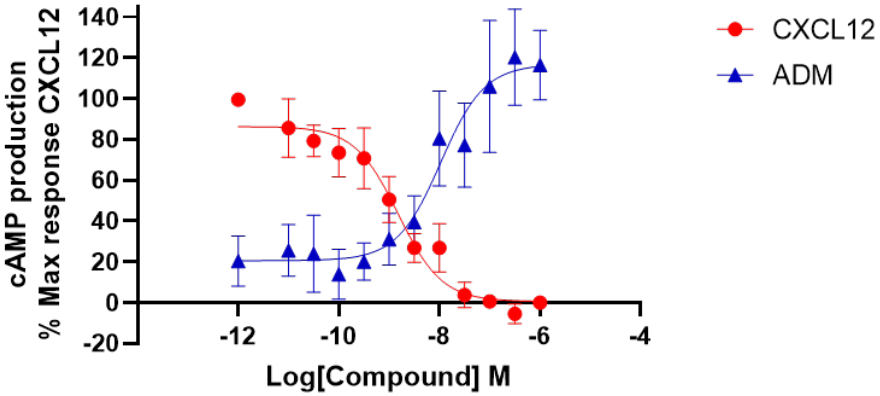


Fig. 4.8. CXCL12 reduces cAMP accumulation in hCXCR4-mEGFP-expressing Flp-In T-REx 293 cells and adrenomedullin reverses this effect. The data show mean ± SEM from n=10 and n=5 independent experiments, respectively, for CXCL12 and ADM. CXCL12 inhibited forskolin-amplified cAMP levels (pEC_{50}: 8.8 ± 0.5) whilst adrenomedullin (pIC50: 8.0 ± 0.7) prevented the effect of an EC_{80} concentration of CXCL12.

As reported in **Fig. 4.8,** hCXCR4 signalling by CXCL12 through $G_{\alpha i/o}$ proteins, reduced forskolin-amplified cAMP levels. When cells pre-treated with ADM for 15 mins before adding a fixed (EC_{80}) concentration of CXCL12 (63 nM, previously calculated from the agonist test), the opposite was observed. This shows that ADM can prevent CXCL12-induced inhibition of adenylyl cyclase, resulting in an increased intracellular cAMP level. Therefore, ADM seems to antagonize CXCL12-mediated G protein activation in a similar way to that observed in [^{35}S]GTPγS incorporation assay. Additionally, in the concentration-response curve of ADM, the vehicle start from a value of 20%. This value is the result of the normalization on the

percentage of the maximal response generated by CXCL12. The vehicle contains only the EC_{80} of CXCL12, but it does not contain ADM. This means that, in the normalization process, a signal is obtained because the effect observed is due mostly to the action of CXCL12. This does not differ substantially from the lowest concentration tested of ADM (10^{-11}, or 0.01 nM) in presence of 63 nM CXCL12 added to cells.

As further control, adrenomedullin was also tested alone, to show if there was any partial activation of the receptor's signalling activity. **Fig. 4.9** shows in a bar graph a comparison between vehicle, adrenomedullin (1 µM) and CXCL12 (1 µM) in generating HTRF signal, without plotting with cAMP standard curve. This shows that ADM was unable to mimic CXCL12. This enforces the outcomes obtained from previous experiments in which adrenomedullin acted as an antagonist for CXCL12-mediated G protein signalling of hCXCR4.

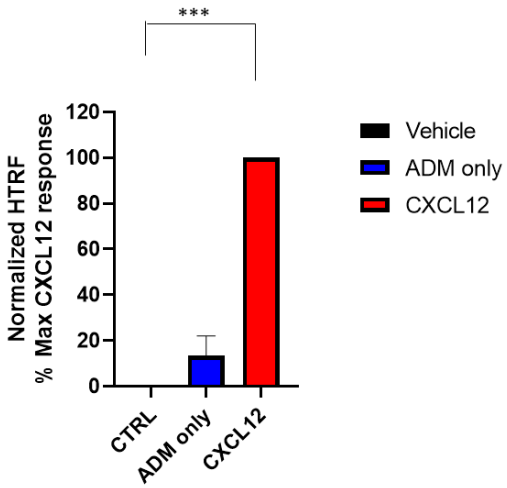


Fig. 4.9. ADM does not directly activate hCXCR4-mEGFP to regulate cAMP levels. Data are means ± SEM of n=3 independent experiments. Both ADM and CXCL12 were tested at the concentration of 1 µM. ADM was not able to generate a statistically significative HTRF signal when compared to vehicle (*, $p < 0.5$, one-way ANOVA).

As further control to assess the quality of this assay in detecting the antagonism of a ligand for CXCR4, the small ligand antagonist IT1t was used as a reference inhibitor of CXCL12 activity.

The results were analysed as previously described and the data are represented in **Fig. 4.10**. IT1t caused an increase of cAMP accumulation at higher concentrations administered to the cells after their pre-treatment with different concentrations of this ligand for 15 mins before adding a fixed concentration (EC80) of CXCL12 agonist. This is a result of the block of the $G_{\alpha i/o}$ protein signalling pathway, as previously reported in literature by Ward et al. (2021).

These outcomes confirm the quality of this assay in investigating $G_{\alpha i/o}$ protein signalling for hCXCR4 and that the effects observed with ADM are reliable.

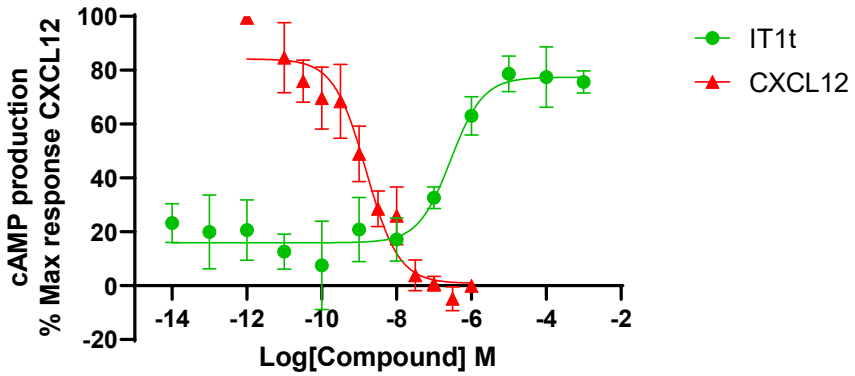


Fig. 4.10. cAMP accumulation assay with IT1t treatment of hCXCR4-mEGFP-expressing Flp-In T-REx cells shows blockade of CXCL12-mediated inhibition of adenylyl cyclase activity. The graph is the analysis of the combination of n=10 and n=4 independent experiments, respectively, for CXCL12 and IT1t. CXCL12 displayed a concentration-dependent effect in blocking the activity of adenylyl cyclase that causes a reduction in intracellular cAMP level (pEC_{50}: 8.8 ± 0.6). IT1t (pIC_{50} 7.2 ± 0.4), added for 15 minutes to the cells followed by a fixed concentration of CXCL12 (EC_{80}), acted to reverse the effect of CXCL12 on adenylyl cyclase activity, leading to an increase of intracellular cAMP levels.

4.6 Adrenomedullin does not antagonize CXCL12-mediated hCXCR4 ß-arrestin interactions

GPCRs can signal through various signalling pathways: previously in this chapter, G protein signalling for CXCR4 was characterized. In this section, β-arrestin

recruitment for hCXCR4 will be assessed. As a consequence of outcomes reported earlier for adrenomedullin, the next step in characterization of hCXCR4-adrenomedullin interaction consisted of assessing whether this ligand might affect β-arrestin recruitment to hCXCR4. I therefore explored interactions between hCXCR4 and β-arrestin-2 by using a bystander Bioluminescence Resonance Energy Transfer (BRET) approach.

As previously described in Chapter 3, when the same assay was used to characterize hACKR3-β-arrestin-2 interaction, and in the relevant paragraph in Materials and Methods chapter, in the 'bystander' BRET assay an untagged receptor, in this case hCXCR4, is used. This was co-transfected with an internal ribosome entry site (pIRES) plasmid that allows expression of two proteins in mammalian cells from the same bicistronic transcript. These were β-arrestin-2-nanoluciferase and mNeonGreen. When the mNeonGreen is expressed, it becomes linked to the plasma membrane because its sequence has been modified to incorporate the fatty acylation motif of the Lyn non-receptor tyrosine kinase. Initially I assessed the effect of varying concentrations of CXCL12 on hCXCR4 mediated β-arrestin-2-nanoluciferase translocation to the vicinity of mNeonGreen in a set of kinetic assays (**Fig. 4.11A**). Concentrations are expressed as nano/micromolar in case of kinetics experiments, while they are expressed in logarithmic scale in case of concentration-response curve. pEC_{50}/pIC_{50} values are the negative logarithm of EC_{50}/IC_{50} values.

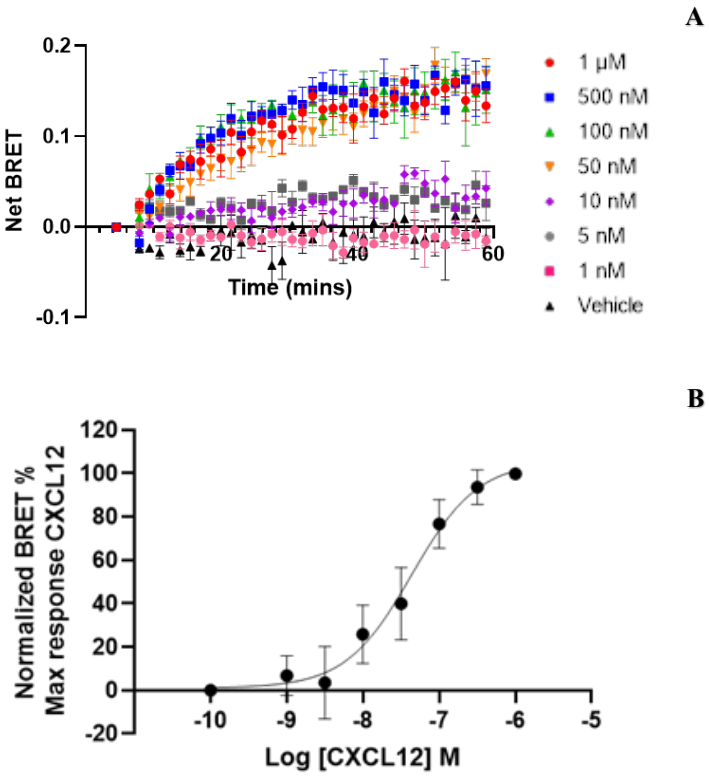


Fig. 4.11. CXCL12-promotes β-arrestin-2 recruitment to hCXCR4. A) Increasing concentrations of CXCL12 were used to treat HEK293T cells transiently co-transfected to express hCXCR4 and with a pIRES vector able to express both β-arrestin-2-nanoluciferase and Lyn11-mNeonGreen. BRET was measured every 90 seconds for 60 minutes after agonist addition. The association kinetics of hCXCR4 and β-arrestin-2 increased with CXCL12 concentration. B) A concentration-response curve was generated by selecting values obtained from the kinetic curves (25 mins after agonist addition). The graph shows a concentration-dependent effect of CXCL12 in recruiting β-arrestin-2 to the receptor. pEC_{50} for CXCL12 in this assay was 7.4 ± 0.3. (n = 9)

These results showed that CXCL12 promoted robust β-arrestin-2 recruitment into proximity with hCXCR4 in a concentration-dependent manner. They also showed that the kinetics of this measurement varied with different concentrations of CXCL12 tested. By taking the net BRET values after exposure to CXCL12 for 25 minutes it was possible to generate a concentration-response curve to CXCL12 with pEC_{50} 7.4 ± 0.3 (**Fig. 4.11B**).

To further characterize the robustness and sensitivity of the assay and its ability to explore the pharmacological profile of hCXCR4, the small ligand antagonist IT1t was also tested in this assay as previously used in other approaches. Therefore, HEK293T cells were transiently co-transfected with untagged hCXCR4 and the pIRES and pre-treated for 15 mins with increasing concentrations of IT1t, before adding a fixed concentration of CXCL12 (EC_{80}, 150 nM, calculated previously from agonist test). It was observed that increasing concentrations of IT1t decreased the effect of CXCL12 in a concentration-dependent fashion (**Fig. 4.12A**). Also in this case as previously seen for CXCL12, the kinetics of these measurements varied with different concentrations of IT1t tested, as shown in **Fig. 4.12A,** with pIC50 7.8 ± 0.4 (**Fig .4.12B**).

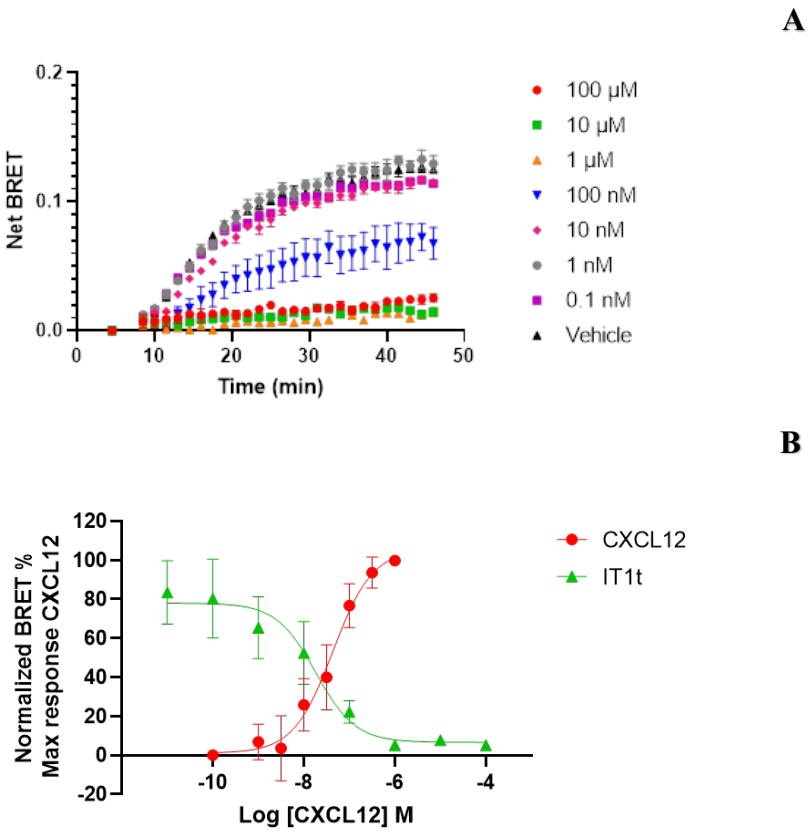


Fig. 4.12. IT1t blocks CXCL12-mediated β-arrestin-2 recruitment to hCXCR4. A) Increasing concentrations of IT1t were used to pre-treat HEK293T cells transiently co-transfected to express hCXCR4 and a pIRES vector able to express both β-arrestin-2-nanoluciferase and Lyn11-mNeonGreen before adding a fixed concentration of CXCL12 agonist (EC_{80}). Samples were measured every 90 seconds for 60 minutes after agonist addition. The association kinetics of hCXCR4 and β-arrestin-2 decreased with increasing IT1t concentrations. B) A concentration-response curve was generated by taking values obtained from the kinetic curves (25 mins after agonist addition). The graph displays a concentration-dependent effect of CXCL12 in recruiting β-arrestin-2 to the receptor (red) and a concentration-dependent effect of IT1t in blocking this recruitment (green). The calculated pEC_{50} for CXCL12 in this assay was 7.4 ± 0.3 (n = 9), while calculated pIC_{50} for IT1t was 7.8 ± 0.4 (n = 4).

These experiments displayed good suitability of the bystander BRET assay in detecting β-arrestin-2 recruitment to hCXCR4, also in presence of an antagonist. Because the experimental conditions required for the quality of the assay were

assessed with the previous experiments, the same conditions and protocol were used to determine whether ADM had an effect in promoting β-arrestin-2 recruitment to hCXCR4. I tested ADM either in absence of CXCL12 or by pre-treating HEK293T cells transiently co-transfected with untagged hCXCR4 and pIRES before adding an EC_{80} concentration of CXCL12.

The outcome of these experiments is reported in **Fig. 4.13**. When ADM was added alone to cells, no significant BRET signal was recorded at any concentration tested, suggesting that this ligand does not promote β-arrestin-2 recruitment to hCXCR4 (**Fig. 4.13A**). When cells were pre-treated with ADM before adding a fixed concentration of CXCL12, the BRET signal due to the presence of agonist was unaffected (**Fig. 4.13B**). This indicates that ADM was not able to block the CXCL12-mediated β-arrestin-2 recruitment to hCXCR4. As ADM was ineffective in this assay but was able to prevent CXCL12-induced G protein activation, this suggests that ADM may act as biased antagonist ligand for hCXCR4.

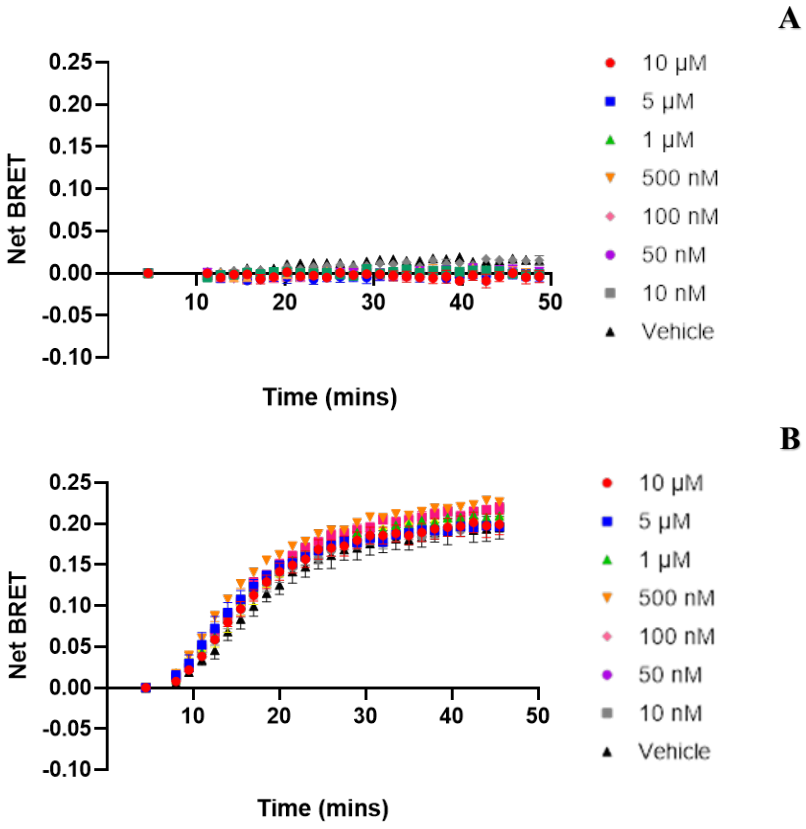


Fig. 4.13. ADM does not alter β-arrestin-2 recruitment to hCXCR4. A) Representative traces of n = 2 independent experiments are shown. Increasing concentrations of ADM were used to treat HEK293T cells transiently co-transfected to express hCXCR4 and a pIRES vector able to express both β-arrestin-2-nanoluciferase and Lyn11-mNeonGreen. Samples were measured every 90 seconds for 50 minutes after ligand addition. ADM did not generate any significant BRET signal, showing no interaction between hCXCR4 and β-arrestin-2. B) Representative traces of n = 2 independent experiments are shown. When added to cells for 15 mins before adding a fixed concentration of CXCL12 (EC_{80}), ADM was not able to block β-arrestin-2 recruitment to hCXCR4 even at the highest concentration of ligand tested. CXCL12 showed a positive BRET response, as previously reported.

4.7 Characterization of adrenomedullin effects on hCXCR4 phosphorylation using phosphosite-specific antisera

Another way to characterize hCXCR4 activation and the effect of its interaction with adrenomedullin that I used employed phosphosite-specific antisera via immunoblots to investigate the phosphorylation of hCXCR4 upon treatment with different ligands. Phosphorylation of intracellular serine and threonine residues is an important post-translational modification of many GPCRs. After agonist exposure, these receptors acquire an active conformation, which is recognized by a family of highly specialized GPCR kinases (GRKs). Agonist-driven phosphorylation by GRKs regulates acute receptor desensitization, arrestin recruitment, internalization, and post-activation signalling.

Phosphosite-specific 7TM antisera are designed to specifically detect agonist-activated GPCRs and were purchased from 7TM antibodies and Invitrogen. The intracellular C-terminal region of hCXCR4 contains several residues that may be subject to phosphorylation: $pSer^{324}/Ser^{325}$, $pSer^{330}$, $pSer^{338}/Ser^{339}$, $pSer^{346}/Ser^{347}$. The localization of these phosphosites is represented in **Fig.4.14.** Since a potential $pSer^{346}/Ser^{347}$ antiserum I had access to did not work very efficiently, no immunoblots are shown using this antiserum. The $pSer^{338}/Ser^{339}$ antiserum (Invitrogen), as well as $pSer^{324}/Ser^{325}$ (7TM antibodies) were already commercially available and I characterized a further antiserum, $pSer^{330}$ (7TM antibodies) to determine whether it was sensitive and suitable to use for such studies.

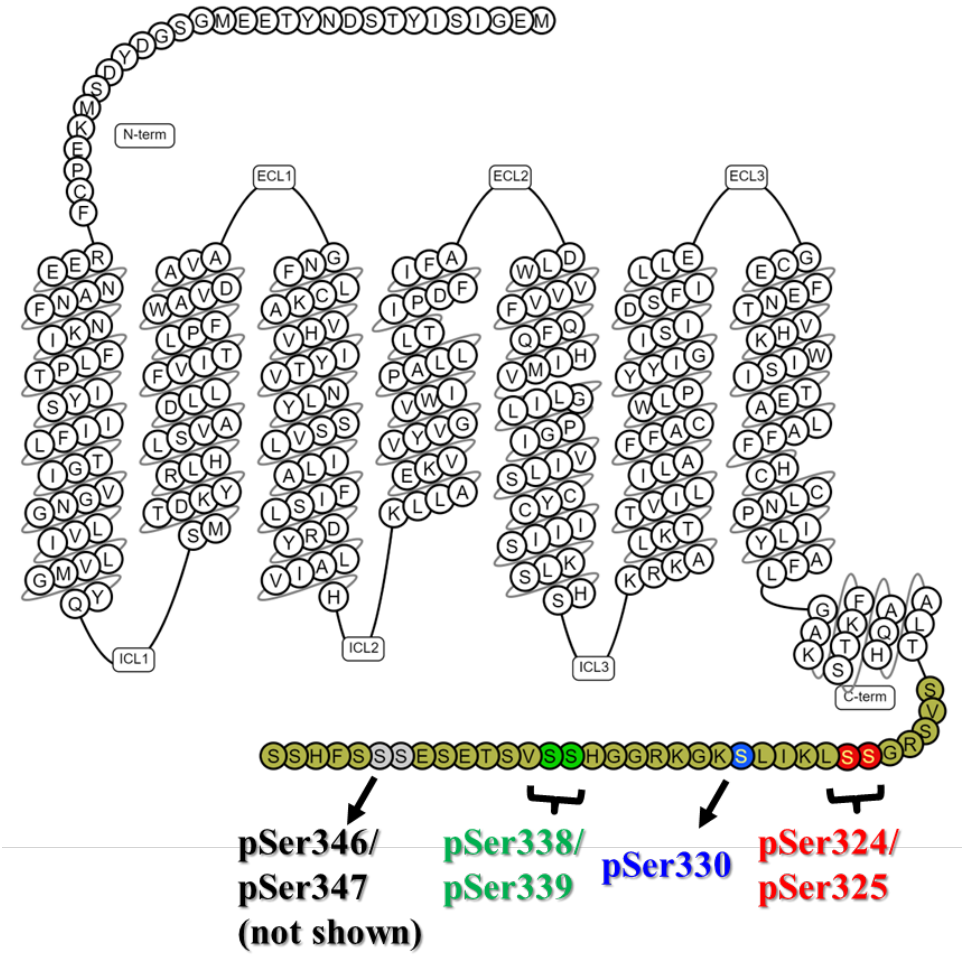


Fig. 4.14. hCXCR4 contains several sites within its C-terminus that may be subject to phosphorylation. Potential phosphosites located in the C-terminus (colored differently than the rest of hCXCR4 structure) are shown: pSer^{324}/Ser^{325} (red), pSer^{330} (blue), pSer^{338}/Ser^{339} (green), pSer^{346}/Ser^{347} (grey, data not shown). Phosphosite-specific antisera targeting these sites were generated in rabbits by commercial providers. The figure was generated by using the GPCR database (https://gpcrdb.org).

To determine whether adrenomedullin would influence phosphorylation of hCXCR4, stable Flp-In T-REx 293 cells expressing hCXCR4-mEGFP after addition of doxycycline were (pre)treated with the ligand of interest (CXCL12 and/or ADM) according to the conditions already described in Chapter 2, Materials and Method section, paragraph 2.5.3 before preparing cell lysates to use for these experiments. I also assessed the effect of IT1t in these studies which represents a good control since this ligand has already been characterized in the past and its effects on hCXCR4 are known.

Since the receptor was tagged with mEGFP, all samples were immunoprecipitated using a GFP-TRAP kit (Chromotek) to enrich their yield and facilitate their detection via immunoblot (as described in Chapter 2, Materials and Methods section, paragraph 2.5.4). I firstly started the characterization of the effects of adrenomedullin on hCXCR4 by separating protein samples by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and successively transferring protein samples onto a nitrocellulose membrane. Each sample loaded onto the gel contains an estimated quantity of 250 μg of lysate, which were originally calculated by using the BCA assay. However, it is hard to quantify the exact concentration of the samples after the immunoprecipitation step, because for the nature of the protocol the amount loaded onto the gel is quantified in total μL rather than μg. However, each lane should contain the same amount of cell lysate once loaded onto the gel. After incubating with blocking buffer, primary antiserum solution and secondary antiserum solutions were added before scanning the membranes using a LICOR system.

Fig. 4.15 displays the effects of CXCL12 (100 nM), IT1t (10 μM) + CXCL12 and ADM (1 μM) + CXCL12 on hCXCR4 phosphorylation after immunoblotting with a structural anti-CXCR4 (not phosphorylation state-dependent) antiserum (**Fig.4.15A**), anti-CXCR4 $pSer^{324}/Ser^{325}$ (**Fig. 4.15B**), anti-CXCR4 $pSer^{330}$ (**Fig. 4.15C**), or anti-CXCR4 $pSer^{338}/Ser^{339}$ (**Fig. 4.15D**) phosphosite-specific antisera.

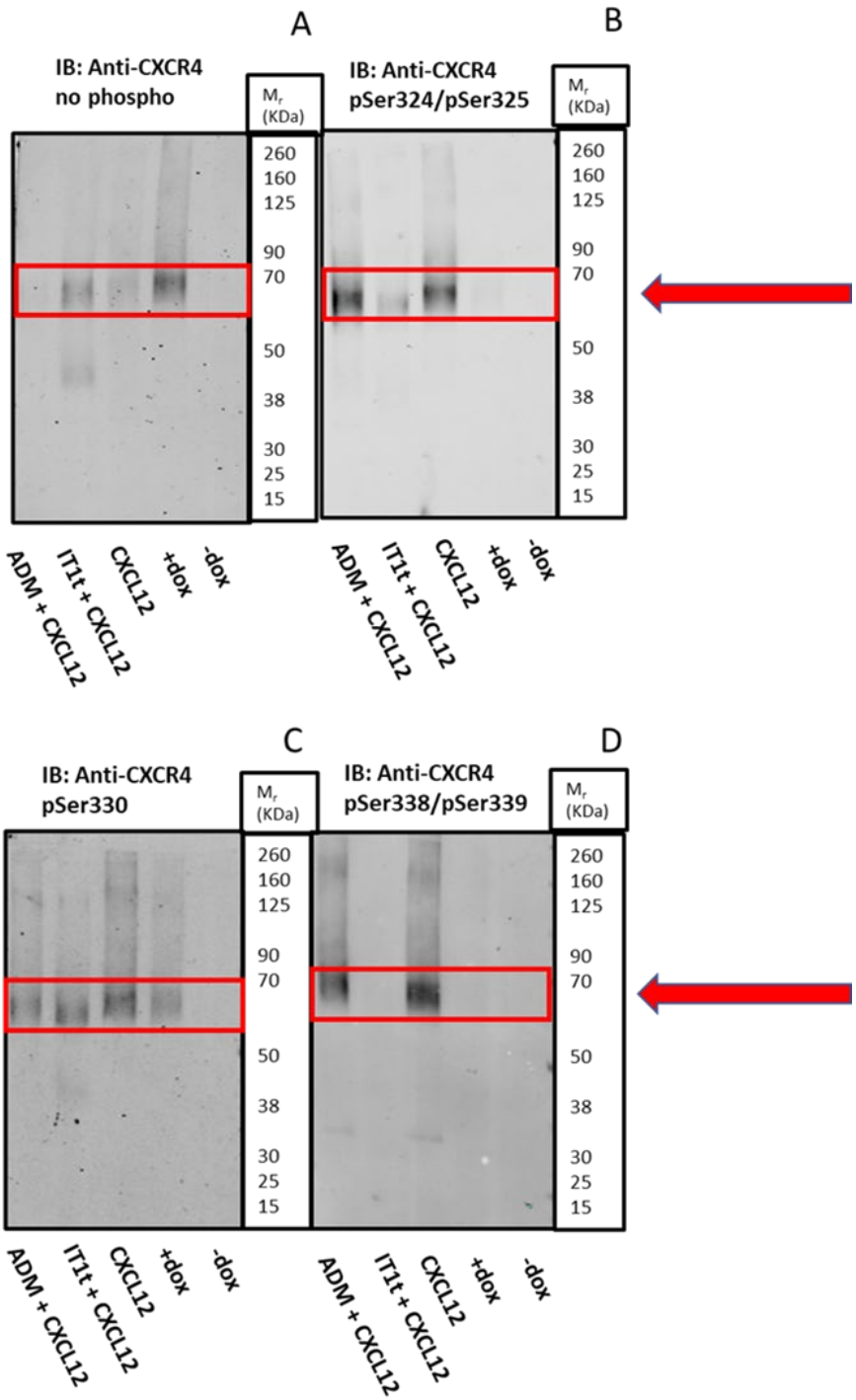


Fig. 4.15: Different ligands display diverse effects on hCXCR4 phosphorylation. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with the indicated ligands as reported in **Table 4.1** before preparing immunoprecipitated lysates from these cells. Samples were resolved by SDS-PAGE and immunoblotted by using anti-CXCR4 not phospho-state antiserum (**panel A**), anti-CXCR4 pSer^{324}/Ser^{325} (**panel B**), anti-CXCR4 pSer^{330} (**panel C**) or anti-CXCR4 pSer^{338}/Ser^{339} (**panel D**). In all blots shown, the samples are (from left to right): ADM (1 μM) + CXCL12 EC_{80} (100 nM), IT1t (10 μM) + CXCL12 EC_{80}, CXCL12 EC_{80}, +doxycycline only, -doxycycline. The figure shows representative traces of n = 3 independent experiments.

While CXCL12 treatment was carried out for 5 mins, pre-stimulation with IT1t and ADM was carried out for 15 mins before adding CXCL12 for the same amount of time as would be used in absence of antagonists. Looking at all immunoblots shown in **Fig. 14.15**, a first feature that is clear is the presence of different phosphorylation levels for different conditions tested when membranes were incubated with an anti-CXCR4 no phospho antiserum (**Fig. 4.15A**). Indeed, the detection of the receptor decreased in presence of treatment with CXCL12, which makes more difficult the detection of hCXCR4 compared to the vehicle condition (+ dox), at the point that the relative band has almost "disappeared". IT1t pre-treatment before adding CXCL12 seems to be comparable to + dox condition, while the pre-stimulation with ADM did not alter the effect of CXCL12 on hCXCR4, making the detection of the receptor as reduced as CXCL12 only condition, although the band relative to ADM is very clear due to the export of the image. These effects observed might be explained by the possibility that CXCL12-mediated phosphorylation of the receptor might alter the ability of the antiserum to identify hCXCR4.

In fact, in **Fig. 4.14**, it is shown the presence of several serine residues along the C-terminal domain of the receptor which may be highly phosphorylated upon CXCL12 binding. The high phosphorylation levels of this region could lead to a conformational change in the receptor which causes a steric hindrance making harder the detection of the receptor by this antiserum. Indeed, antisera provided by 7TM are generated based on C-terminus side of the receptor used as tagging epitope for receptor detection once these antisera are produced in the species of interest. However, because IT1t effectively blocks CXCL12-mediated hCXCR4 activation, it is reasonable to think that IT1t restores the phosphorylation state of the receptor to a level comparable to +dox. On the contrary, this effect is not observed in presence of ADM, which does not seem to alter the phosphorylation state of hCXCR4, and it could fail in reversing CXCL12-mediated effects on antiserum detection.

Fig. 4.15B shows the effects of ligands on hCXCR4 in membranes immunoblotted with anti-CXCR4 $pSer^{324}/pSer^{325}$ phosphosite-specific antiserum. This was already commercially available when I started the characterization of the phosphorylation of hCXCR4. In this case, CXCL12 showed an activation of the receptor, confirmed by the higher intensity of the band representing treatment with this agonist when compared to vehicle (+dox). This activation was effectively blocked by IT1t, while ADM did not alter this activation.

I characterized the newly generated anti-CXCR4 pSer^{330} phosphosite-specific antiserum, whose results are showed in **Fig. 4.15C**. Here it is still present a minor activation of hCXCR4 mediated by CXCL12, but the increase observed in this case is minimal when compared to +dox condition. This might be due to constitutive phosphorylation of the receptor on this phosphosite, which may be CXCL12-independent. Besides, the reduction in signal observed by IT1t pre-stimulation was less relevant compared to that observed with anti-CXCR4 pSer^{324}/pSer^{325} phosphosite-specific antiserum. Similar conclusion can be done for ADM pre-stimulation, which did not differ substantially from CXCL12 only condition.

Lastly, immunoblot with anti-CXCR4 pSer^{338}/pSer^{339} phosphosite-specific antiserum is shown in **Fig. 4.15D**. By using this antiserum from Invitrogen, it was possible to observe the agonist-dependent activation of hCXCR4 upon CXCL12 stimulation. This activation was not affected by ADM pre-treatment, which did not differ from CXCL12 addition only. No bands are detected for +dox and IT1t + CXCL12, showing that this antiserum correctly detects phosphorylated forms of hCXCR4 upon agonist addition.

Moreover, differences in the mobility were observed in each immunoblots upon IT1t pre-stimulation before adding CXCL12 agonist. Although in SDS-PAGE proteins should be resolved according to their negative charges, the presence of serine-enriched regions can cause a higher molecular weight which could slow down proteins' mobility on SDS-PAGE (Kinoshita et al., 2006; Kirkwood et al., 2013; Zhu et al., 2023). Therefore, CXCL12, phosphorylating the receptor might lead to a higher band compared to IT1t, which blocks the phosphorylation of hCXCR4 and can affect the molecular weight of the receptor on SDS-PAGE. However, a good quantification of phosphorylation levels upon ligands addition was harder with the anti-CXCR4 no phospho antiserum.

So, for this reason, membranes were also incubated in parallel with an anti-GFP control antibody, which is shown in **Fig. 4.16**. In this case all samples, including adrenomedulliun, show a comparable intensity and molecular weight except joint IT1t + CXCL12 treatment. This was consistently found in n = 3 different experiments and it may be due to the possible effects on IT1t in increasing the receptor density of hCXCR4 at higher concentrations and in presence of long-term treatment, as explored in the past by Ward et al. (2021). Indeed, during the wash steps carried out during immunoprecipitation of samples, IT1t was kept in the wash buffer for the

entire duration of the process, leading to longer times of treatment. Thus, this could result into a higher intensity band, while other ligands do not show a similar effect. The presence of specific bands for each sample confirms that in the previous immunoblots the samples are all present in the same amount in the gels. Because the construct expressed by these cells is hCXCR4-mEGFP, where "m" stands for monomeric (Ward et al., 2021), the absence of the upper band is clearly due to the specific detection only of the monomeric form of the GFP.

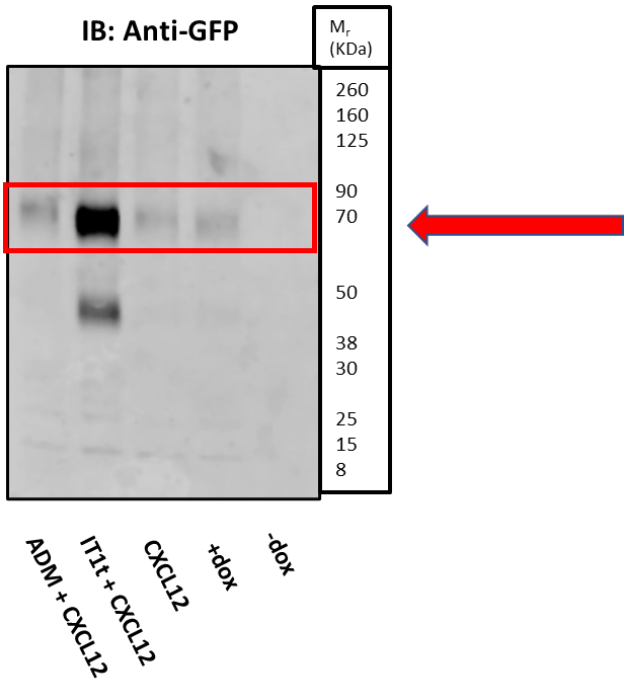


Fig. 4.16: Immunoblot with anti-GFP antibody as control shows the same band intensity for all immunoprecipitated samples but IT1t + CXCL12 condition. The figure is a representative of n=3 separate experiments carried out in parallel with other immunoblots previously shown. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with the indicated ligands as previously reported, before preparing immunoprecipitated lysates from these cells. Samples were resolved by SDS-PAGE and immunoblotted by using an anti-GFP antibody. Samples are, from left to right: ADM (1 µM) + CXCL12 EC_{80}, IT1t (10 µM) + CXCL12 EC_{80}, CXCL12 EC_{80} (100 nM), +doxycycline only, -doxycycline.

The anti-GFP antibody used as control helped the quantification of the phosphorylation levels detected by different phosphosite-specific antisera and confirmed the considerations made earlier in this paragraph. In fact, **Fig. 4.17** reports

as bar graph a combination of n = 3 independent experiments, in which the values were normalized based on the luminescence percentages generated by the anti-GFP antibody. Data expressed as normalized mean values ± SEM were analysed by using one-way ANOVA test and where */**/*** are shown, they mean that the data observed are statistically significant.

Anti-CXCR4 pSer^{324}/Ser^{325} (**Fig. 4.17A**) antiserum showed statistically significant (*, $p < 0.5$, one-way ANOVA) increases in luminescence upon CXCL12 (100.0 ± 0.5%) and CXCL12+ADM (113.0 ± 23.0%, **, $p < 0.5$, one-way ANOVA) treatments when compared to -dox (0.1 ± 1.4%) and +dox (0.7 ± 1.5%). This means that the phosphorylation at this phosphosite is agonist-dependent upon CXCL12-mediated stimulation. Also, the decreased luminescence of CXCL12 observed in presence of IT1t pre-stimulation was statistically significant (*, $p < 0.5$, 6.4 ± 8.3%), showing the efficacy of IT1t in blocking the CXCL12-promoted phosphorylation of the receptor at this site. The effect of the pre-stimulation with ADM was statistically significant only compared to IT1t pre-treatment (**, $p < 0.5$), but not when compared with CXCL12 only, showing that ADM did not affect either positively or negatively the agonist-dependent phosphorylation of hCXCR4, as previously suspected after carrying out a bystander BRET assay where no affection of the β-arrestin recruitment to hCXCR4 was recorded.

The same statistical analysis was performed with combined data deriving from membranes incubated with anti-CXCR4 pSer^{330} antiserum, but no statistically significant data were observed (**Fig. 4.17B**) overall. In particular, CXCL12 treatment did not statistically increase the phosphorylation of the receptor at this phosphosite (100.0 ± 0.1%) when compared to -dox (-0.2 ± 18.6%) and +dox (85.5 ± 25.7%), although a partial increase was observed. This can be due to either the high statistical error, or to the possibility that this phosphosite phosphorylation might be agonist-independent or constitutive. However, IT1t pre-stimulation caused a decrease in general phosphorylation of the receptor (-2.6.0 ± 20.8%), and ADM seemed to diminish the phosphorylation level when compared to CXCL12 (49.9 ± 11.4%), although these decreases were not statistically significant.

Finally, the anti-CXCR4 pSer^{338}/Ser^{339} (**Fig. 4.17C**) antiserum showed comparable statistically significant data and outcomes as seen for anti-CXCR4 pSer^{324}/Ser^{325} antiserum, and this means that this phosphosite phosphorylation is agonist-dependent upon CXCL12 addition (99.9 ± 0.1%). Indeed, this activation was higher than -dox

(2.2 ± 0.1%, *, p < 0.5) and +dox (-0.1 ± 4%, **, p < 0.5) conditions. Once again, the CXCL12-mediated activation of hCXCR4 could be effectively blocked by IT1t pre-stimulation (-8.6 ± 5.8%, **, p < 0.5). However, even in this case, ADM pre-treatment did not affect the phosphorylation of CXCL12 (105.6 ± 15.1%,***, p < 0.5), confirming the previous consideration about the role of ADM as biased antagonist for hCXCR4.

Additionally, no oligomeric form of the receptor was detected, because hCXCR4 is tagged with mEGFP, where "m" stands for monomeric. Since the anti-GFP antibody used is a home-made antibody (produced in our lab), it is possible that the oligomeric form of the receptor is not easily detected because of steric hindrance reasons. Indeed, steric hindrance could affect the recognition by the antibody of the epitope that was used for the generation of the anti-GFP antibody.

Another explanation about the absence of oligomers in the immunoblot can be that when FLAG-hCXCR4-mEGFP expressed by Flp-In T-REx 293 cells forms oligomers, the GFP tagged to the receptor in the protomeric form enters in contact with the other GFP tagged to another hCXCR4 protomer. Therefore, they could be able to form oligomeric complexes of GFP as a result of the oligomerization of hCXCR4. These complexes, however, are not recognized by the specific anti-GFP antibody used, because the GFP recognized at the molecular mass relative to hCXCR4 is monomeric.

The characterization of these phosphosite-specific antisera showed that ADM is not involved in hCXCR4 phosphorylation, and this is in agreement with the outcome of bystander BRET assay, in which ADM did not show any effect in β-arrestin recruitment to hCXCR4 upon CXCL12-mediated activation of the receptor.

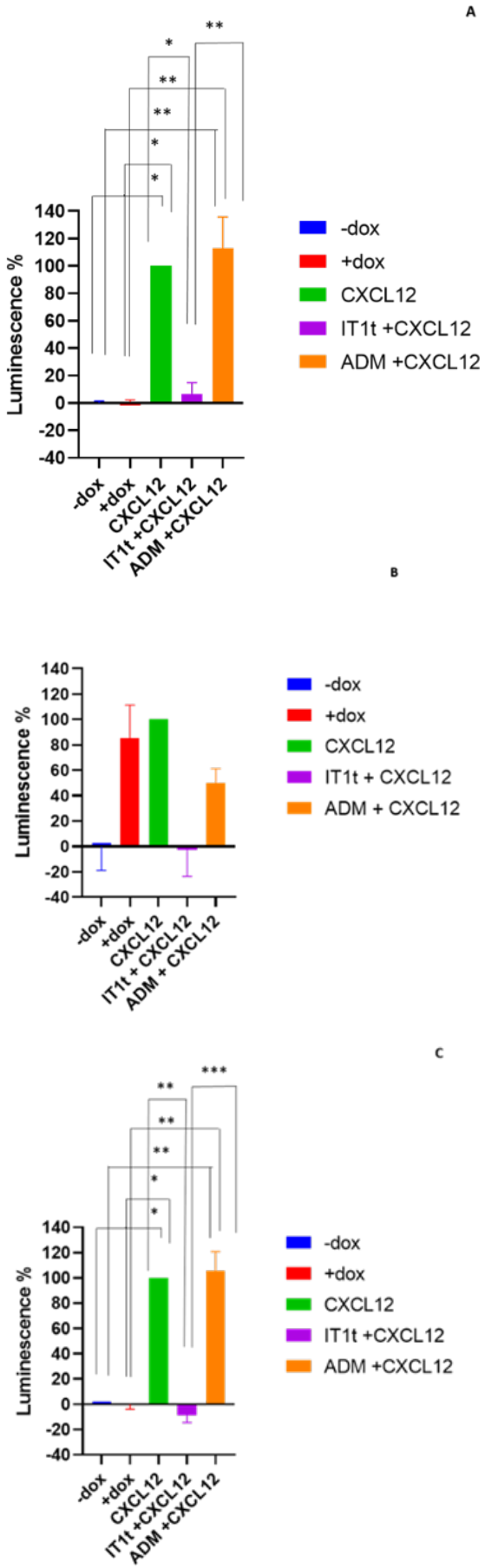


Fig. 4.17: The effects of ligands on hCXCR4 phosphorylation at different sites. The figure is the combination of n=3 separate experiments carried out in parallel with other immunoblots previously shown. Here, a quantitative analysis is reported for the different effects observed when different ligands were added to cells expressing FLAG-hCXCR4-mEGFP upon doxycycline treatment. Combined data from immunblots with anti-CXCR4 pSer^{324}/Ser^{325} are shown in **Fig. 4.17A**. **Fig. 4.17B** shows combined data of immunoblots with anti-CXCR4 pSer^{330}. **Fig. 4.17C** shows the combination of experiments carried out with pSer^{338}/Ser^{339} antiserum. Data represents normalized means ± SEM and were analysed by using one-way ANOVA test on GraphPad Prism. Data were normalized based on the luminescence percentages generated by the anti-GFP antibody, as shown in **Fig. 4.16.**

When working with phosphorylation, a good negative control is represented by treating samples with an enzyme called λPPase (lambda protein phosphatase), a Mn^{2+}-dependent protein phosphatase with activity towards phosphorylated serine, threonine, and tyrosine residues. This enzyme can be used to release phosphate groups from these phosphorylated residues present in proteins. Therefore, this treatment can provide insights about whether the phosphorylation is agonist-mediated or not.

As further control to check the specificity of the phosphorylation due to CXCL12-mediated effects, I carried out SDS-PAGE experiments with samples treated or not with this enzyme and CXCL12, whose results are shown in **Fig. 4.18**. Although this treatment has been extensively used in our lab (Marsango et al., 2022; Barki et al., 2023) and the protocol executed was effective for other GPCRs, in case of hCXCR4 antisera, this treatment did not give consistent results. Indeed, in presence of samples treated with this enzyme, what I would have expected to see was a reversion of CXCL12 effect observed in other immunoblots, demonstrating the specificity of the phosphorylation of hCXCR4 dependent on CXCL12 administration, detected by these phosphosite-specific antibodies.

This outcome did not happen consistently in any of the immunoblots reported with all the antisera used for these experiments. A reason behind these negative results could be that, according to the protocol used, samples are incubated for 90 mins at 30 °C before elution in SDS-PAGE sample buffer. Samples are then heated for 10 mins at 60 °C before resolving them on an SDS-PAGE gel. This long time at these temperatures might degrade samples and making difficult to quantify possible reductions of phosphorylation for this receptor. When incubated for longer times, membranes did not show any sample left, since they were all degraded in each blot (data not shown).

Another key point could be due to the number of units used for λPPase to treat samples (normally 10 units/μL), which might not be enough specifically for this receptor. Therefore, further optimizations of the protocol used would be required to determine the specificity of the phosphorylation observed in immunoblots shown in this paragraph.

However, although further controls and additional experiments might be required to fully demonstrate this hypothesis, it sounds reasonable to say that a trend was

observed in this approach about the effects of adding adrenomedullin to hCXCR4-mEGFP-expressing Flp-In T-REx 293 cells. Indeed, according to data collected up to this point, this ligand seems to be unable to affect hCXCR4 phosphorylation, maintaining its G protein-biased antagonist behaviour towards this receptor.

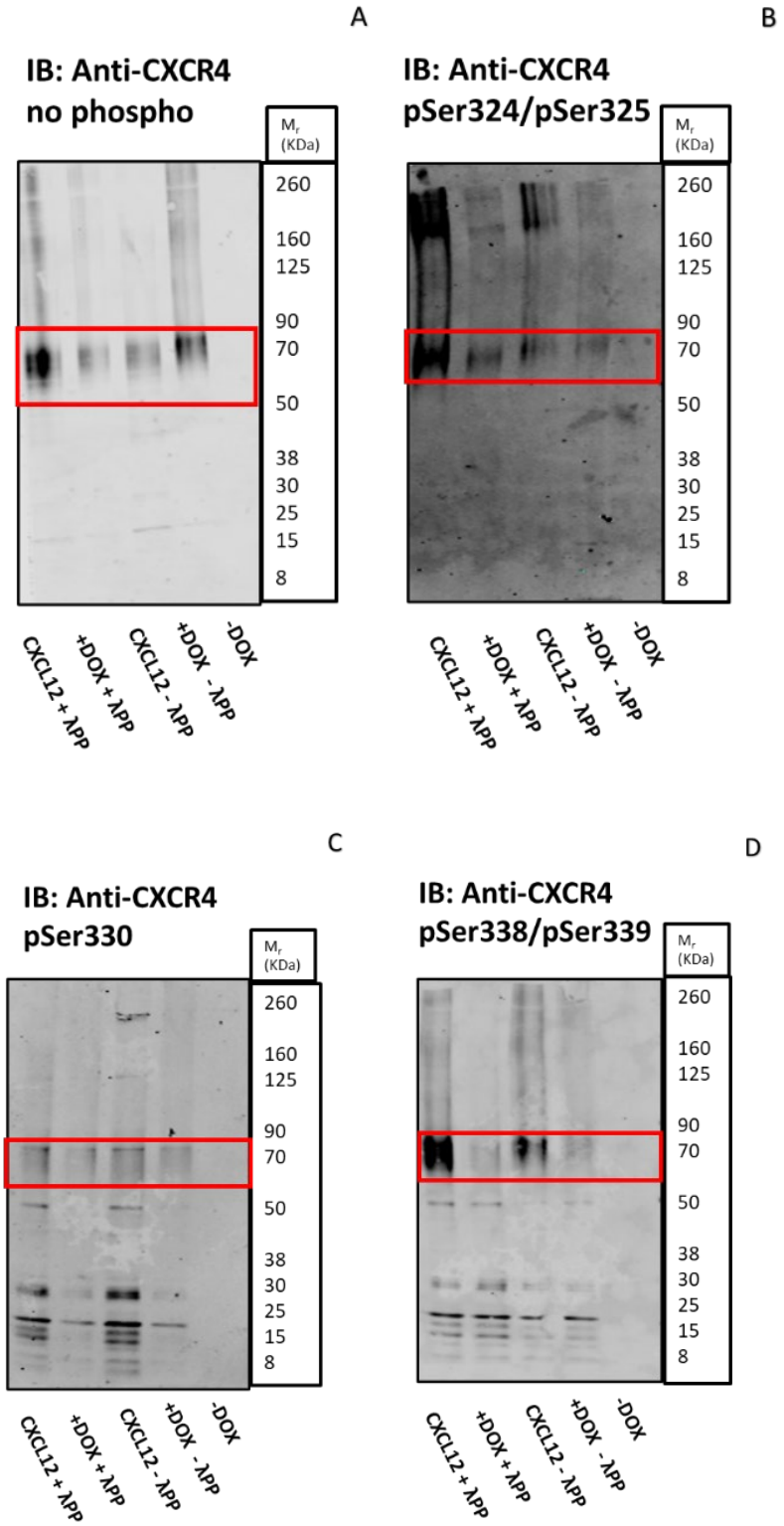


Fig. 4.18: The effects of λPP treatment on hCXCR4 phosphorylation at different sites. The figure shows representative traces of n=2 separate experiments, in which Flp-In T-REx cells induced to express hCXCR4-mEGFP, either in presence or absence of CXCL12 treatment, were treated or not with λPP enzyme before eluting in SDS-PAGE sample buffer. Immunoblots with different anti-CXCR4 antisera (no phospho, **Fig. 4.18A**; $pSer^{324}/Ser^{325}$, **Fig. 4.18B**; $pSer^{330}$, **Fig. 4.18C**; $pSer^{338}/Ser^{339}$, **Fig. 4.18D**) are displayed. Red box represents the expected molecular mass for FLAG-hCXCR4-mEGFP, where it is possible to observe the band detected by these antisera.

4.8 Characterization of adrenomedullin effects on hCXCR4 homo-oligomerization using Blue Native PAGE (BN-PAGE) and phosphosite-specific antisera

Another additional explanation for the effects of adrenomedullin as a biased antagonist for hCXCR4 previously observed and discussed in this chapter was related to a possible role of ADM in influencing the homo-oligomerization state of hCXCR4. Therefore, to further characterize the effects of the interactions between adrenomedullin and hCXCR4, I used the same phosphosite-specific antisera described and characterized in this chapter to investigate whether adrenomedullin might influence the homo-oligomerization state of hCXCR4. For this type of study, a blue native PAGE approach was used. Here proteins are analysed in native conditions, avoiding their denaturation. Instead of Coomassie Blue, an analogue dye is used, called G-250. By using this approach, it is possible to study protein-protein interactions in native conditions and, therefore, the homo-oligomerization state of GPCRs. Using this approach, Ward et al. (2021) showed that the antagonist IT1t was able to selectively disrupt the oligomerization state of hCXCR4. I carried out a similar study with adrenomedullin to determine whether this ligand might affect hCXCR4 homo-oligomerization, since an interaction between this ligand and the CXCR4 receptor was already hypothesised in previous paragraphs of this chapter.

Firstly, I assessed the effects of CXCL12 on hCXCR4 homo-oligomerization by immunoblotting lysates derived from stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP. **Fig. 4.19** shows a concentration-response curve of CXCL12 treatment of these cells and following immunoblots with anti-hCXCR4 no phospho antiserum (**Fig. 4.19A**) and the anti-hCXCR4 $pSer^{324}/pSer^{325}$ antiserum

(**Fig. 4.19B**). As shown in **Fig. 4.19A**, hCXCR4 was detected by anti-CXCR4 no phospho antiserum as a mixture of oligomers and monomers, with a major presence of the former. With addition of increasing concentrations of CXCL12, this relationship changes, with an increase presence of monomeric forms and a consequential decrease in oligomeric forms of the receptor once CXCL12 concentration reaches 100 nM.

When immunoblotted with anti-CXCR4 $pSer^{324}/pSer^{325}$ antiserum (**Fig. 4.19B**), the situation is quite different. This antiserum recognizes, conceptually, the phosphorylated forms of hCXCR4. In the condition +doxycycline (when, thus, no ligand is added), the ratio between oligomers and monomers shows a clear preference for the latter in this case, suggesting that monomeric forms of hCXCR4 are the most phosphorylated forms in the basal state. This feature is kept until 100 nM CXCL12 agonist are added to cells, which cause an overall decrease in signal detected, with still a major presence of monomers observed.

The reduction in band intensity observed at 100 nM has been observed frequently in BN-PAGE experiments. This outcome may depend on the high level of phosphorylation present in C-terminus side of hCXCR4. Indeed, as observed in **Fig. 4.14**, several residues (especially serine residues) are present upstream of the phosphorylation site detected by this antiserum. These residues, upon CXCL12-dependent phosphorylation, may lead to steric hindrance which can cause a lower detection of the receptor by the antiserum. Moreover, this site is very important for the regulation of the phosphorylation of hCXCR4 since it represents one of the key areas in which CXCL12 exert an effect. Indeed, these residues are rapidly phosphorylated by protein kinase C and GRK6 upon CXCL12 treatment, with a kinetic that is faster than other sites phosphorylated upon CXCL12 treatment (Busillo et al., 2010).

In parallel, as a control, the same cells lysates were resolved on an SDS-PAGE experiment and immunoblotted with the same antisera (**Fig. 4.20**). A concentration-response curve is shown in **Fig. 4.20B**, where it was observed that 100 nM is the minimum concentration required to see an agonist-mediated activation, and so phosphorylation of hCXCR4. Indeed, lower concentrations of agonist do not differe substantially from +dox. The same concentration causes a decrease in signal with anti-CXCR4 no phospho antiserum (**Fig. 4.20A**), as already noticed and discussed

previously in paragraph 4.7 of this chapter and as already observed for other GPCR as well, such as GPCR35 (Ganguly et al., 2023).

The phenomenon observed in BN-PAGE was not observed in SDS-PAGE, where CXCL12 causes an increase in phosphorylation of hCXCR4, leading instead to a major detection of the receptor upon agonist treatment. This is probably due to the nature of the assay, since in BN-PAGE samples are not heated before resolving them in a gel, while in SDS-PAGE a key step is represented by heating the samples to allow the denaturation of the receptor. By this way, it is possible to break the weak chemical bonds and help the SDS-PAGE sample buffer to bind the sample and cover them with negative charges. Therefore, the steric hindrance that may cause detection problems in BN-PAGE might not be that relevant in SDS-PAGE, because CXCL12-mediated phosphorylated forms of hCXCR4 are the monomeric ones.

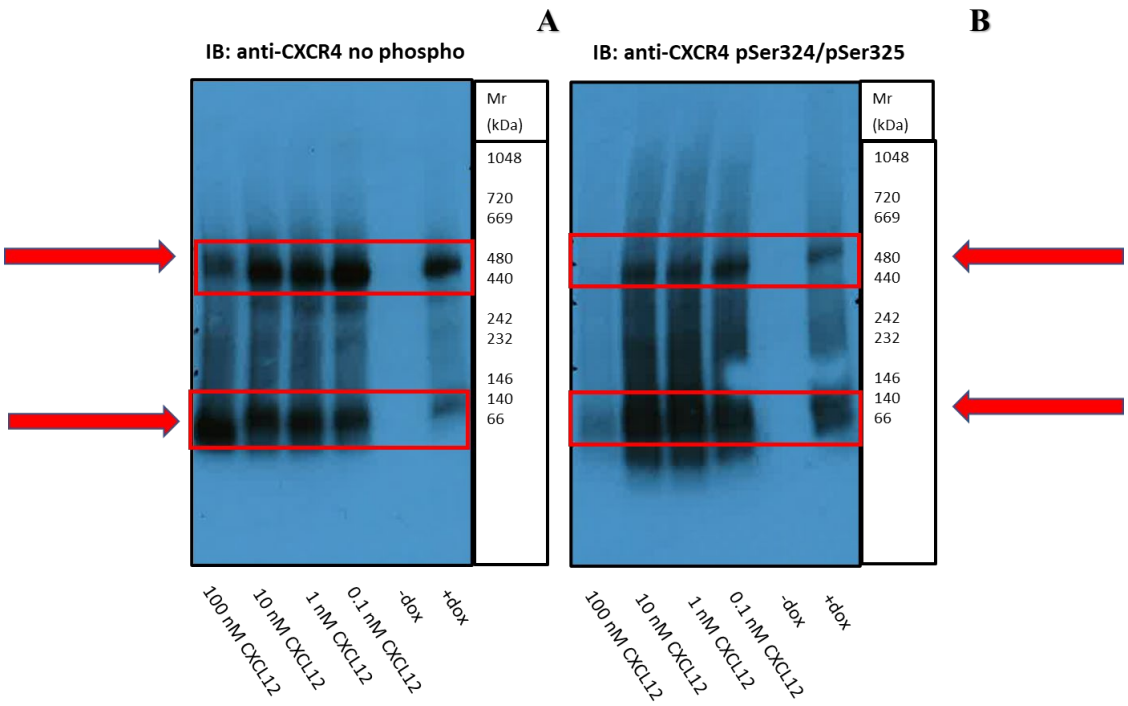


Fig. 4.19: CXCL12 affects oligomerization state of hCXCR4 increasing the quantity of monomeric form of the receptor. The figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with different concentrations of CXCL12 agonist as reported in the figure (from 0 to 100 nM) before preparing lysates from these cells. Samples were resolved by BN-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**panel A**), anti-CXCR4 $pSer^{324}/Ser^{325}$ (**panel B**). hCXCR4 is physiologically expressed in a co-existence of oligomers (upper arrow) and monomers (lower arrow), with a major presence of the former in the not phosphorylated form of the receptor, while monomer proportion is higher when the receptor is phosphorylated.

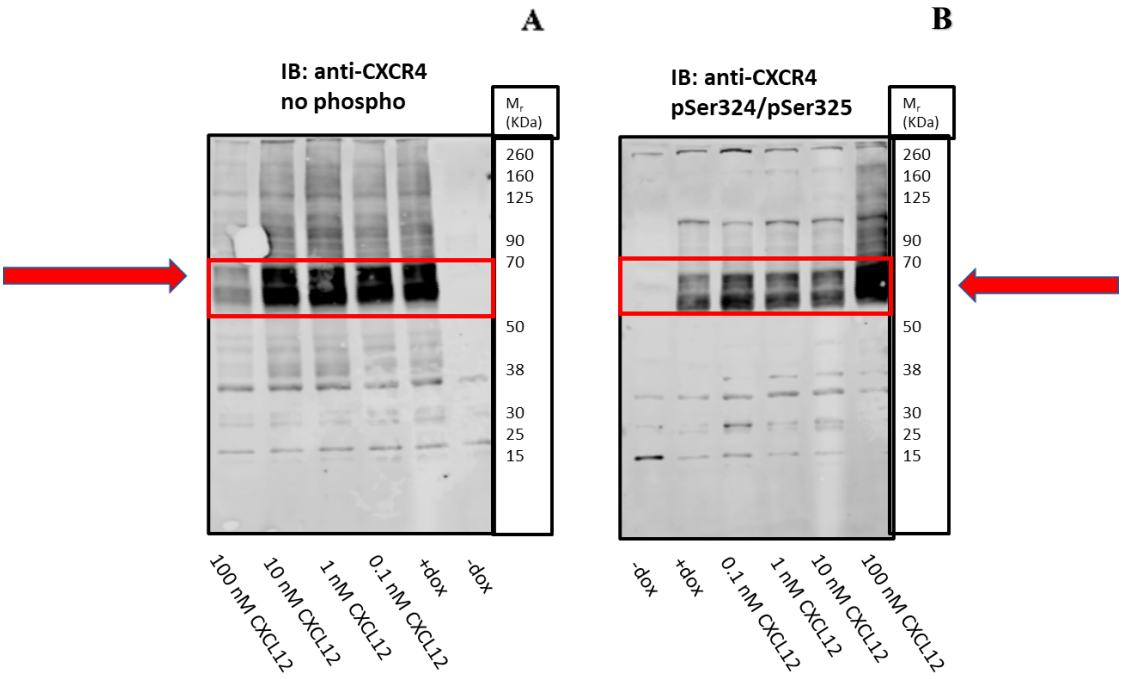


Fig. 4.20: 100 nM CXCL12 is the minimum concentration required to detect agonist-mediated phosphorylation of hCXCR4. The figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with different concentrations of CXCL12 agonist as reported in the figure (from 0 to 100 nM) before preparing lysates from these cells. Samples were resolved by SDS-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**panel A**) or anti-CXCR4 pSer^{324}/Ser^{325} (**panel B**). In **panel B**, anti-CXCR4 pSer^{324}/Ser^{325} shows an agonist-mediated activation of the receptor only at 100 nM.

However, the information gained from these immunoblots is limited by the absence of a proper quantification. Firstly, for the nature of the assay it is hard to quantify correctly BN-PAGE experiments in terms of size of the oligomeric complex and the molecular mass shown in the marker is indicative. Also, it is hard to say whether oligomers are dimers or trimers or higher-order complexes because of the presence of post-translational modifications, such as glycosylation, which may influence the molecular mass detected for both oligomers and monomers. In addition, no immunoblots with a housekeeping protein was carried out as a further control, although it would have been helpful for the quantification of both types of immunoblots.

The reason behind these missing experiments is that this part of the project was mostly explorative (as it is possible to see from the number of biological replicates reported here) and it would have been time consuming in the late stage of the project. Finally, the sensitivity of BN-PAGE as an approach is more limited than other approaches used for characterizing oligomerization of GPCR, such as BRET or FRET, for the nature of the assay itself. In fact, a limitation for BN-PAGE is that it requires clean and robust antibodies that can detect the protein in its native form. Antibodies based on denatured antigens of the protein may have trouble detecting proteins on a BN-PAGE. Also, the lack of resolution between protein complexes could also represent an issue and would require further optimization of the gradient gel parameters. Furthermore, the Coomassie dye is not totally inert to protein-protein interactions, which could lead to some disruption of native complexes and, in addition, the presence of salts or other solutes may cause protein smearing, lowering the quality of the gel. Lastly, this approach would eventually provide information about the homo-oligomerization of hCXCR4 upon ligand stimulation, but not about the hetero-oligomerization of the receptor, which would require more sensitive and specific approaches.

Once the effects of CXCL12 on hCXCR4 oligomerization were determined, also adrenomedullin effects were assessed by using the same approach. **Fig. 4.21** reports the result of samples derived from same cell line stably expressing hCXCR4 immunoblotted with the same antisera used before. In the immunoblot with anti-CXCR4 no phospho antiserum (**Fig. 4.21A**), the outcome of treating these cells with adrenomedullin is pretty similar to what was seen previously with CXCL12 treatment. Therefore, there is an evident major presence of monomeric forms of the receptor in presence of CXCL12 treatment which differs from +doxycycline only where the prevalence of oligomers is more evident. When 1 μM ADM only and 100 nM CXCL12 only are compared, both oligomers and monomers seem to increase upon ADM treatment compared to CXCL12 treatment, with still a major presence of monomers. However, compared to + doxycycline, the presence of monomers in ADM treatment in absence of CXCL12 seems to be increased. In the immunoblot with anti-CXCR4 $pSer^{324}/Ser^{325}$ (**Fig. 4.21B**), although both + doxycycline and 1 μM ADM show a similar trend, with a major presence of oligomeric forms of hCXCR4, the band corresponding to 1 μM ADM seems to be slightly more intense than + doxycycline. Moreover, pre-stimulating cells with ADM before adding a fixed concentration of CXCL12, the only effect observed was an increase in monomers

formation as already described with CXCL12 treatment in previous BN-PAGE experiments. Although ADM seems to exert some effects on oligomerization state of hCXCR4, it is hard to say whether this could be true, due to a lack of a quantification system which would allow a suitable statistical analysis and more specific interpretation of these data. Additionally, the effect observed could be related to a steric hindrance issue caused by adrenomedullin, which would help the antisera to better recognize the receptor, rather than a specific ligand-dependent effect.

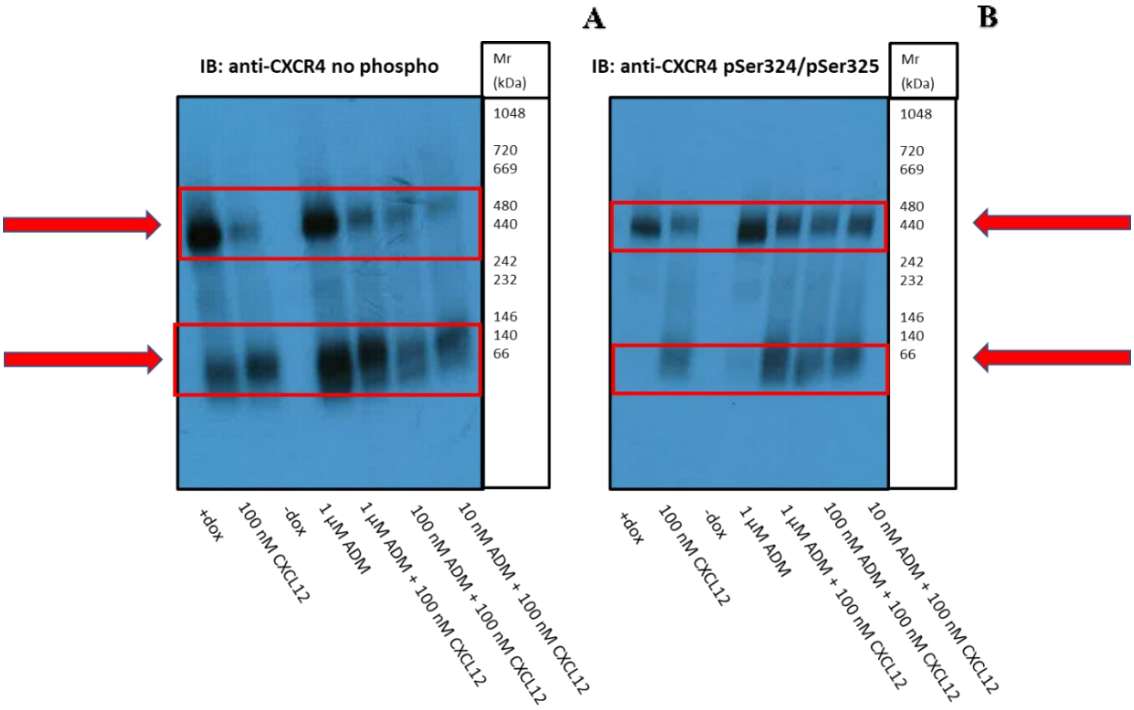


Fig. 4.21: ADM does not affect the oligomerization state of hCXCR4 neither in presence nor absence of CXCL12. The figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were pre-treated with different concentrations of ADM as reported in the figure (from 0 to 1 µM) before adding a fixed concentration of CXCL12 (100 nM) and preparing lysates from these cells. Samples were resolved by BN-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**panel A**), anti-CXCR4 pSer^{324}/Ser^{325} (**panel B**). Lower arrows indicate monomers, while the higher arrows represent oligomers.

In parallel, as a control, the same cell lysates were resolved on an SDS-PAGE experiment and immunoblotted with the same antisera (**Fig. 4.22**). A concentration-response curve of samples pre-stimulated with ADM for 15 minutes before adding a fixed concentration of CXCL12 (100 nM) is shown in **Fig. 4.22A** and Fig. **4.22B.** In

both cases, it was observed that 1 μM ADM is not sufficient to either stimulate receptor phosphorylation or block CXCL12-mediated hCXCR4 phosphorylation.

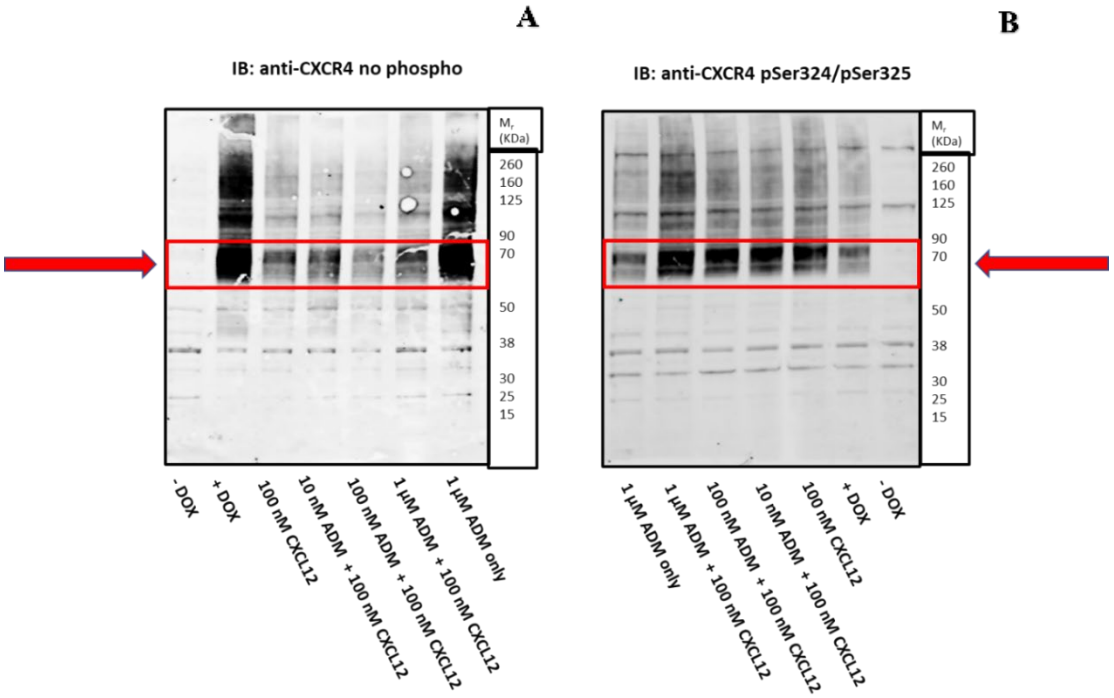


Fig. 4.22: ADM does not affect agonist-mediated phosphorylation of hCXCR4. The figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with different concentrations of ADM as shown in the figure before adding a fixed concentration of agonist (100 nM CXCL12) before preparing lysates from these cells. Samples were resolved by SDS-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**Fig. 4.22A**) or anti-CXCR4 pSer^{324}/Ser^{325} (**Fig. 4.22B**).

Although it is hard to clearly define the outcome of these data relative to ADM effects on hCXCR4 because of the explanation provided earlier in this paragraph, these data seem to show a trend which could be on the same page with the results obtained with bystander BRET assay and with SDS-PAGE data. Therefore, ADM did not seem to affect in any way either the recruitment of β-arrestin recruitment to hCXCR4 or the phosphorylation of hCXCR4 and, potentially, neither the oligomerization state of hCXCR4.

As further control, I re-assessed via BN-PAGE and SDS-PAGE also the activity of the small antagonist IT1t, to determine the sensitivity of the approach used and confirm the robustness of the assay used to justify the data observed for ADM. Data

relative to IT1t treatment of cells deriving from same cell line are shown in **Fig. 4.23**, where a BN-PAGE approach was used to confirm the disruption of oligomerization of hCXCR4 as already demonstrated and published by Ward et al. (2021).

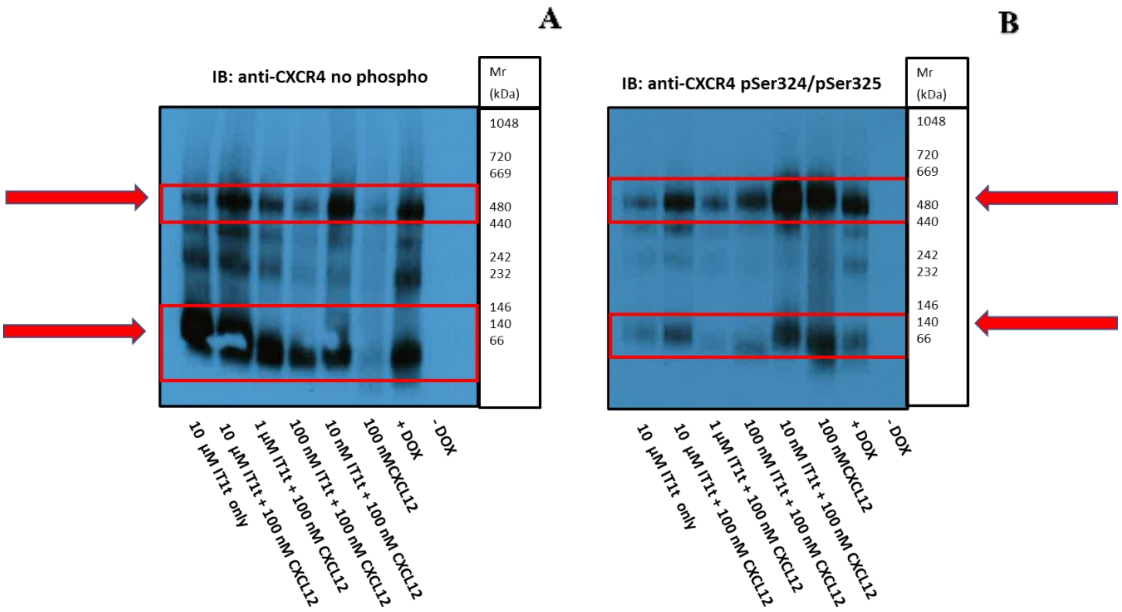


Fig. 4.23: IT1t disrupts oligomerization of hCXCR4. The figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were pre-treated with different concentrations of IT1t as reported in the figure (from 0 to 1 µM) before adding a fixed concentration of CXCL12 (100 nM) and preparing lysates from these cells. Samples were resolved by BN-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**panel A**), anti-CXCR4 $pSer^{324}/Ser^{325}$ (**panel B**). Upper arrows represent oligomeris, while lower arrows indicates monomers.

As observed in **Fig.4.23A**, IT1t displayed its disrupting effect on oligomeric forms of hCXCR4, increasing the quantity of monomers when cells were stimulated for 15 minutes with this ligand. This disruption was effective even in presence of CXCL12 added to cells, but it showed a concentration-dependent effect. **Fig. 4.23B** reports the outcome of immunoblotting lysates treated with different concentrations of IT1t and a fixed concentration of CXCL12. In this case, there is still a definite presence of oligomeric form of the receptor, with a major reduction of monomeric forms. This seems to suggest that IT1t tends to affect phosphorylation of hCXCR4 in a

monomeric form, rather than the oligomeric form. Moreover, differences in running mobility of the receptor were observed, depending probably on post-translational modifications.

Besides, these immunoblots showed variability in phosphorylation states of both oligomeric and monomeric forms of the receptor upon IT1t and CXCL12 treatments. This variability can be due to the cell environment, in which, some oligomers might be still active even thought IT1t was added. In fact, this ligand may exert a better effect in blocking phosphorylation on monomeric forms of hCXCR4, affecting, in some ways, the detection of the receptor with these antisera.

As further control, also an SDS-PAGE experiments was carried out as seen for CXCL12 and ADM. The data deriving from this experiment are shown in **Fig. 4.24**. These outcomes show what was already observed in other SDS-PAGE immunoblots with IT1t and its ability to block the phosphorylation of hCXCR4 (**Fig. 4.24B**) with a minimum concentration of 100 nM. Instead, when immunoblotting with anti-CXCR4 no phospho antiserum, decreasing concentrations of IT1t added to cells in presence of a fixed concentration of CXCL12 agonist show an overall decrease in signal detected, as already seen for CXCL12 in previous SDS-PAGE immunoblots experiments (**Fig. 4.24A**).

Even in this case, previous considerations that have been explained about differences between BN-PAGE and SDS-PAGE about the effects observed upon different ligands, especially for CXCL12 can be applied also to IT1t.

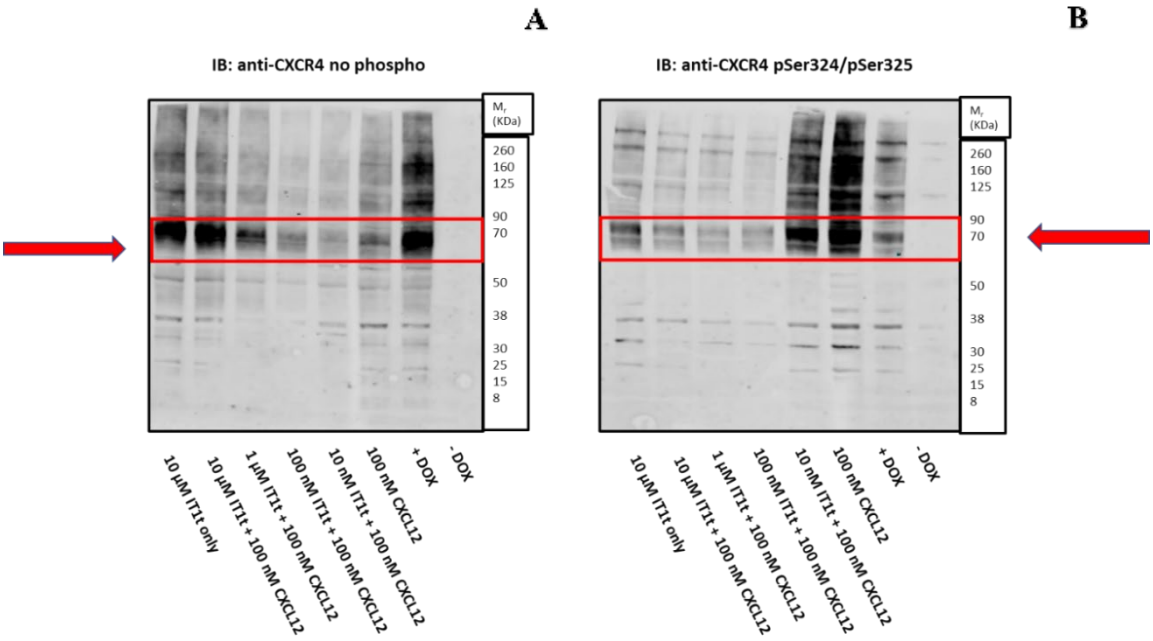


Fig. 4.24: IT1t blocks the agonist-mediated phosphorylation of hCXCR4. This figure shows representative traces of n = 2 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with different concentrations of IT1t as shown in the figure before adding a fixed concentration of agonist (100 nM CXCL12) before preparing lysates from these cells. Samples were resolved by SDS-PAGE and immunoblotted by using anti-CXCR4 no phospho antiserum (**Fig. 4.24A**) or anti-CXCR4 $pSer^{324}/Ser^{325}$ (**Fig. 4.24B**).

These results show that the approach used could be helpful to investigate the homo-oligomerization of GPCRs upon ligands treatments, although further optimization might be needed, and additional controls should be considered to better clarifying the role of these ligands in affecting GPCRs quaternary structure.

4.9 Is adrenomedullin a competitive or non-competitive modulator of hCXCR4-mediated G protein activation?

Earlier I showed that ADM may act as a 'biased' antagonist of CXCR4. I further investigated whether ADM was a competitive or non-competitive modulator of the effects of CXCL12 to activate G protein-signalling via hCXCR4. To address this question, a Schild regression analysis was carried out. In this method concentration–response curves to agonist are generated in the absence of antagonist and in presence of a series of increasing antagonist concentrations. This should give a surmountable shift in agonist potency (in case of competitive antagonism) or a reduction in efficacy (in the case of non-competitive antagonist).

To explore this, I used a cAMP accumulation assay to test ADM because the antagonism of this ligand was only observed in G protein signalling-based assays. In **Fig. 4.25,** where generation of HTRF signal was measured, several concentrations of ADM (0, 100 nM, 500 nM, 1 μM, 5 μM and 10 μM) were tested in the presence of increasing concentrations of CXCL12. The results showed a decline in efficacy of CXCL12 with increasing ADM concentration whilst the observed pEC_{50} of CXCL12 did not change significantly.

This indicates that ADM acts as a non-competitive antagonist/ negative allosteric modulator, because it is able to inhibit the G protein signalling of hCXCR4. Although there is currently no information on the binding site of ADM it must be to a distinct binding site from the region occupied by CXCL12.

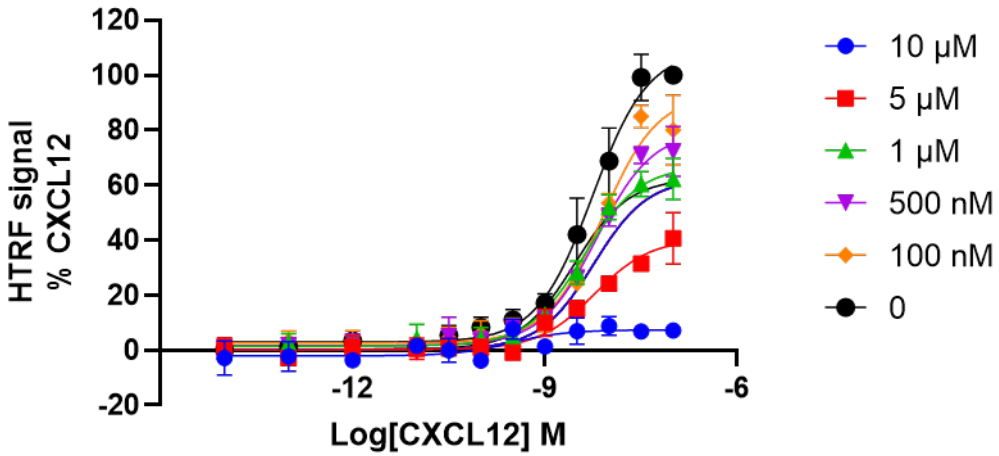


Fig. 4.25: Adrenomedullin acts as a non-competitive antagonist and negative allosteric modulator for hCXCR4 in cAMP inhibition assay. Data derive from n=3 independent experiments. Stable Flp-In T-REx 293 cells expressing FLAG-hCXCR4-mEGFP were treated with six concentrations of ADM (for 15 min) as indicated before increasing concentrations of CXCL12 were added.

4.10 Discussion

As discussed earlier in this chapter, the aim of this sub-project was determining a possible role of adrenomedullin ligand at hCXCR4. This ligand, acting as an endogenous agonist for calcitonin-like receptor complexed with RAMP2 (forming the adrenomedullin-1 receptor) or RAMP3, which creates the adrenomedullin-2 receptor. In the previous chapter I investigated its role as a possible agonist for hACKR3. Therefore, it sounded reasonable the hypothesis of a possible role of adrenomedullin also for hCXCR4, which strictly interacts with hACKR3 via heterodimerization and with which it shares some ligands, such as CXCL12, endogenous agonist for hCXCR4. Data from the xCELLigence RTCA assay described in this chapter, in which Flp-In T-REx 293 stable cells expressing FLAG-hCXCR4-mEGFP were treated with adrenomedullin, either in presence or absence

of CXCL12, showed a decrease of CXCL12-mediated response after pre-treatment with this ligand. This demonstrates that adrenomedullin can affect the adhesion properties of cells expressing hCXCR4 influencing this receptor activity at the concentration of 1 μM, even though already 100 nM ADM is sufficient to partially reduce CXCL12-mediated effect. The possibility that adrenomedullin could affect hCXCR4 functionality has been already investigated by Tanaka and colleagues (2021). Indeed, this study originally aimed to test the therapeutic potential of the adrenomedullin-RAMP2 system after laser-induced choroidal neovascularization, a mouse model of age-related macular degeneration. The authors observed that adrenomedullin intravitreal injections improved pathological conditions of mice tested and that fibrosis-related molecules, including tgfb, cxcr4, ccn2, and thbs1, were all downregulated by adrenomedullin. Therefore, this recent study seems to open up the possibility of a connection between adrenomedullin and CXCR4, among other receptors and proteins.

To further characterize the effects of interactions between adrenomedullin and hCXCR4 on receptor signalling, functional assays to investigate G protein signalling pathways were carried out, such as [^{35}S]GTPγS incorporation and cAMP accumulation assays, where adrenomedullin showed an inhibitory effect on CXC12-mediated $G\alpha_{i/o}$ signalling of hCXCR4 in a concentration-dependent manner, with a oupIC_{50} of 7.8 ± 0.8. The effects observed are specific of adrenomedullin, since when cells were not induced with doxycycline to express hCXCR4, no inhibition adrenomedullin-mediated was seen, strengthening the idea of a specific effect of this ligand for this receptor. Moreover, the small antagonist IT1t was used as positive control to assess the robustness of the assay; because IT1t exerted its antagonist activity already described in literature (Ward et al., 2021), this suggests that data obtained were strong enough to hypothesize the antagonism of adrenomedullin on hCXCR4.

The signalling of hCXCR4 was furtherly characterized via bystander BRET assay to determine whether adrenomedullin might have an impact on arrestin-3 recruitment to hCXCR4. In this assay no effect was observed when adrenomedullin was added to cells either in presence or absence of CXCL12 agonist and, specifically, no blockade of CXCL12-mediated arrestin-3 recruitment was observed, while IT1t used as reference antagonist was efficient in blocking this recruitment. This outcome disagreed with data from functional assays investigating the influence of adrenomedullin on G protein signalling of hCXCR4, suggesting a possibility of this

ligand acting as a biased antagonist on hCXCR4, with a preference for G protein signalling rather than arrestin-3 recruitment. The first evidence of biased signalling for GPCRs were originally explored by Kenakin (1995) and only later the first ligands were classified as biased, with the development of the concept of functional selectivity. Functional selectivity simply indicates a difference in efficacy for one or more functional readouts, while ligand bias usually involves a formal analysis of opposing pathways and can be quantitative (Tan et al., 2018).

However, so far no specific technology has been developed for easily identifying biased ligands. In general, these ligands are identified from an initial bias hypothesis that one pathway should be favored over a second one to improve the candidate behavior. Therefore, two functional assays can be used to investigate different pathways, with the objective to identify ligands biased for a pathway A over a pathway B. Considering the data from the different functional assays carried out, a further step in the analysis of the potential biased behaviour of adrenomedullin could consist in the calculation of the bias by measuring the $\Delta\Delta\log(\tau/K_A)$ (Kenakin et al., 2012) between two different pathways. In this case, τ represents the operational efficacy of the ligand in the receptor system, and K_A which represents the dissociation constant of the ligand for the receptor. This measurement would probably give a more quantifiable insight on the biased behaviour of adrenomedullin towards hCXCR4.

Because arrestin-3 recruitment is often related to phosphorylation in GPCRs, also phosphosite-specific antisera targeting hCXCR4 at a different number of sites were used. These sites are phosphorylated as consequence of receptor activation upon agonist binding (Busillo et al., 2010), so it was expected to see an inhibition of phosphorylation of the receptor once adrenomedullin was used to stimulate cells expressing hCXCR4. However, while IT1t displayed an inhibition of the receptor's phosphorylation when used as a control to assess the quality of the approach used, adrenomedullin did not cause any consistent reduction in phosphorylation. These outcomes strengthen and complement the data collected from bystander BRET assay to investigate the arrestin-3 recruitment on hCXCR4 and, therefore, the idea of adrenomedullin as biased antagonist for hCXCR4. However, these antisera used in this project presented some limitations. Indeed, as already observed for other GPCRs studied in our lab, such as GPR35 (Divorty et al., 2022; Ganguly et al., 2023), the antiserum generated for the detection of hCXCR4 independently on phosphorylation state of the receptor did not show these properties. Instead, upon ligand addition to

cells, such as the agonist CXCL12, the detection of the receptor was affected with a loss of signal relative to the receptor detected. Even with an “home-made” anti-GFP antibody, which should recognize the receptor with no differences depending on its phosphorylation state, a similar effect was consistently observed with IT1t antagonist, which blocks the phosphorylation of the receptor with a preference of its monomeric forms. The effect of IT1t observed may be due to its property in increasing the receptor density of hCXCR4 at higher concentrations and in presence of long-term treatment, as explored in the past by Ward et al. (2021).

A limit of studying phosphorylation sites with phosphosite-specific antisera is that phosphorylation sites are often used as binding domains for adaptors and scaffold proteins (Skolnik et al., 1993). If an adaptor or scaffold protein binds tightly to a phosphorylated amino acid residue in a protein, this adaptor may prevent the reaction by the antibody with its phospho-epitope. An optimization of this approach could consist of using saponin and Triton-X-100 as permeabilizing agents and/or other fixatives such as methanol that may solve some steric hindrance problems (Brumbaugh et al., 2017). Therefore, because of everything that has been said before, other additional studies may be required to further understand whether adrenomedullin might affect the phosphorylation of hCXCR4 with either more efficient antisera or with other approaches.

Of these phosphosite-specific antisera, the best characterized from the ones provided from 7TM Antibodies company is the anti-CXCR4 pSer^{324}/Ser^{325} antiserum and the anti-CXCR4 no phospho antiserum. Therefore, these were used to highlight possible effects mediated by adrenomedullin on homo-oligomerization of hCXCR4. In blue native PAGE experiments, although adrenomedullin did not show any influence on homo-oligomerization of hCXCR4, CXCL12 and IT1t, which induced monomerization of the receptor, showed similar limitations in allowing the detection of the receptor with these antisera because of a possible impairment in the C-terminal tail of the receptor due to its high phosphorylation state upon ligand addition. This consideration, already explained for SDS-PAGE experiments, can be extended to BN-PAGE experiments as well, therefore, even in this case, the homo-oligomerization state of hCXCR4 may require further analysis with different systems to better highlight the outcomes explained in this chapter.

Finally, it was determined with a Schild regression analysis that adrenomedullin, additional to its role as a biased antagonist, act as a negative allosteric modulator (NAM) for hCXCR4. This means that this ligand might bind the receptor in a

different site than CXCL12, but it can still exert its antagonist role, fixing the receptor in a specific conformation which could inhibit the binding of G proteins. This information might help in clarifying the biased behaviour of this ligand towards G protein signalling pathways with proper additional studies.

In recent years, increasing attention has been paid to new biased ligands, because of their selective effect in improving the quality of new therapeutics developed. Indeed, biased ligands may change the functional consequences of different pathways activation, even when these pathways could be subject to convergent regulation by different mechanisms. Moreover, these ligands might show a preference for determined active states of receptors that they bind, shifting their equilibrium and therefore, also affecting their pharmacological and physiological profiles. hCXCR4 is known to have some biased ligands, such as the clinical candidate plerixafor (Jørgensen et al., 2021) or the synthesis small peptide antagonist X4-2-6 (Hitchinson et al., 2021) and the ability to form hetero-complexes with other GPCRs might partially explain the results observed with adrenomedullin, which itself can bind several GPCRs in hetero-complexes as explained earlier.

Although biased agonists are overall more common than biased antagonists, some examples of the latters have been reported in literature, such as 6β-naltrexol for μ-opioid receptor (Sadee et al., 2020), 1-(3-(3-chlorophenyl)-1H-pyrazol-4-yl)-1,4-diazepane 2c against 5-HT_7R (Kwag et al., 2021), peptide R321 for CCR3 (Grozdanovic et al., 2019). Therefore, it could be possible for that other ligands may act as biased antagonist for hCXCR4, since the GPCR family is quite various and intricated in terms of signalling and information about some receptors is still limited. Additionally, adrenomedullin was firstly reported as a possible agonist towards hACKR3. Whilst little is still known about its possible interaction with hCXCR4, other examples exist of ligands and small molecules that act as antagonist for hCXCR4 but as agonists for hACKR3, such as ADM3100 (Kalatskaya et al., 2009) and TC14012 (Gravel et al. 2010). Then, adrenomedullin could potentially show a similar behaviour as these ligands, due to its structural or chemical features.

Another important detail about adrenomedullin is its size, which is relatively small compared to CXCL12 and other chemokines. Indeed, it is known that hCXCR4, as the most of chemokine receptors, possesses a large binding pocket to accommodate its cognate chemokine ligand CXCL12 (Mysinger et al., 2012; Kalinina et al., 2013). This difference in the size of adrenomedullin compared with CXCL12 might play a role in the way this ligand could bind hCXCR4 and affect its pharmacological

behaviour, added to the high versatility of this ligand in binding several receptors. Indeed, this high tendency to interact with GPCRs of different nature might by affected by the molecular weight of adrenomedullin and its size, which could cause steric hindrance with negative effects for other ligands to bind their cognate receptors. This would also explain the outocome reported by Meyrath and colleagues (2021) for hACKR3, for which adrenomedullin is a putative agonist. Indeed, in their work it is shown how proadrenomedullin N-terminal 20 peptide (PAMP) and even more evidently PAMP-12 had a stronger potency toward hACKR3 than adrenomedullin, to underline that the size of this family of peptides has a relevance that needs to be considered in pharmacology and drug discovery.

Because little is still known about this ligand and its possible interaction with hCXCR4, the study presented in this chapter represents a first step in understanding the molecular mechanisms and the effects of this interactions. Different methods such as radioligand binding assay or molecular docking approaches combined to recent deep learning-based protein structure predictions (Lee et al., 2022) could be used to better clarify the aspects that are still to enlighten, to determine the exact region allowing the allocation of adrenomedullin on hCXCR4 and explain its biased behaviour on hCXCR4. These outcomes and these plans would be helpful in drug discovery research on hCXCR4 pharmacology, with the hope of developing new therapeutics based on adrenomedullin ligands, acting efficiently on specific signalling pathways of hCXCR4 which may be used to treat the multiple pathological conditions to which this receptor is associated.

5. CHAPTER 5 (Results part 3) - Study of GPCRs kinases (GRKs) recruitment and GRKs-mediated phosphorylation of hCXCR4 and hACKR3

GRKs are a group of seven different soluble (except for GRK5 and GRK6 which are membrane tethered, as explained later in details) proteins whose main function is to phosphorylate at Ser or Thr residues within the intracellular loops and/or carboxyl-terminal tail (C-tail) of GPCRs upon agonist-promoted activation of the receptor. This phosphorylation represents an essential step in promoting receptor desensitisation. GRK 2, 3, 5 and 6 are ubiquitously expressed (Pitcher et al., 1998): GRK2 and GRK3 localize to the plasma membrane in response to receptor activation and thanks to the pleckstrin homology (PH) domain they can bind the $G_{\beta\gamma}$ subunit of the G proteins. The binding of this subunit recruits GRK2/3 to the membrane, where the receptors reside, to allow the kinases to cause phosphorylation (Haga K. and Haga T., 1992; Pitcher et al. 1992; Pitcher et al., 1995).

The GRK4/5/6 subfamily lacks the PH domain as well as the C-terminal prenylation that allows GRK1 and GRK7 to remain anchored to the plasma membrane. (Gurevich et al., 2012). Instead, these GRKs associate with the plasma membrane *via* palmitoylation of specific C-terminal cysteines (Cys^{561}, Cys^{562}, Cys^{565}) and/or *via* an amphipathic helix interacting with the membrane phospholipids (Gurevich et al., 2012; Gurevich V.V. and Gurevich E.V., 2019, Stoffel et al., 1994). These make them constitutively localized to the plasma membrane. The increasing interest in understanding the mechanisms by which phosphorylation works on specific amino acid residues is related to the interesting concept of how such phosphorylation may differ in the identity or order of sites involved, generating the so-called GPCR phosphorylation barcode that determines β-arrestin recruitment, receptor intracellular fate, and signalling outcomes (Reiter et al., 2012).

In recent years, several studies have shown the important role of GRKs, especially GRK2 and GRK6 in phosphorylating Ser residues in the C-terminal region of CXCR4, with distinct and non-overlapping effects on a cellular and disease phenotype-levels (Busillo et al., 2010; Mueller et al., 2013; Balabanian et al., 2008). ACKR3 GRKs-mediated phosphorylation is still poorly characterized due to lack of

effective mass spectrometry analysis. Indeed, only one study carried out by using astrocytes showed a GRK2-mediated ACKR3 phosphorylation, with no involvement of other GRKs. Moreover, this phosphorylation was essential for subsequent ACKR3-operated activation of ERK1/2 and AKT pathways (Lipfert et al., 2013), suggesting a cell type–dependent phosphorylation of ACKR3. In this chapter, the role(s) of GRK2/3/5/6 in hCXCR4 phosphorylation was explored after CXCL12 and ADM stimulation and the ability of hACKR3 to recruit the same GRKs after stimulation with the same ligands was determined.

5.1 Investigation of GRKs-dependent phosphorylation of hCXCR4 using phosphosite-specific antisera and small molecule antagonists

Previous works investigated the phosphorylation of hCXCR4 and reported that several serine residues, including Ser^{324}, Ser^{325}, Ser^{330}, Ser^{338}, Ser^{339}, and all serine residues between Ser^{346} and Ser^{352} are subject to phosphorylation upon CXCL12 agonist binding, and with different kinetics and hierarchy (Busillo et al., 2010). In first instance, roles of GRK2/3/5/6 were investigated by immunoblotting with phosphosites-specific antisera immunoprecipitated lysates prepared from Flp-In T-REx cells that stably express FLAG-hCXCR4-mEGFP after doxycycline addition and treatment with the endogenous agonist CXCL12. Moreover, two small molecule inhibitor ligands, compound 101 and compound 19, were used to assess the dependency of the phosphorylation on GRK subtypes. Compound 101 (3-[[[4-methyl-5-(4-pyridyl)-4H-1,2,4-triazole-3-yl] methyl] amino]-N-[2-(trifluoromethyl) benzyl] benzydaminehydrochloride) is a membrane-permeable, small-molecule inhibitor of GRK2 and GRK3, which was originally produced by Takeda (Lowe et al., 2015), while compound 19 ((S)-N2-(1-(5-chloropyridin-2-yl)ethyl)-N4-(5-ethyl-1H-pyrazol-3-yl)-5-methoxyquinazoline-2,4-diamine) is one of a series of several GRK5/6 inhibitors reported by Uehling et al. (2021) in their recent paper. When cells stably expressing FLAG-hCXCR4-mEGFP were pre-stimulated with a fixed concentration of either compound 101 or compound 19 (each at 10 μM for 30 mins) before adding CXCL12 (100 nM for 5 mins), in all phosphosites studied there was a clear reduction of phosphorylation mediated by CXCL12, supporting the involvement of these GRKs in hCXCR4 phosphorylation stimulated by CXCL12 (**Fig. 5.1**).

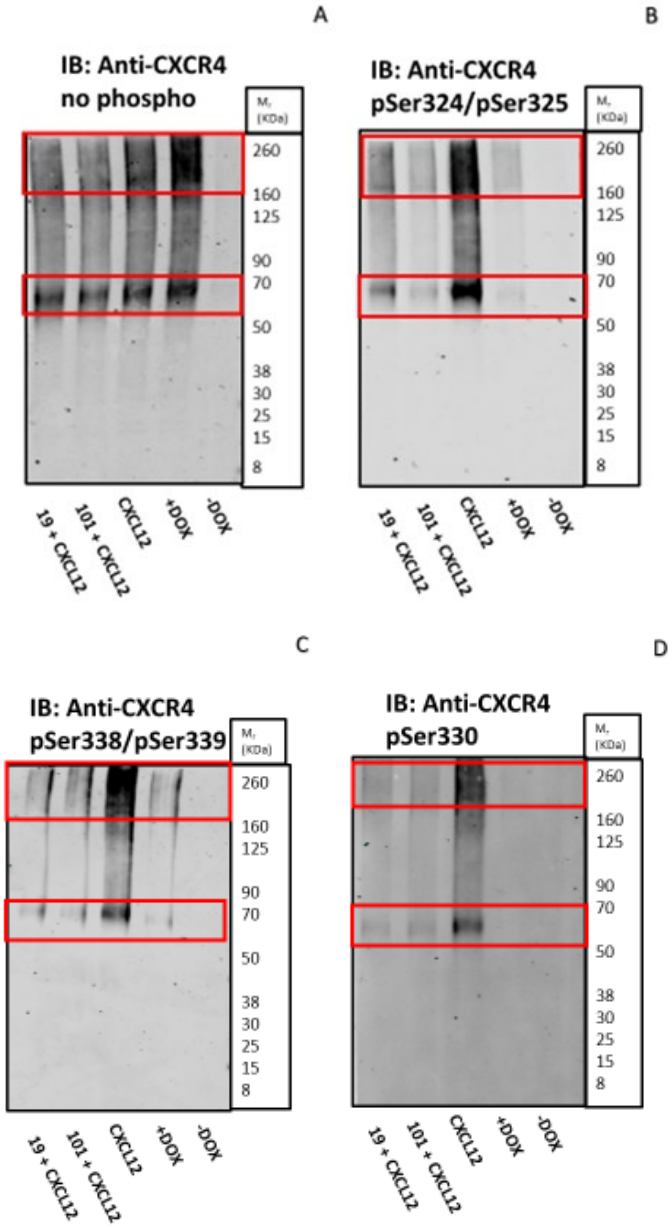


Fig. 5.1 Compound 101 and compound 19 limit phosphorylation of hCXCR4 in Flp-In T-REx cells stably expressing FLAG-hCXCR4-mEGFP. The figure consists of representative traces of n=2 independent experiments. Flp-In T-REx 293 cells developed to be able to express FLAG-hCXCR4-mEGFP on addition of doxycycline (+ DOX) were pre-treated with 10 μM of compound 101 or compound 19 for 30 minutes before adding CXCL12 (100 nM for 30 mins). Samples immunoprecipitated with GFP-TRAP kit were resolved by SDS-PAGE and immunoblotted using a non-phospho anti-CXCR4 antiserum (**Fig. 5.1A**) or antisera targeting $pSer^{324}/pSer^{325}$ (**Fig. 5.1B**), $pSer^{330}$ (**Fig. 5.1C**), or $pSer^{338}/pSer^{339}$ (**Fig. 5.1D**). The anticipated molecular mass of the receptor construct is 68 kDa (lower red rectangle). The immunoreactivity corresponding to the receptor construct was detected also at higher molecular mass (see upper red rectangle). All phosphosites are phosphorylated upon CXCL12 treatment.

As previously stated in Chapter 3 and Chapter 4, a proper quantification of the intensity of the bands observed is impossible because these samples are immunoprecipitated with an GFP-Trap kit, which enriches samples for the presence of GFP, avoiding the detection of unspecific bands. Additionally, a control experiment with a housekeeping protein is missing, which could have helped for a proper quantification of the increase/decrease observed for the differend bands corresponding to hCXCR4. Lastly, a statistical evaluation was not carried out even because of the number of biological replicates (n=2).

5.2 Investigation of GRKs recruitment to hCXCR4 via NanoBiT technology

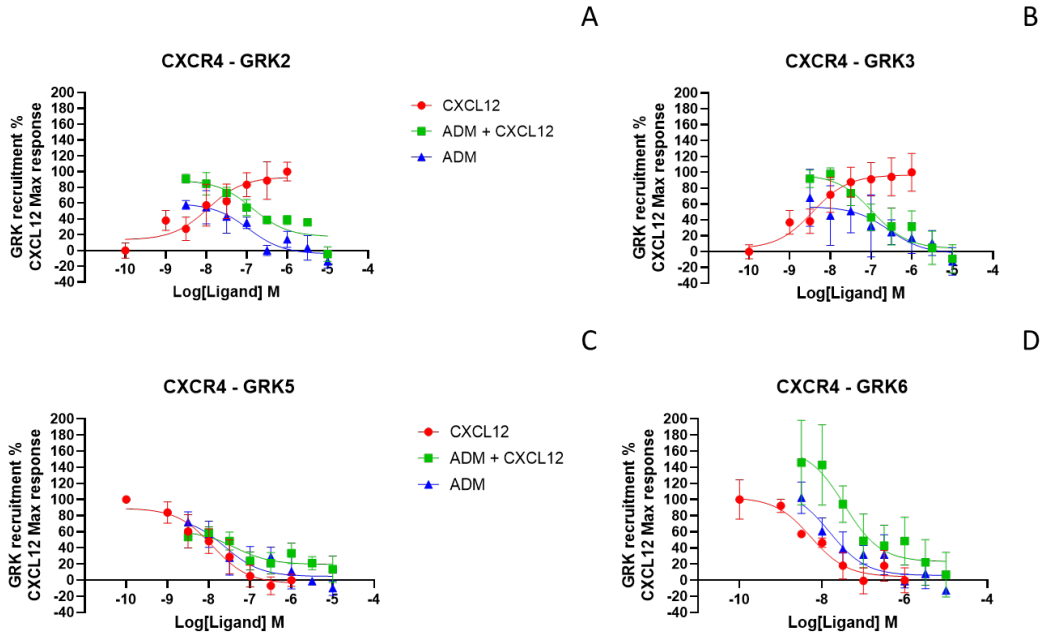
To highlight the role of GRKs in hCXCR4 phosphorylation, an important aspect to consider is the recruitment of these kinases to the receptor. An established approach that has been developed to pursue this goal, especially for chemokine receptors, is described by Palmer et al. (2022). This assay is called the nanoluciferase-based complementation assay (NanoBiT), which represents a simple and standardized approach for systematic profiling of GRKs-GPCRs interactions based on the complementation of the split nanoluciferase enzyme. In this assay, the GRK of interest is N-terminally tagged with a fragment of the nanoluciferase (NLuc) enzyme (18 kDa), called Large-BiT (LgBiT), while the GPCR of interest is C-terminally tagged with the complementary 11 amino acid fragment, called Small-Bit (SBiT). Upon ligand binding and G protein dissociation, the active conformation of the receptor can accommodate a specific GRK allowing the Nluc complementation. Cells supplemented with the NLuc substrate produce strong bioluminescence, which can then easily be measured. Because the inhibitor studies suggested possible roles for each of GRK2/3/5/6 or combinations of these kinases in hCXCR4 phosphorylation, the NanoBiT assay was carried out by transiently co-transfecting a pNEB2 plasmid allowing the expression of hCXCR4-SBiT and a pNEB2 vector for GRK-LgBiT subtype expression.

Fig. 5.2 shows the outcome of stimulation of HEK 293 cells in which expression of all GRKs was eliminated via CRISPR-Cas9. These cells were kindly provided by Prof. Carsten Hoffmann, University of Jena. These were transfected to transiently express hCXCR4-SBiT and the GRK-LgBiT of interest. Concentrations are

expressed in logarithmic scale in case of concentration-response curve. pEC_{50}/pIC_{50} values are the negative logarithm of EC_{50}/IC_{50} values. CXCL12 and ADM were then added, either separately or together. GRK2 (pEC_{50} = 8.0 ± 0.1) and GRK3 (pEC50 = 8.4 ± 0.1) showed concentration-dependent recruitment to hCXCR4 upon CXCL12 stimulation (**Fig. 5.2A**, **Fig. 5.2B**). There was a level of potential constitutive interaction of hCXCR4 with each of GRK2 and GRK3 as detected by luciferase signal in the absence of addition of CXCL12 (**Fig. 5.2A**, **Fig. 5.2B**). When cells transfected to co-express hCXCR4-SBiT and the GRK-LgBiT were treated with various concentrations of ADM in the absence of CXCL12 a concentration-dependent reduction in luciferase signal was observed for both GRK2 (**Fig. 5.2A**, pEC_{50} = 6.6 ± 0.1) and GRK3 (**Fig. 5.2B,** pEC_{50} = 7.0 ± 0.1). This is at least consistent with ADM acting as a hCXCR4 inverse agonist. To extend this I explored the effect of ADM on the ability of CXCL12 to promote hCXCR4-SBiT interactions with LgBiT-tagged forms of GRK2 and GRK3. ADM inhibited in a concentration-dependent manner (GRK2, **Fig. 5.2A**, pIC_{50} = 6.9 ± 0.1; GRK3, **Fig. 5.2B**, pIC_{50} = 7.0 ± 0.1) the effect of an EC_{80} concentration of CXCL12 on these two GRK.

Similar studies employing LgBiT-tagged forms of GRK5 and GRK6 were complicated by the high constitutive interaction recorded as luciferase activity. However, in each case addition of CXCL12 resulted in a concentration-reduction in signal for both GRK5 (**Fig. 5.2C,** pEC_{50} = 7.9 ± 0.1) and GRK6 (**Fig. 5.2D,** pEC_{50} = 8.3 ± 0.1) displayed a concentration-dependent effect of CXCL12. Surprisingly, addition of ADM also results in a concentration-dependent decrease in nanoluciferase signal (**Fig. 5.2C-D**) with pEC_{50} for GRK5 (**Fig. 5.2C,** pEC_{50} = 7.6 ± 0.1) and GRK6 (**Fig. 5.2D,** pEC_{50} = 7.8 ± 0.1). Additionally, as previously performed for GRK2 and GRK3, I explored the effect of ADM on the ability of CXCL12 to promote hCXCR4-SBiT interactions with LgBiT-tagged forms of GRK5 and GRK6. Again, a concentration-dependent decrease was observed in nanoluciferase signal for both GRK5 (**Fig. 5.2C**, pIC_{50} = 7.4 ± 0.1) and GRK6 (**Fig. 5.2D**, pIC_{50} = 7.5 ± 0.2), even in presence of EC_{80} CXCL12 stimulation. For a better visualization of the pEC_{50}/pIC_{50} values a table is reported in **Fig. 5.2**. This figure is a representation of combined data of n = 3 independent experiments. Statistical analysis was carried out by using a one-way ANOVA analysis (*, $p < 0.5$), but all data were not statistically significant due to the high error measured.

Even though a decrease of signalling is observed for GRK5 and GRK6, it is still unclear to the authors of the paper reporting this method on the reason behind this behaviour, which was observed for many GPCRs, belonging to different subfamilies, such as the μ-opioid receptor, the β2-adrenoceptor, and the atypical arrestin-biased chemokine receptor hACKR3, all of which were shown in the same published work. So, it does not surprise that a similar trend was observed for hCXCR4. According to Palmer et al. (2022), the interpretation provided for different orientation of the curves obtained for GRK2/3 and GRK5/6 resides in the different localizations that these GRKs have at a cellular level, since GRK5 and GRK6 are membrane-anchored, or it can be due to a constitutive interaction and competition from other effectors or regulators (Zheng et al., 2012; Laganà et al., 2021). Additionally, in the same published work, Palmer and colleagues showed that GRK5 and GRK6 recruitment seemed to be much slower than GRK2 and GRK3, and that it decreased over time, as already shown by Verweij and colleagues (2020). When agonists are used to stimulate GRKs recruitment to GPCRs, the different orientation of curves for GRK2/GRK3 and GRK5/GRK6 may make the interpretation of results more difficult and less immediate to understand. This can become especially true in presence of antagonists. Therefore, the authors conclude that the NanoBiT assay is more sensitive for the recruitment of GRK2/GRK3 rather than GRK5/GRK6.



GRK	CXCL12	ADM + CXCL12	ADM
GRK2	8.0 ± 0.4	6.9 ±0.4	7.0 ± 0.3
GRK3	8.4 ± 0.5	7.0 ± 0.6	6.6 ± 0.4
GRK5	7.9 ± 0.4	7.4 ± 0.3	7.6 ± 0.2
GRK6	8.3 ± 0.5	7.5 ± 0.8	7.8 ± 0.9

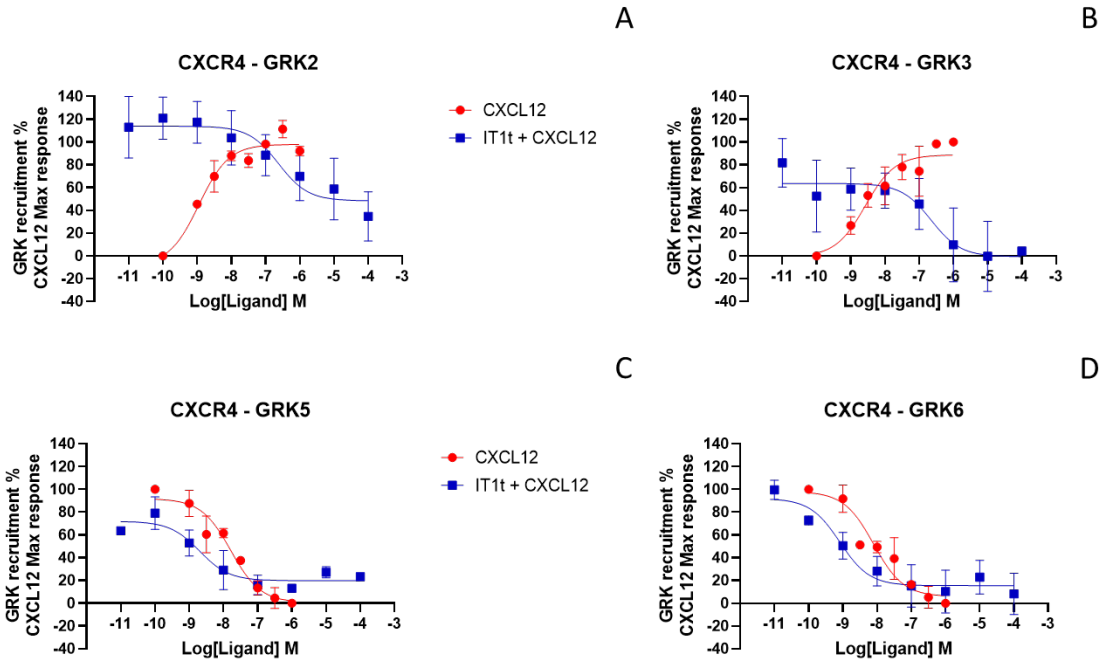
Fig.5.2 Assessment of GRK subtype recruitment to hCXCR4 using NanoBiT assays in GRK knock-out 293 cells. HEK293 cells subjected to CRISPR-Cas9 to eliminate expression of all GRKs were co-transfected with hCXCR4-SmBiT and the specific GRK-LgBiT of interest and treated with increasing concentrations of CXCL12, ADM or pre-preteated with increasing concentrations of ADM before adding a fixed concentration of CXCL12 (EC_{80}). Concentration-dependent effects of CXCL12 for each GRK were recorded in n = 3 independent experiments. Signal corresponding to GRK2 and GRK3 recruitment was recorder upon CXCL12 stimulation (**Fig. 5.2A-B,** pEC_{50} = 8.0 ± 0.4, and pEC_{50} = 8.4 ± 0.5) and for GRK5/6 (**Fig. 5.2C-D,** pEC_{50} = 7.9 ± 0.4, and pEC_{50} = 8.3 ± 0.5). Data correspondint to GRK2 and GRK3 recruitment upon ADM stimulation are shown in **Fig. 5.2A-B,** pEC_{50} = 7.0 ± 0.1, and pEC_{50} = 6.6 ± 0.1**, Fig. 5.2C,** pEC_{50} = 7.6 ± 0.1 and **Fig. 5.2D** pEC_{50} = 7.8 ± 0.1. GRKs recruitment was also measured when cells were pre-stimulated with ADM for 15 minutes before adding a fixed concentration of CXCL12 agonist (EC_{80}) for GRK2 and GRK3 (**Fig. 5.2A-B,** pEC_{50} = 6.9 ± 0.4, and pEC_{50} = 7.0 ± 0.6) and for GRK5 and GRK6 recruitment (**Fig. 5.2C-D,** pEC_{50} = 7.4 ± 0.3, and pEC_{50} = 7.5 ± 0.3). Statistical analysis was carried out by using one-way ANOVA (*, $p < 0.5$) but all the results were not statistically significant.

As an additional control, also the hCXCR4 antagonist IT1t was tested in this assay to validate the antagonist test previously shown with ADM. **Fig. 5.3** shows a combination of n = 2 independent experiments on the outcome of experiments carried out with this ligand, in which the same GRKs were analysed. Once again, in case of GRK2 (**Fig. 5.3A,** pEC_{50} = 8.9 ± 0.2) and GRK3 (**Fig. 5.3B,** pEC_{50} = 8.6 ± 0.2), the stimulation with increasing concentrations of CXCL12 agonist led to a concentration-dependent effect in recruiting GRK2-LgBiT and GRK3-LgBiT to hCXCR4-SmBiT. When cells were pre-stimulated for 15 mins with increasing concentrations of IT1t before adding EC_{80} CXCL12, IT1t seemed to block the GRK2-LgBiT (**Fig. 5.3A**, pIC50 = 6.6 ± 0.2) and GRK3-LgBiT (**Fig. 5.3B**, pIC_{50} = 6.7 ± 0.2) recruitment to hCXCR4-SmBiT in a concentration-dependent manner.

In case of GRK5 (**Fig. 5.3C,** pEC_{50} = 7.8 ± 0.2) and GRK6 (**Fig. 5.3D,** pEC_{50} = 8.1 ± 0.2) the stimulation with CXCL12 gave a similar outcome as observed in previous experiments in this chapter with this assay, with a concentration-dependent reduction of the luminescent signal recorded along CXCL12 concentration increases. In presence of IT1t pre-stimulation and EC_{80} CXCL12, a decrease in luminescence was observed in a concentration-dependent manner for both GRK5-LgBiT (**Fig. 5.3C**,

pIC_{50} = 8.7 ± 0.2) and GRK6-LgBiT (**Fig.5.3D**, pIC_{50} = 9.1 ± 0.2) recruitment to hCXCR4-SmBiT. Additionally, a slight shift in the potency of CXCL12-mediated GRK5 and GRK6 recruitment was observed (**Fig. 5.3C-D**). For a better visualization of the pEC_{50}/pIC_{50} values, a table is reported in **Fig. 5.3**.

Because of the number of biological replicates carried out (n = 2), no statistical analysis has been carried out, although a trend for each GRK tested in presence of IT1t was observed. This may be explained with the ability of IT1t antagonist to negatively affect the phosphorylation of CXCR4, as widely reported in literature. Because of the reasons explained earlier about the nature of this assay, it is hard to determine whether for GRK5 and GRK6 it is possible to see a block of recruitment to the receptor. However, this approach shows a higher sensitivity and robustness for detecting GRK2 and GRK3 recruitment to GPCRs, as explained by Palmer et al. in their paper relative to the NanoBiT assay (2022).



GRK	CXCL12	IT1t + CXCL12
GRK2	8.6 ± 0.3	6.2 ±0.5
GRK3	8.6 ± 0.4	6.7 ± 0.5
GRK5	7.8 ± 0.2	8.7 ± 0.2
GRK6	8.1 ± 0.2	9.1 ± 0.2

Fig.5.3: IT1t affects GRK recruitment to hCXCR4 in GRK knock-out cells 293 cells using the NanoBiT assay. GRK knock-out 293 cells were co-transfected with hCXCR4-SmBiT and the specific GRK-LgBiTof interest and pre-exposed to increasing concentrations of IT1t before adding a fixed concentration of CXCL12 agonist (EC_{80}). Interaction of each GRK tested was modulated upon CXCL12 stimulation but distinct differences were recorded for GRK2/3 compared to GRK5/6. In case of GRK2 (**Fig. 5.3A,** $pIC_{50} = 6.6 \pm 0.5$) and GRK3 (**Fig. 5.3B,** $pIC_{50} = 6.7 \pm 0.5$), a concentration-dependent effect of IT1t was observed in blocking the GRK2 and GRK3 recruitment to hCXCR4. In case of GRK5 (**Fig. 5.3C,** $pIC_{50} = 8.7 \pm 0.2$) and GRK6 (**Fig. 5.3D,** $pIC_{50} = 9.1 \pm 0.2$), there was no difference in the orientation of the curve obtained from CXCL12-promoted GRK5 and GRK6 recruitment to hCXCR4 with IT1t, but a shift was observed comparing the two curves. Data shown are means ± SEM values derived from a combination of n=2 independent experiments.

5.3 Investigation of the effects of compound 101 and compound 19 on hCXCR4 GRKs recruitment via NanoBiT technology

As next step in the characterization of GRK-mediated phosphorylation of hCXCR4, after I investigated the recruitment of GRK2/3/5/6 to hCXCR4, I further studied the roles of GRKs on hCXCR4 phosphorylation by using NanoBiT assay and small antagonists such as compound 101 and compound 19 previously mentioned in this chapter. **Fig. 5.4** shows the outcome of stimulation of HEK 293 cells in which expression of all GRKs was eliminated by via CRISPR-Cas9 and that were transfected to transiently express hCXCR4-SBiT and the GRK-LgBiT of interest. As control, HEK 293 parental cells (PAR) were used. When CXCL12 was added to parental cells, GRK2 (**Fig. 5.4A**, $pEC_{50} = 8.2 \pm 0.3$) and GRK3 (**Fig. 5.4B**, pEC50 = 8.3 ± 0.4) showed concentration-dependent recruitment to hCXCR4 upon CXCL12 stimulation. The same result was observed in GRK knock out cells transiently transfected to express hCXCR4-SmBiT and GRK2-LgBiT (**Fig. 5.4A**, $pEC_{50} = 8.0 \pm 0.2$) or GRK3-LgBiT (**Fig. 5.4B**, $pEC_{50} = 8.3 \pm 0.3$). Once again, there was a level of potential constitutive interaction of hCXCR4 with each of GRK2 and GRK3 as detected by luciferase signal in the absence of addition of CXCL12 (**Fig. 5.4A, Fig. 5.4B**).

When GRK knock out 293 cells transfected to co-express hCXCR4-SBiT and the GRK-LgBiT of interest were pre-treated with a fixed concentration of compound 101 (10 μM for 30 mins) before adding increasing concentrations of CXCL12, it was

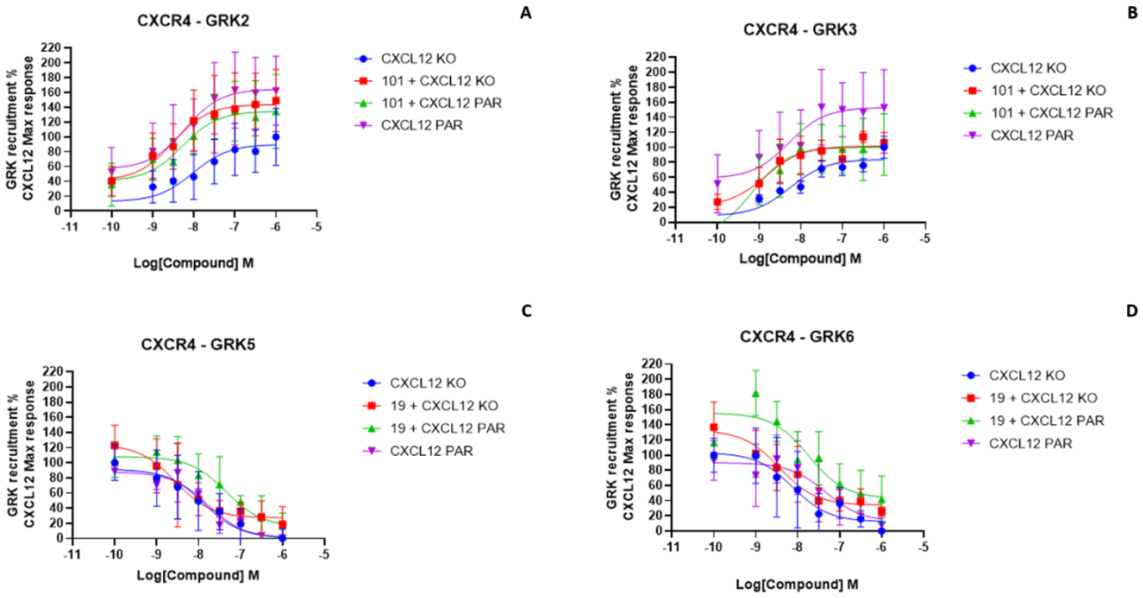
observed that compound 101 seemed to increase CXCL12 efficacy to recruit GRK2 (**Fig. 5.4A**, pEC_{50} = 8.5 ± 0.7) and GRK3 (**Fig. 5.4B**, pEC_{50} = 8.8 ± 0.7). This outcome, although not statistically significant (data were analyzed by using one-way ANOVA, $p \leq 0.5$), is graphically represented in **Fig. 5.4** by a shift in CXCL12 concentration-response curve. In parental cells, the same pre-stimulation with compound 101 before adding increasing concentrations of CXCL12 resulted in a decrease of CXCL12 efficacy in recruiting GRK2 (**Fig. 5.4A**, pEC_{50} = 8.3 ± 0.6) and GRK3 **(Fig. 5.4B**, pEC_{50} = 9.1 ± 0.6). Although even this result is not statistically significant, the trend observed is represented graphically in **Fig. 5.4** by a shift in CXCL12 concentration-response curve.

Then, I carried out similar experiments to determine whether compound 19 was able to affect GRK5 and GRK6 recruitment to hCXCR4, by using NanoBiT assay in parental and GRK knock out 293 cells. The outcomes of these experiments are shown in **Fig. 5.4**. As previously seen by using this assay, stimulating GRK knock out 293 cells transfected to express hCXCR4-SmBiT and GRK5-LgBiT or GRK6-LgBiT with increasing concentrations of CXCL12 resulted in a concentration-dependent decrease of luminescent signal recorded for both GRK5 (**Fig. 5.4C**, pEC_{50} = 7.9 ± 0.6) and GRK6 (**Fig. 5.4D**, pEC_{50} = 8.1 ± 0.6). A similar trend was observed when parental cells were stimulated with different concentrations of CXCL12 (GRK5, **Fig. 5.4C**, pEC_{50} = 7.8 ± 0.5; GRK6, **Fig. 5.4D**, pEC_{50} = 7.3 ± 0.6).

In a similar way as described for compound 101, GRK knock out 293 cells expressing hCXCR4-SmBiT and GRK5-LgBiT or GRK6-LgBiT were pre-stimulated with a fixed concentration of compound 19 (10 μM, incubated for 30 mins) before adding increasing concentrations of CXCL12. In this case, it was observed for both GRK5 (**Fig. 5.4C**, pEC_{50} = 8.6 ± 0.6) and GRK6 (**Fig. 5.4D**, pEC_{50} = 8.4 ± 0.7) a better recruitment upon compound 19 treatment, although these outcomes are not statistically significant. A similar trend was observed when similar experiments were executed with parental cells (GRK5, **Fig. 5.4C**, pEC_{50} = 7.3 ± 0.5; GRK6, **Fig. 5.4D**, pEC_{50} = 7.7 ± 0.6). For a more clear visualization of pEC_{50} values, a table in **Fig. 5.4** is reported to sum up.

Taking into consideration the results of these experiments, it seems likely that compound 101 and compound 19 do not affect the recruitment of different GRKs to hCXCR4, since their action consists in blocking the activity of different GRKs. However, the high variability of the data, although derived from n = 3 independent experiments, resulted in non statistically significant values when analysed with a one-

way ANOVA analysis ($p \leq 0.5$). This variability can find its reasons in the nature of the assay, the quality of the cell culture and the experimental conditions used, included the quality of the luminescent substrate used for this assay. Therefore, the hypothesis connected to the findings of these experiments reported in this paragraph would require further analysis and different technologies to (dis)prove these results.



GRK	CXCL12 Knock-out	CXCL12 Parental	101/19 Knock-out	101/19 Parental
GRK2	8.0 ± 0.2	8.2 ±0.3	8.5 ± 0.7	8.3 ±0.6
GRK3	8.3 ± 0.3	8.3 ± 0.4	8.8 ± 0.7	9.1 ± 0.6
GRK5	7.9 ± 0.6	7.8 ± 0.5	8.6 ± 0.6	7.3 ± 0.5
GRK6	8.1 ± 0.6	7.3 ± 0.6	8.4 ± 0.7	7.7 ± 0.6

Fig. 5.4. Effects of compound 101 and compound 19 in GRKs recruitment to hCXCR4 via NanoBiT assay. GRK knock-out and parental HEK 293 cells were co-transfected with hCXCR4-SmBiT and the specific GRK-LgBiT of interest and pre-stimulated for 30 minswith 10 µM compound 101 or compound 19 before adding increasing concentrations of CXCL12 agonist or in presence of CXCL12 agonist only. The graphs represent the mean values ± SEM of n=3 independent experiments. All data were analysed by using one-way ANOVA analysis ($p \leq 0.5$).

5.4 Investigation of GRKs recruitment to hACKR3 via NanoBiT technology

Currently, no good phosphosite-specific antisera for hACKR3 are commercially available, making difficult studying the effects of GRK-mediated phosphorylation of hACKR3 via immunoblots as done for hCXCR4. However, the NanoBiT assay was used as a first effort to characterize the GRKs recruitment to this receptor upon CXCL12 and ADM stimulation and the effects of compound 101 and compound 19 in possibly influencing this recruitment. Firstly, I assessed that GRK2/3/5/6 were all recruited to hACKR3 upon CXCL12 stimulation, as reported in **Fig. 5.5**. GRK knock out 293 cells co-transfected to express hACKR3-SmBiT and GRK-LgBiT of interested showed a similar outcome already seen for hCXCR4. Indeed, in presence of GRK2 (**Fig. 5.5**, pEC_{50} = 7.6 ± 0.1) or GRK3 (**Fig. 5.5**, pEC50 = 7.5 ± 0.1), an increase in GRK recruitment was observed along the concentration of CXCL12 added to cells. Similar to what was described for hCXCR4, in presence of increasing concentrations of CXCL12 agonist in GRK knock out 293 cells co-transfected to express the receptor and GRK5 or GRK6, a concentration-dependent decrease in luminescence was observed along the concentration of CXCL12 increased (**Fig. 5.5**, GRK5 pEC_{50} = 8.1 ± 0.1; GRK6 pEC_{50} = 8.0 ± 0.1).

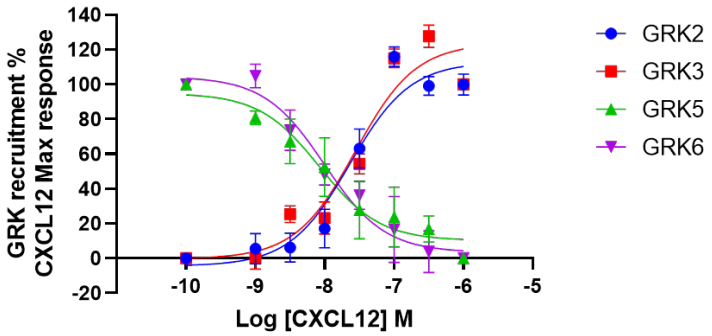


Fig. 5.5. Assessment of GRK recruitment to hACKR3 via NanoBiT assay in GRKs knock out cells with CXCL12 agonist. The graph represents mean values ± SEM of n=3 independent experiments. HEK293 cells subjected to CRISPR-Cas9 to make them knock out for GRKs expression were co-transfected with hACKR3-SmBiT and the specific GRK-LgBiT of interest and stimulated with increasing concentrations of CXCL12. As previously observed for other GPCRs, the curve for GRK2 (pEC_{50} = 7.6 ± 0.1) and GRK3 (pEC_{50} = 7.5 ± 0.1) showed an increase in GRKs recruitment in a concentration-dependent fashion. Instead, a decrease in luminescence was recorded when increasing concentrations of CXCL12 were added in GRK knock out cells co-transfected with GRK5 (pEC_{50} = 8.1 ± 0.1) or GRK6 (pEC_{50} = 8.0 ± 0.1).

Successively, I used the same approach to investigate the effects of adrenomedullin on hACKR3 via NanoBiT, as shown in **Fig. 5.6**. which showed differences with CXCL12. Surprisingly, when GRK knock out 293 cells co-transfected with the receptor in presence of GRK2 or GRK3, a concentration-dependent decrease in luminescence was recorded upon stimulation with increasing concentrations of ADM agonist for both GRK2 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1) and GRK3 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1). Instead, for GRK5 (**Fig. 5.6**, pEC_{50} = 6.4 ± 0.1) and GRK6 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1), as previously observed for hCXCR4, a concentration-dependent decrease in luminescent signal was recorded in presence of increasing concentrations of ADM. According to the paper published by Palmer at al. (2022), GRK2 and GRK3 recruitment to hACKR3 seems to be negatively affected by ADM addition. Moreover, this outcome might partially explain why no arrestin-3 recruitment was observed for hACKR3 expressing HEK cells at a concentration lower than 1 μM (**Fig. 3.9**). However, this hypothesis would require further analysis to complement and support these findings.

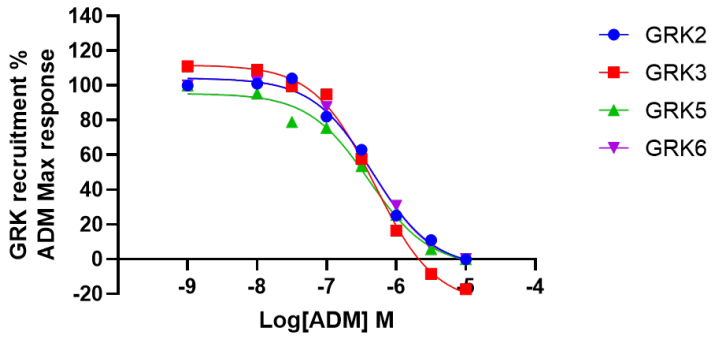


Fig. 5.6. Assessment of GRK recruitment to hACKR3 via NanoBiT assay in GRKs knock out cells with adrenomedullin agonist. The graph reports mean values ± SEM of n=3 independent experiments. GRK knock out 293 cells were co-transfected with hACKR3-SmBiT and the specific GRK-LgBiT of interest and stimulated with increasing concentrations of adrenomedullin (ADM). In presence of GRK2 or GRK3, a concentration-dependent decrease in luminescence was recorded upon stimulation with increasing concentrations of ADM agonist for both GRK2 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1) and GRK3 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1). A similar outcome was obtained for GRK5 (**Fig. 5.6**, pEC_{50} = 6.4 ± 0.1) and GRK6 (**Fig. 5.6**, pEC_{50} = 6.3 ± 0.1), for which a concentration-dependent decrease in luminescent signal was recorded in presence of increasing concentrations of ADM.

5.5 Investigation of the effects of compound 101 and compound 19 on hACKR3 GRKs recruitment via NanoBiT technology

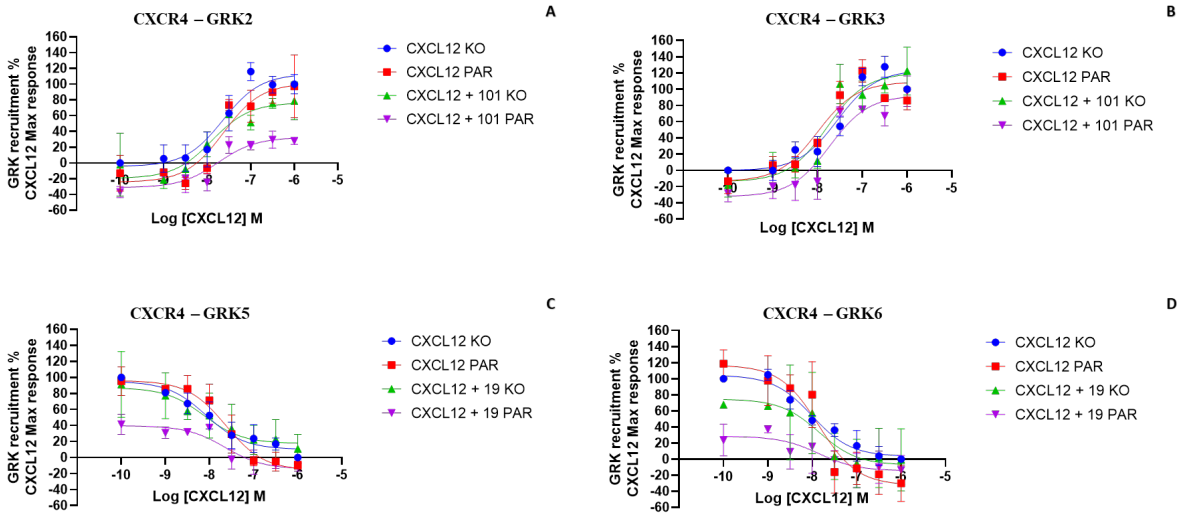
As successive step after assessing the effects of CXCL12 and adrenomedullin on GRK2/3/5/6 recruitment to hACKR3 via NanoBiT, I also tested both compound 101 and compound 19 properties via NanoBiT assay to define whether these small GRKs inhibitors could affect the recruitment of these kinases to hACKR3. These experiments were conducted in parallel in GRKs knock out cells and parental HEK 293 cells as shown in **Fig. 5.7** (CXCL12) and **Fig. 5.8** (ADM, adrenomedullin). As seen for hCXCR4, compound 101 and compound 19 (both 10 μM) were used to pre-stimulate for 30 minutes cells expressing the receptor and GRK of interest before adding increasing concentrations of agonist. When GRK knock out cells co-transfected with hACKR3-SmBiT and GRK2-LgBiT or GRK3-LgBiT were stimulated with increasing concentration of CXCL12, a concentration-dependent effect was observed for CXCL12 in favouring the recruitment of GRK2 (**Fig. 5.7A**, pEC_{50} = 7.6 ± 0.6) and GRK3 (**Fig. 5.7B**, pEC_{50} = 7.6 ± 0.7). A similar result was obtained in parental cells for GRK2 (**Fig. 5.7A**, pEC_{50} = 7.6 ± 0.6) and GRK3 (**Fig. 5.7B**, pEC_{50} = 8.0 ± 0.7).

In presence of pre-stimulation with compound 101 in GRK knock out 293 cells, a decrease was observed in the efficacy of CXCL12 to recruit GRK2 (**Fig. 5.7A**, pEC_{50} = 7.9 ± 0.6) and GRK3 (**Fig. 5.7B**, pEC_{50} = 7.8 ± 0.7). Although this finding seemed interesting, the result was not statistically significant when analyzed by using a one-way ANOVA analysis. This was probably due to the high statistical error measured with these values. A similar outcome was recorded in parental cells pre-stimulated with compound 101 for GRK2 (**Fig. 5.7A**, pEC_{50} = 7.7 ± 0.6) and GRK3 (**Fig. 5.7B**, pEC_{50} = 7.7 ± 0.7), which again gave non statistically significant results on a one-way ANOVA analysis.

When GRK knock out 293 cells co-transfected with the receptor and GRK5-LgBiT or GRK6-LgBiT were stimulated with different CXCL12 concentrations, a concentration-dependent decrease in luminescence was recorded for both GRK5 (**Fig. 5.7C**, pEC_{50} = 8.1 ± 0.5) and GRK6 (**Fig. 5.7D**, pEC_{50} = 8.0 ± 0.4). A similar result was found out in parental cells for GRK5 (**Fig. 5.7C**, pEC_{50} = 7.6 ± 0.5) and GRK3 (**Fig. 5.7D**, pEC_{50} = 7.9 ± 0.4).

When GRK knock out 293 cells co-transfected with receptor and GRK5 or GRK6 were pre-stimulated with compound 19 before adding increasing concentrations of CXCL12, a decrease in efficacy of CXCL12 to recruit GRK5 and GRK6 was

recorded, graphically represented by a shift. This decrease involved both GRK5 (**Fig. 5.7C**, pEC_{50} = 8.1 ± 0.4) and GRK6 (**Fig. 5.7D**, pEC_{50} = 7.8 ± 0.5). This outcome, even though not statistically significant when analyzed by using a one-way ANOVA method, was observed also in parental cells, were instead it was more evident for both GRK5 (**Fig. 5.7C**, pEC_{50} = 7.6 ± 0.4) and GRK 6 (**Fig. 5.7D**, pEC_{50} = 7.9 ± 0.6). However, all these results are not statistically significant, probably due to high levels of variability recorded. For a better visualization of the values measured for pEC_{50}, a table to sum up is shown in Fig. 5.7. As explained before, it is difficult to say whether this variability depends on a single factor or on many additional issues. These issues could be related to the sensitivity of the assay, the quality of the cell culture, the experimental conditions, the viability of the cells, the quality of the transfection and the functionality of the fluorescent substrate used, which could be partially degraded.



GRK	CXCL12 Knock-out	CXCL12 Parental	101/19 Knock-out	101/19 Parental
GRK2	7.6 ± 0.6	7.6 ±0.6	7.9 ± 0.6	7.7 ±0.6
GRK3	7.6 ± 0.7	8.0 ± 0.7	7.8 ± 0.7	7.7 ± 0.7
GRK5	8.1 ± 0.5	7.6 ± 0.5	8.1 ± 0.4	7.6 ± 0.4
GRK6	8.0 ± 0.4	7.9 ± 0.4	7.8 ± 0.5	7.9 ± 0.6

Fig. 5.7: Investigation of the effects of compound 101 and compound 19 in GRK recruitment to hACKR3 via NanoBiT assay upon CXCL12 stimulation. The graph represents the mean values of n=3 independent experiments analysed via one-way ANOVA. HEK293 cells subjected to CRISPR-Cas9 to make them knock out for GRKs expression and parental HEK293 cells were co-transfected with hACKR3-SmBiT and the specific GRK-LgBiT of interest and pre-stimulated with compound 101 or compound 19 (10 μM, 30 mins incubation) before adding increasing concentrations of CXCL12 agonist.

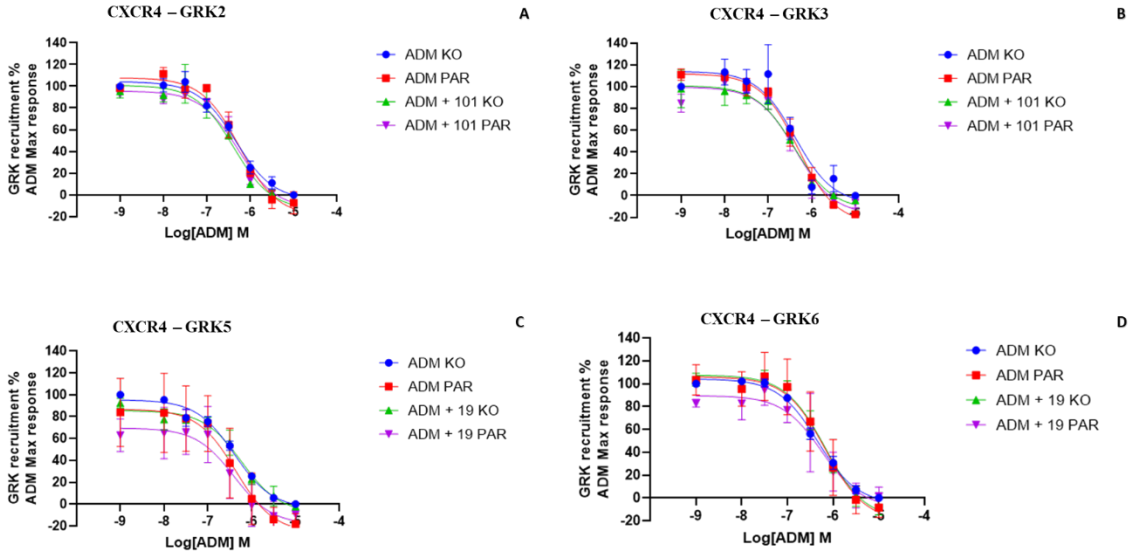
Equally as done for CXCL12, I determined the effects of compound 101 and compound 19 after stimulating GRK knock out and parental HEK293 cells expressing hACKR3 and a GRK of interest with adrenomedullin, as shown in **Fig. 5.8**.

Fig.5.8A shows data relative to GRK2: this kinase was able to decrease the luminescent signal in a concentration-dependent fashion along the concentration of ADM increased in both GRK knock out 293 cells (pEC_{50} = 6.3 ± 0.1) and parental cells (pEC_{50} = 6.2 ± 0.1). Pre-treatment with compound 101 did not affect the luminescent signal relative to GRK2 and generated by stimulating cells with ADM in both GRK knock out 293 cells (pEC_{50} = 6.4 ± 0.1) and parental cells (pEC_{50} = 6.3 ± 0.1). **Fig. 5.8B** shows that GRK knock out 293 and parental cells co-transfected with the receptor and GRK3 showed a decreased luminescent signal at higher concentration of ADM in a concentration-dependent fashion (knock out cells, pEC_{50} = 6.4 ± 0.2; parental cells, pEC_{50} = 6.3 ± 0.2), with no major differences. However, when cells were pre-treated with compound 101 before adding ADM at different concentrations, a small decrease in the curve generated by GRK3 was recorded in both GRK knock out 293 cells (pEC_{50} = 6.4 ± 0.3) and parental cells (pEC_{50} = 6.4 ± 0.3). However, even in this case the values were not statistically significant.

When GRK knock out cells co-transfected with hACKR3-SmBiT and GRK5-LgBiT were stimulated with CXCL12, the luminescent signal deriving from this interaction decreased with a concentration-dependent fashion (**5.8C**, pEC_{50} = 6.4 ± 0.3). The same effect was observed in parental cells (**5.8C**, pEC_{50} = 6.4 ± 0.5). After pre-stimulation of 10 μM compound 19 for 30 mins, a decrease in signal was recorded for GRK5 recruitment in GRK knock out cells (**Fig. 5.8C**, pEC_{50} = 6.2 ± 0.3) and parental cells (**Fig. 5.8C**, pEC_{50} = 6.4 ± 0.5). Although this decrease in signal measured was more evident in parental cells, data were not statistically significant. GRK knock out cells were co-transfected with the receptor and GRK6 and stimulated with CXCL12. The outcome was like GRK5, with a concentration-dependent decrease in signal recorded upon higher concentrations of agonist (**Fig. 5.8D**, pEC_{50} = 6.3 ± 0.3). As control, in parental cells the same experiment gave the same result (**Fig. 5.8D**, pEC_{50} = 6.2 ± 0.4). In presence of compound 19 pre-treatment, a little decrease in the efficacy of ADM in recruiting GRK6 was observed, showed as lowering of the curve obtained in absence of compound 19, in GRK knock out 293 cells (**Fig. 5.8D,** pEC_{50} = 6.2 ± 0.2). The same effect was observed in parental cells

used as control (**Fig. 5.8D**, $pEC_{50} = 6.2 \pm 0.5$). Although there seemed to be a specific effect, this was not statistically significant. All the pEC_{50} values are collected in a table represented in **Fig. 5.8** for a better visualization of the data.

Even if it looks like compound 101 and compound 19 might affect the GRKs recruitment to hACKR3, influencing the action of CXCL12 and ADM in promoting this process, statistical analysis executed on these data gave back non statistically significant results. This issue could be due to the overlapping of all concentration-response curve for adrenomedullin. So, there is not a clear difference which would suggest a different interpretation for the recruitment of these GRK enzymes to hACKR3 upon small inhibitors stimulation. Therefore, further studies are required to determine whether these small ligands might affect not only the activation of the GRKs recruited to the receptor, but also GRKs recruitment as a possible consequence of different phosphorylation patterns. Data reported in this paragraph relatively to compound 101 and compound 19 effect on hACKR3-GRKs interactions may be cell-type dependent and other cellular effectors may play a crucial role in helping the recruitment process of specific GRKs to hACKR3.



GRK	CXCL12 Knock-out	CXCL12 Parental	101/19 Knock-out	101/19 Parental
GRK2	6.3 ± 0.1	6.2 ± 0.1	6.4 ± 0.1	6.3 ± 0.1
GRK3	6.4 ± 0.2	6.3 ± 0.2	6.4 ± 0.3	6.4 ± 0.3
GRK5	6.4 ± 0.3	6.4 ± 0.5	6.2 ± 0.3	6.4 ± 0.5
GRK6	6.3 ± 0.3	6.2 ± 0.4	6.2 ± 0.2	6.2 ± 0.5

Fig.5.8: Study of compound 101 and compound 19 influence on GRK recruitment to hACKR3 via NanoBiT assay upon adrenomedullin stimulation. The graph represents the mean values of n=3 independent experiments analysed via one-way ANOVA. HEK293 cells subjected to CRISPR-Cas9 to make them knock out for GRKs expression and parental HEK293 cells were co-transfected with hACKR3-SmBiT and the specific GRK-LgBiT of interest and pre-stimulated with compound 101 or compound 19 (10 μM, 30 mins incubation) before adding increasing concentrations of adrenomedullin.

5.6 Determining the recruitment of GRK1, GRK4 and GRK 7 to hCXCR4 and hACKR3 via NanoBiT technology

To further validate the assay, also GRK1, GRK4 and GRK7 were tested as additional control for both hCXCR4 and hACKR3. GRK1 and GRK7 are expressed respectively in retinal rods and cones, while the expression of GRK4 is limited to cerebellar, testicular and kidney tissues. Therefore, their recruitment upon agonist stimulation in this cell system used might not be relevant. **Fig. 5.9** shows that none of these GRKs are recruited to the cognate receptor upon CXCL12 agonist treatment.

Fig. 5.9A shows that, in GRK knock out 293 cells co-transfected with hCXCR4-SmBiT and GRK-LgBiT of interest, CXCL12 stimulation (1 μM) did not give any significant GRK1/4/7 recruitment to hCXCR4, since the levels of recruitment recorded for GRK1 and GRK7 are, respectively, 10% and 20%, while GRK4 gave a negative level of recruitment, calculated as a -20%. All the values were normalized on the response generated by treating vehicle (GRK knock out cells expressing the receptor only, but not GRKs) with the ligand of interest. Analysing data which were collected for hACKR3, it was evident that nor 1 μM CXCL12 (**Fig. 5.8B**) or 10 μM adrenomedullin (ADM, **Fig. 5.8C**) were able to induce the recruitment of GRK1/4/7 to hACKR3. In this case, GRK1 gave a negative signal (-20%) upon CXCL12 stimulation, while GRK4 and GRK7 gave a positive signal (respectively 15% and 30%). In case of ADM stimulation, all GRKs gave negative signals (GRK1, -100%, GRK4, -120%, GRK7, -90%).

Therefore, these GRKs are not recruited in HEK cells upon agonist stimulation and the assay could be suitable for the detection of their recruitment.

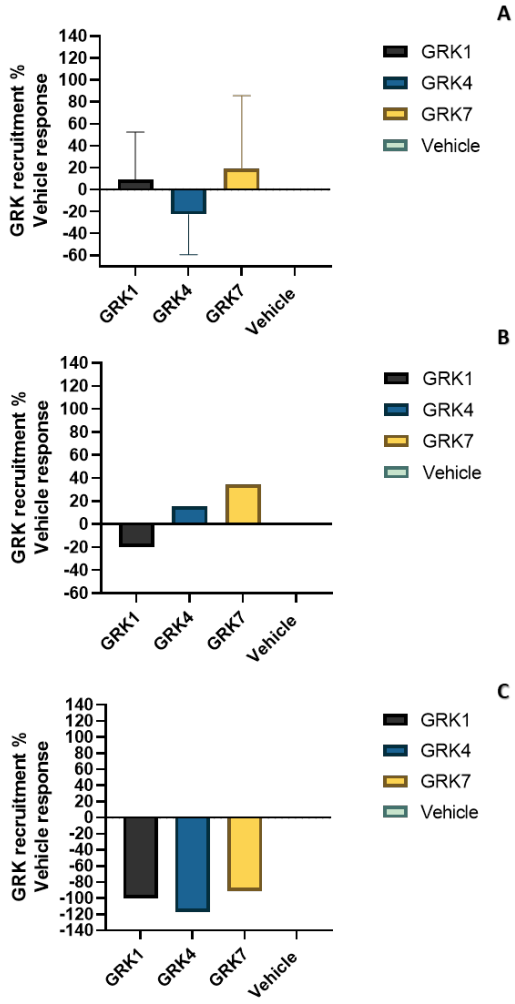


Fig. 5.9. Assessment of GRK1/4/7 recruitment to hCXCR4 and hACKR3 via NanoBiT assay in GRKs knock out cells upon CXCL12 and adrenomedullin stimulation. The graph shows representative traces of n = 2 experiments. HEK293 cells subjected to CRISPR-Cas9 to make them knock out for GRKs expression were co-transfected with hCXCR4-SmBiT and the specific GRK-LgBiT of interest and stimulated with 1 μM CXCL12 or 10 μM ADM. None of GRKs tested was able to be recruited to hCXCR4 (**Fig. 5.8A**) or hACKR3 (**Fig. 5.8B-C**) upon agonist stimulation. All the values were normalized to the response generated by treating vehicle (GRK knock out cells expressing either hCXCR4 or hACKR3 only, but not GRKs) with the ligand of interest.

5.7 Discussion

In recent years, several published works reported the crucial role of specific GRKs in GPCRs life cycle, with a great emphasis on GRK2, GRK3, GRK5, GRK6 (Ferguson et al., 2001; Gurevich et al., 2012; Pitcher et al., 1992) and their importance in regulating important processes such as desensitization and internalization of GPCRs.

The variety by which GRKs and other cellular effectors, such as arrestins and other kinases, makes the understanding of the phosphorylation mechanisms of GPCRs still not fully clarified. Indeed, different kinases can phosphorylate serine and threonine residues in the third intracellular loop or in C-terminus of GPCRs in different ways, establishing a *phosphorylation signature* that led to the conceptualization of the "*barcode theory*" (Tobin, 2008). This theory implies that there are multiple conformations of active arrestin energetically similar, and that different receptor phosphorylation patterns can increase the probability of arrestin to adopt a particular active conformation (Kim et al., 2005; Nobles et al., 2011; Kaya et al., 2020).

Therefore, the research investigating about this topic focusses on highlighting the phosphorylation mechanisms regulating different GPCRs functionality and biology, in a drug discovery and therapeutic perspective.

The increasing interest in investigating the role of GRKs in GPCRs pharmacology represented the rational for the execution of the experiments reported in this chapter. About hCXCR4, firstly the role of GRK2/3/5/6 in phosphorylating the receptor was assessed. Indeed, Busillo and colleagues (2010) reported that these GRK enzymes are involved in the phosphorylation of hCXCR4, with GRK2 and GRK3 phosphorylating distal Ser/Thr sites on the C-tail, whereas GRK6 phosphorylating more membrane proximal Ser/Thr sites. Additionally, GRK2/3-promoted phosphorylation of phosphosites located at the distal C-tail of hCXCR4 but not membrane proximal phosphosites is a required step for the CXCL12-mediated phosphorylation of FAK (focal adhesion kinase) (Zhuo et al., 2022). The phosphorylation of FAK, together with the β-arrestin 1-STAM1 (signal-transducing adaptor molecule) interaction are important for regulating the chemotaxis toward CXCL12 in HeLa cells (Alekhina and Marchese, 2016) and for the lysosomal trafficking and signalling of hCXCR4 (Malik and Marchese, 2010).

The role of GRK enzymes in hCXCR4 phosphorylation was firstly assessed via SDS-PAGE with phosphosite-specific antisera combined with the use of small inhibitors. These are called compound 101, a GRK2 and GRK3 inhibitor originally developed from Takeda (Ikeda et al., 2007, Thal et al., 2011; Lowe et al., 2015) and compound 19, which represents one of a series of GRK5 and GRK6 inhibitors developed by Uehling and colleagues (2021).

These experiments showed how different serine residues localized in the C-terminal region of the receptor are subject to phosphorylation upon CXCL12 agonist treatment. This activation was inhibited by pre-stimulating Flp-In T-REx cells stably expressing

hCXCR4-mEGFP with compound 101 and compound 19, confirming that these GRK enzymes are crucial for hCXCR4 phosphorylation.

To support these results, the NanoBiT assay was used to study the recruitment of these GRKs to hCXCR4. All GRKs tested were able to be recruited in a concentration-dependent manner to hCXCR4 (but GRK1/4/7 as expected, because of their limited expression in human body to specific areas (Watari et al., 2016)) upon CXCL12 stimulation. Even IT1t, antagonist for hCXCR4, and adrenomedullin displayed some interesting effects on GRKs recruitment. Both these ligands showed an inhibition of GRK2/3 recruitment, but unclear it is the result obtained with GRK5/6 because of the difficult interpretation of NanoBiT assay data. In fact, the orientation of GRK2/3 and GRK5/6 curves differ in an opposite way, and this is explained by Palmer et al. (2022) as a possible consequence of membrane-anchored localization of GRK5/6, a constitutive interaction and the possible competition with other cellular effectors with GRKs. If this interpretation is confirmed, the inhibition observed for adrenomedullin on GRK2/3 recruitment may find an explanation in adrenomedullin antagonist effects on G protein signalling of hCXCR4 reported in Chapter 3 of Results section in this thesis. Indeed, the $G_{\beta\gamma}$ subunit can play a role in GRK2 and GRK3 activation in many GPCRs families (Daaka et al., 1997) in a ligand-dependent way. Therefore, by blocking CXCL12-mediated G protein signalling of hCXCR4, adrenomedullin might avoid the recruitment of GRK2 and GRK3 to hCXCR4 and this might also explain why bystander BRET assay did not show any arrestin-3 recruitment to hCXCR4 upon adrenomedullin addition to HEK cells expressing hCXCR4. However, these results and this hypothesis should be better clarified in the future with more sensitive and specific approaches to determine the importance of GRKs in hCXCR4 phosphorylation and whether adrenomedullin might influence the recruitment of these GRKs. Indeed, the NanoBiT assay presents some limitations: the assay requires the smallBiT and largeBiT tags, thus necessitates the use of recombinant proteins. Additionally, the composition of the buffer used in the assay might affect the quality of the experiments. For example, it has been observed that the presence of some detergents can interfere with the generation of the luminescent signal (Shetty et al., 2020).

For what concerns about hACKR3, since no good phosphosite-specific antisera are commercially available, the only way used to assess the recruitment of GRKs to hACKR3 was the NanoBiT assay. Also for hACKR3 the studied GRKs were GRK2/3/5/6 because they are widely expressed in the human body and the best

characterized. Indeed, GRK2 and GRK5 are the most known GRKs considered important for the phosphorylation of the C-terminal side of hACKR3 (Chen et al., 2023; Schafer et al., 2023).

Even in this case, GRK2/3/5/6 were able to be recruited to hACKR3 in a concentration dependent-manner upon CXCL12 stimulation, with curves of GRK2/3 and GRK5/6 orientated in an opposite way as seen already for hCXCR4. Adrenomedullin was tested as well as an agonist, since it is a putative agonist for this receptor, and while it looked like there was a trend in recruitment of GRK2/3 in a concentration-dependent manner, the curves obtained for GRK5/6 had the same orientation of GRK2/3, making harder once again the interpretation of the data. However, GRK1/4/7 have not shown any recruitment upon agonist treatment once again, as already seen for hCXCR4. Therefore, the role of adrenomedullin in recruiting GRKs to hACKR3 is still unclear and requires further studies with different approaches to reach a consistent conclusion.

Finally, compound 101 and compound 19 were tested in NanoBiT assay to complement the information collected via immunoblots. The assay was carried out in GRK knock out HEK 293 cells and parental cells, to confirm that the effects observed were specific for GRKs activity and not due to other external factors. However, these small GRK inhibitors showed different trends, depending on different cell types used. Although for hCXCR4 a reduction in GRKs recruitment was observed for both knock out and parental cells, the entity of this decrease was not consistent among the GRKs, with differences in decreases recorded varying according to whether knock out cells or parental cells were used. Instead, for hACKR3 the major decrease in GRKs recruitment after CXCL12 stimulation was observed for GRK2 and GRK6 in both GRKs knock out and parental cells, but also in parental cells a diminished GRK5 recruitment was observed, suggesting that different cellular effectors or other endogenous GRKs might have a role as well in hACKR3 phosphorylation. Moreover, with adrenomedullin no significant change in GRKs recruitment after pre-stimulation with compound 101 and compound 19 was observed, except for GRK5 in parental cells. Therefore, the considerations done previously for CXCL12 are also valid for adrenomedullin.

As explained earlier, another complication of the NanoBit assay is that the signal measured is dependent on the complementation between the two recombinant proteins of this assay. In the case of hCXCR4, this is endogenously expressed at a certain level in HEK 293 cells (Atwood et al., 2011). Therefore, the variability found in the assay

could also depend on an background effect, because CXCL12 addition is going to activate both the endogenous and the modified receptors, with an effect on the luminescent signal recorded. The same issue could be applied in case of hACKR3 transfection in GRK knockout cells, where endogenous hCXCR4 is still present, possibly affecting the GRK recruitment measured on this assay. A possible approach to overcome this problem would require using GRK knockout cells which have been made knockout also for hCXCR4 and/or hACKR3. In this way, the baseline used for the normalization analysis should be more precise and specific for the activity of the tagged receptors.

Additionally, when comparing GRK knockout with parental cells, we need to consider that compound 101 and compound 19 are going to block the selective activation of GRK2/3 or GRK5/6. This becomes more relevant in parental cells where GRKs blocked may be "replaced" by the other ones because of a balancement or redundancy mechanisms adopted by cells. In fact, by using single GRK or multiple GRKs knockout cells, some GPCRs have been identified that interact with β-arrestins via the overexpression of specific GRKs even in the absence of agonists (Drube et al., 2022). Examples of these GPCRs which can be regulated by individual GRKs even in absence of agonists are reported in these work, such as angiotensin and vasopressin receptor. It is possible that, with some extent, also for chemokine receptors such as hACKR3 this might happen, explaining the effects observed for CXCL12 and adrenomedullin.

Lastly, although compound 101 and compound 19 block the activation of these GRK kinases, they do not avoid the recruitment to the receptor. The variations observed in this recruitment might depend on the phosphorylation state of the receptor, which could assume a conformation in which the binding to other cellular effectors than GRKs, such as arrestins, might affect the agonist-mediated signalling of the receptor. This signalling, on the other end, is also regulated by other kinases which have not been investigated here, such as PKA or PKC. Therefore, an additional understanding on whether the phosphorylation mediated by these other kinases could also affect the recruitment of both GRKs and arrestins.

All these data reported in this chapter represent an interesting addition to the current knowledge on GRKs-mediated hCXCR4 and hACKR3 phosphorylation and a first effort in understanding the molecular mechanisms behind this phosphorylation. This should provide some help in highlighting the barcoding of these receptors upon

treatment with different ligands, such as adrenomedullin, for which many molecular mechanisms of action and physiological effects are still poorly characterized.

6. CHAPTER 6 – FINAL DISCUSSION

6.1 OUTCOMES ON ACKR3-ADRENOMEDULLIN INTERACTIONS

The rationale behind this project was determining whether the non-chemokine peptide adrenomedullin would exert an effect on hCXCR4 and hACKR3. In the past, some works published have reported examples of interactions between adrenomedullin and hACKR3, for which this receptor may act as a scavenger receptor (Szpakowska et al., 2018; Meyrath et al., 2021). However, the mechanism by which hACKR3 regulates adrenomedullin availability by interacting with this ligand is still unclear and the mechanism needs to be clarified (Sigmund et al., 2023). What makes the characterization of the effects of these interactions difficult is the poor knowledge we currently have about this receptor. Indeed, only recently the first models of hACKR3 structure bound to some ligands (e.g. CXCL12) has been published and this seems to open new path in understanding the mechanisms by which this receptor binds the ligands for which it acts as a scavenger receptor, but also with other scaffold proteins involved in regulation of receptor signalling (Schafer et al., 2023; Chen et al., 2023; Sarma et al., 2023).

Because of the biased nature of hACKR3, the most common way to determine the effects of a ligand on this receptor consists of using β-arrestin recruitment assays, which provide insights on β-arrestin recruitment upon agonist activation. However, adrenomedullin in bystander BRET assay did not show any recruitment at concentrations lower than 1 μM. Although this information might be interesting, it is important to consider the physiological relevance of this finding. In fact, adrenomedullin physiological levels in human plasma are found to be in an average value of 2.5 pmol/liter, with a consistent increase in presence of pathological conditions such as heart failure (from 3.5 to 8.7 pmol/liter, according to which type of heart failure is considered) (Nishikimi et al., 1995). Therefore, in terms of pharmacological profile, what was observed in bystander BRET assay, with adrenomedullin requiring high concentrations to activate and exert a functional effect on hACKR3 may be dependent on other cellular factors, such as type of assay, cell line, buffer used, rather than a real dependency on ligand-receptor interactions.

Another important consideration to keep in mind is also the size of adrenomedullin ligand and its nature as a highly "promiscuous" ligand. Indeed, adrenomedullin is known for its ability to bind other GPCRs, such as human calcitonin-like receptor (hCLR), in complex with one of three human receptor activity-modifying proteins (hRAMPs) and, specifically, for hCLR complexed with hRAMP2 or hRAMP3, which create the human adrenomedullin-1 (hAM1) or human adrenomedullin-2 (hAM2) receptors (Weston et al., 2016). This versatility in interacting with different GPCRs represents an interesting feature and could be related to the relatively small size of this peptide (52 amino acids, while CXCL12 is 93 amino acids long), which could help it to fit in the binding pocket of several GPCRs. **Fig. 6.1** displays the different structures of CXCL12 and adrenomedullin, with some arginine residues (R8 and R12) on adrenomedullin which may have the same function of R8 and R12 on CXCL12, with a role in binding chemokine receptors hACKR3 and hCXCR4.

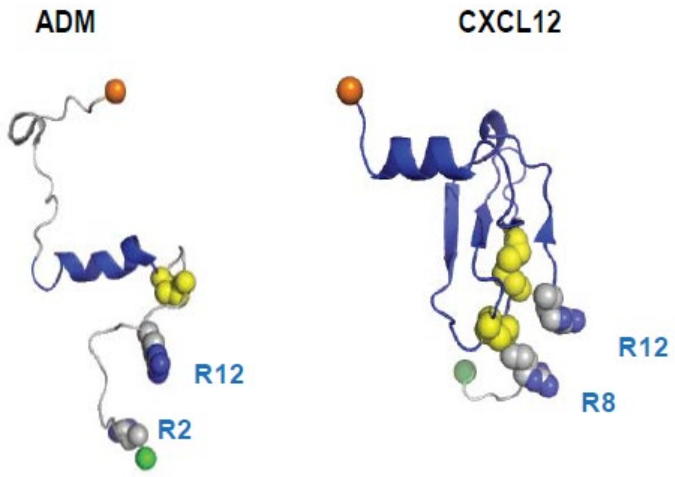


Fig. 6.1. Comparison of adrenomedullin and CXCL12 structures. The figure displays the comparison between CXCL12, which act as an agonist for hACKR3 and represents the endogenous agonist for hCXCR4, and adrenomedullin. The size of CXCL12, 93 amino acids, is almost the double of adrenomedullin size, 52 amino acids. However, arginine residues R2 and R12 could correspond to R8 and R12 on CXCL12 and might allow the interaction between adrenomedullin and ACKR3/CXCR4 (adapted from Capoferri, 2020).

These structural features may justify the outcome of xCELLigence RTCA assay, in which an effect of adrenomedullin on hACKR3 was observed, (although only after 1 μM) upon stimulation of hACKR3-expressing cells with adrenomedullin. However, for the nature of xCELLigence, it is hard to say whether this activation began signalling cascades or not and, in case, which ones these signalling cascades are.

The final point about hACKR3-adrenomedullin interactions is the co-localization of hACKR3 and ADM. The first studies about the correlation between this peptide and this receptor started with Klein et al. (2014). Their work showed that loss of hACKR3 resulted in postnatal lethality in mice due to aberrant cardiac development and myocyte hyperplasia and that dosage and signalling of adrenomedullin is controlled by hACKR3. Although this model could represent a good way to investigate the role of adrenomedullin-hACKR3 interactions, also the expression levels in different tissues could represent a limit for the effects generated by this interaction and which were observed.

Fig. 6.2 show protein expression overview of, respectively, hACKR3 (**Fig. 6.2A**) and adrenomedullin (**Fig. 6.2B**). These graphs provide information about where both receptor and adrenomedullin are expressed at higher levels in terms of different organs and tissues. Both hACKR3 and adrenomedullin are highly expressed in thyroid glands, bronchi, pancreas, kidney, fallopian tubes, placenta, and appendix. These data collected about the protein expression levels narrows the investigations to those regions where the interactions ligand-receptor may occur and exert an effect in a physiological condition because of the presence of both hACKR3 and hCXCR4.

However, all the studies carried out in this thesis were performed in an in vitro system represented by Flp-In T-REx 293 cells, which are derived from HEK (Human Embryonic Kidney) 293 cells. Therefore, it would be interesting and crucial to determine whether with an *ex vivo* or *in vivo* approach data would differ from the one I reported in this thesis and maybe considering possible differences between systems where experiments are performed and whether interactions between adrenomedullin and hACKR3 might be physiologically relevant and not due to other cellular effectors.

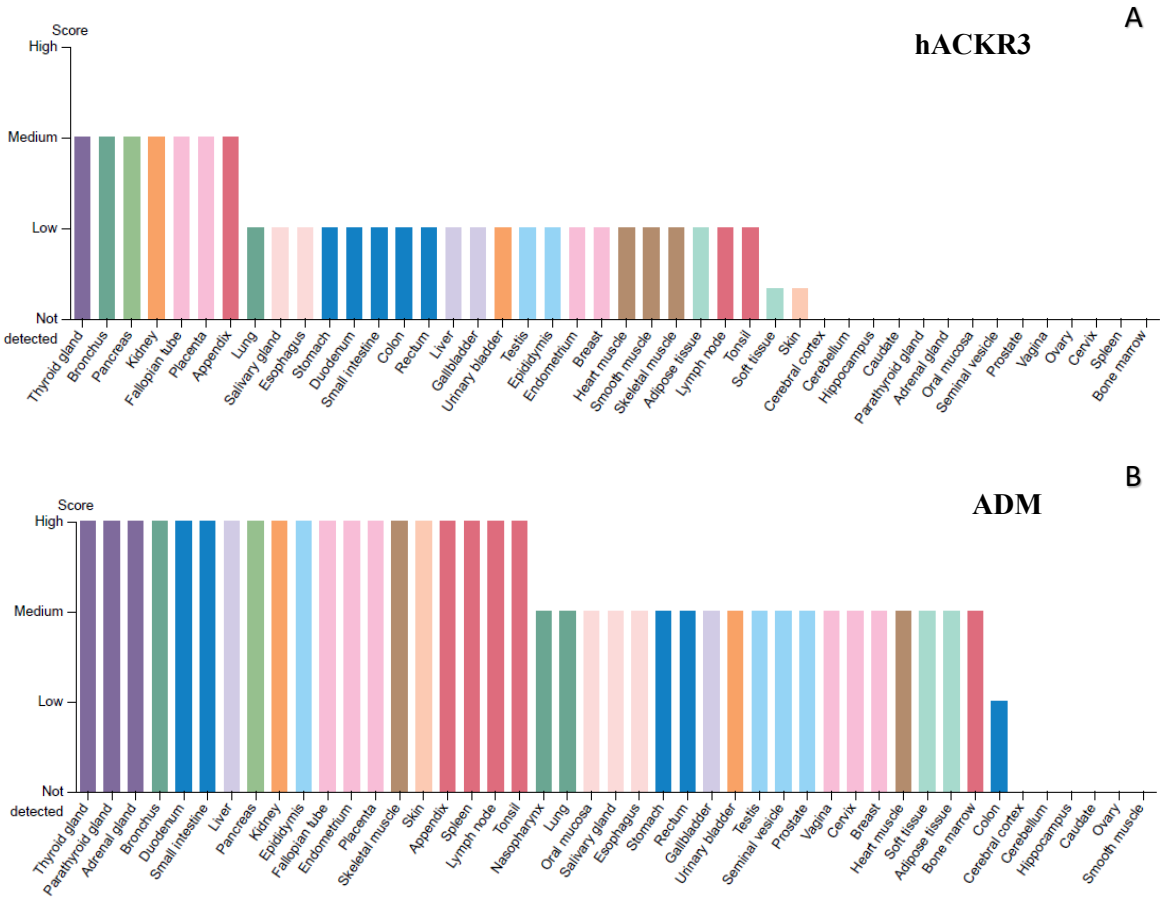


Fig. 6.2. Comparison of hACKR3 and adrenomedullin protein expression in tissue. The figure displays the comparison between hACKR3 (**panel A**) and adrenomedullin (**panel B**) protein expression levels in different organs and tissues. Thyroid glands, bronchi, pancreas, kidney, fallopian tubes, placenta and appendix are the organs where both hACKR3 and adrenomedullin are physiologically highly expressed (adapted from proteinatlas.com).

6.2 OUTCOMES ON hCXCR4-ADRENOMEDULLIN INTERACTIONS

In terms of hCXCR4-adrenomedullin interactions, not so much was reported in literature. One of the aims of this project was determining whether adrenomedullin could exert an effect on hCXCR4 as a consequence of known interactions between this receptor and hACKR3, with which hCXCR4 shares CXCL12 as main agonist ligand. Although hACKR3 and hCXCR4 may look similar, one of the crucial differences is the possibility of hCXCR4 to signal via G protein as the majority of GPCRs. This helped in assessing the effects of adrenomedullin on this receptor. As seen for hACKR3, an xCELLigence RTCA approach was used also to determine whether adrenomedullin would have influenced hCXCR4. It was demonstrated that adrenomedullin could reduce the positive signal recorded upon CXCL12 stimulation

in presence of pre-stimulation with this peptide before adding CXCL12 agonist to Flp-In TRE-x 293 cells expressing hCXCR4-mEGFP. Therefore, this was the first evidence of an effect of adrenomedullin on this receptor, for which it was acting as a blocker for CXCL12-induced response.

Then, during the characterization of G protein signalling pathway for hCXCR4, adrenomedullin showed an inhibitory effect on the agonist-dependent G protein activation for this receptor. Indeed, when Flp-In T-REx cells expressing hCXCR4 were stimulated with adrenomedullin before adding CXCL12, a concentration-dependent inhibition of this signalling pathway was observed. This phenomenon was appreciated in [^{35}S]GTPγS incorporation assay and cAMP accumulation assay, in which G protein activation was measured either by using a GTP analogue which is not hydrolysed during the normal G protein signalling cascade and which was labelled with a [^{35}S] group, or by measuring the accumulation of the intracellular second messenger cAMP, as a consequence of the inhibition of the adenylyl cyclase enzyme upon $G_{\alpha i/o}$ protein activation.

However, the blocking effect of adrenomedullin was not observed in bystander BRET assay, where β-arrestin recruitment was measured. In this case, the CXCL12-mediated β-arrestin recruitment was not affected either negatively or positively by adrenomedullin. For the first time, these outcomes suggested the possibility of adrenomedullin as a possible biased antagonist for hCXCR4. This idea was confirmed via SDS-PAGE by using phosphosite-specific antisera targeting specific serine residues present in the C-terminus side of hCXCR4 which are phosphorylated upon agonist-mediated activation of the receptor. Indeed, even in this case adrenomedullin did not show any reduction of phosphorylation, suggesting that this peptide might be a real biased antagonist for hCXCR4 towards G protein signalling pathway.

However, a point to underline was the limit represented by this, since the anti-CXCR4 no phospho antiserum showed differences in detecting the receptor upon CXCL12 addition. These differences were noticed also with an anti-GFP antibody, where instead IT1t increased the signal of the band relative to hCXCR4. The explanation provided is that the efficiency of these antisera may be affected by the presence of phosphorylated residues that are placed in the C-terminal side of the receptor. These can cause a steric hindrance with strong influence on the detection of the highly phosphorylated receptor. In case of hCXCR4, also the receptor density and the oligomerization state of the receptor could affect the recognition by these antisera since some ligands such as CXCL12 and IT1t seem to prefer hCXCR4 in its

monomeric form rather than oligomeric form. Similar problems with no phospho antisera were found in other GPCRs, such as GPR35 (Ganguly et al., 2023). Here problems in differences in receptor detection upon ligand addition were solved by adding in frame at the C-terminal side of GPR35 a HA-tag, in this case correcting the steric hindrance problems encountered before. The choice of the C-terminus relies on the location of the residues phosphorylated upon agonist activation in most of the GPCRs, and, therefore, this is the critical part where steric hindrance issues may arise in case of phosphorylation of the receptor, rather than the N-terminal side. However, the modification of the C-terminus side of GPCRs has always a percentage of risk of causing a loss of functionality of the receptors, because of the important role of this region in the regulation of the receptors life cycle and signalling. This makes more complicated the study of the phosphorylation of the receptor via immunoblots, since other approaches and additional controls may be needed to further highlights this aspect of receptor signalling.

In the process of understanding the mechanism by which adrenomedullin could act as inhibitor for hCXCR4, by carrying out a Schild regression analysis in cAMP accumulation assay it was demonstrated that adrenomedullin could be a negative allosteric modulator for hCXCR4, since a shift in the efficacy of CXCL12 in generating HRTF signal was recorded with increasing concentrations of adrenomedullin used. Therefore, this ligand it may bind the receptor in a different binding pocket from CXCL12 to exert its effects.

In recent years, in pharmacology, the existence of biased ligands has changed old concepts and opened new insights about the mechanisms of action of different molecules towards their receptors. In general, biased agonists which show a preference in activating a signalling pathway rather than another represents most of biased ligands currently known. Examples are α2-adrenergic receptors agonist, which show differences in the type of G protein that they stimulate (Eason et al., 1994), or dopaminergic D2 receptor agonists, which do not affect β-arrestin recruitment (Allen et al., 2011), or μ opioid receptor agonist morphine which is biased towards G protein signalling (Bohn et al. 2004). However, also few examples of biased antagonists are known, which make our knowledge about the biased ligands behaviour still not fully clear. Examples of these cases are some antagonists against 5-HT7R (serotonin receptor) (Kwag et al., 2021), the β-arrestin-biased β_2 adrenergic receptor agonist carvedilol, and also the peptide antagonist X4-2-6 for CXCR4, which is derived from transmembrane helix 2 and extracellular loop 1 of this receptor and that blocks G

protein-dependent chemotaxis but not β-arrestin1/2 recruitment (Hitchinson et al., 2018).

The fact that biased antagonists exist and one for hCXCR4 has already been characterized in the past, enforces the idea that adrenomedullin could be added to this list for the peculiar behaviour towards this receptor. Moreover, many ligands which are antagonists for hCXCR4, such as IT1t or TC14010, act as agonists for hACKR3. Since adrenomedullin is a putative agonist for hACKR3, acting similarly to the ligands listed before, it may behave as antagonist for hCXCR4 because of its structural features and its ability to activate hACKR3, because of the possible heterodimerization of hCXCR4-hACKR3. This may be especially true in pathological situations, where GPCRs heterodimerization could occur because of their transient expression in regions where normally they are not present in the body (Zagzag et al., 2006; Romain et al., 2014; Tiwari et al., 2021).

Crystal structures of the receptor fused to the T4 lysozyme including the structure with IT1t and CVX15 (Wu et al., 2010), CXCL12 (Xu et al., 2013) and AMD3100 (Adlere et al., 2019) have been described in the recent years, showing that hCXCR4, even if it displays a high similarity with other GPCRs in terms of structure, it presents some peculiarities which make different its binding to its ligands compared to the mechanisms adopted by other GPCRs. Moreover, to make this phenomenon even more intricated, also the tendency of hCXCR4 to form homodimers but also heterodimers with other GPCRs can affect and explain the possible binding of adrenomedullin to this receptor with consequent pharmacological effect. It has already been discussed that adrenomedullin binds to members of hCLR and hRAMPs when they heterodimerize, so it could be possible that this peptide could exert its effect after binding hCXCR4 in specific conformations, which are dictated by its structural flexibility. Indeed, Bailey at al. (2019) reported that hRAMP1 and hRAMP2 are endogenously expressed in HEK 293 cells, with a higher level of the former. Since hRAMP2, when oligomerizing with hCLR forms the adrenomedullin receptor, it may be possible that the presence of endogenously expressed hRAMP2 receptor could contribute to the role of adrenomedullin as biased antagonist for hCXCR4. This could depend either on the oligomerization of these (or more) receptors, or on structural interactions between adrenomedullin and hRAMP2, which may form a complex with inhibitory effects on hCXCR4. Therefore, it would be interesting to investigate more about the hetero-dimerization of these two receptors in relation to adrenomedullin to

determine whether there might be a role of other elements in adrenomedullin-hCXCR4 interactions.

Another aspect that could explain the biased antagonism of adrenomedullin towards G protein could be the conformation which hCXCR4 might assume upon cellular effectors binding, such as G proteins, arrestins or GRKs. Paradis et al. (2022) hypothesized the existence of two different conformations of hCXCR4 quaternary structure, which are called open-dimer and closed-dimer conformations, by using a QUESTS approach (QUaternary rEceptor STate design for Signaling selectivity). This method consists of building active and inactive states of GPCRs, which are docked to identify possible modes of protomer associations. This is used therefore to design binding interfaces and generate quaternary structures with distinct dimer stabilities, conformations, and propensity to recruit and activate specific intracellular signalling proteins (Paradis et al., 2022). In this study, it was put in evidence that the steric hindrance prevents the correct interaction between the finger loop of β-arrestin with the residues localized in the intracellular binding groove of hCXCR4 when the receptor occupies the closed-dimer conformation. In this conformation, the area of close contacts formed between β-arrestin and the open-dimer hCXCR4 (such as helix 8 of hCXCR4 monomer 2 with the C-tip of β-arrestin, and ICL2 of hCXCR4 monomer 1 with the C-loop of β-arrestin) would be disrupted and this could explain the biased behaviour of some ligands, which may show a preference for activating or inhibiting specific pathways because of steric hindrance and conformational reasons.

The inhibition effect of adrenomedullin could also depend on the different phosphorylation state of hCXCR4. Although SDS-PAGE experiments with phosphosite-specific antisera did not show any influence of adrenomedullin on phosphorylation of hCXCR4 upon CXCL12 addition, in NanoBiT complementation assay used to study the interactions between GRKs and hCXCR4 interesting data were found. Indeed, in case of this receptor, adrenomedullin seemed to inhibit the recruitment of GRK2 and GRK3 to hCXCR4, which might affect the phosphorylation pattern required for a correct functioning of the receptor. Since the importance of the interactions with other cellular effectors, such as arrestins, has been shown for many GPCRs regulation and signalling using different approaches, it could be possible a correlation between the block of G protein signalling mediated by adrenomedullin and the block of GRKs. In the past, it has been reported a connection between GRKs and Gβγ subunit of G proteins, especially for GRK2 and GRK3 (Eichmann et al., 2003; Sterne-Marr et al., 2003). Indeed, GRK2/3, normally localized in the cytosol, rely on

free Gβγ subunits to translocate to the membrane to active receptor. This Gβγ-mediated GRK2/3 recruitment is important to make the receptor able to be phosphorylated to enhance β-arrestin2 recruitment. This would explain even more the biased nature of adrenomedullin ligand towards G protein signalling.

Therefore, since it is not fully understood yet the mechanisms through which adrenomedullin can act as a biased antagonist for hCXCR4, further analysis, which involve molecular docking, structural analysis and mutagenesis approaches shall be used to clarify the key residues of the binding pocket of this ligand on hCXCR4 and the study performed in this thesis represents a first step for the identification of new therapeutic agents targeting hCXCR4.

6.3 FUTURE PERSPECTIVE

Both hACKR3 and hCXCR4 still represent important targets for the treatment of several pathological conditions related to dysfunction of immune system. Moreover, these receptors are widely expressed in the body and might represent a target also for other conditions where inflammation might play an important role. Adrenomedullin, because of its heterogeneity in binding to different GPCRs, could represent an important lead molecule in a drug discovery perspective to obtain new treatments. Therefore, it would be interesting developing new and effective phosphosite-specific antisera to investigate the phosphorylation pattern determined by adding adrenomedullin to cells expressing hACKR3 and highlight which are the effects of this peptide on hACKR3.

Also, by molecular docking on model of hACKR3, hCXCR4, CXCL12 and adrenomedullin structures, it would be interesting assessing which are the region involved in the binding and formation of ligand-receptor complexes. This in particular could be more complicated for hCXCR4, for which adrenomedullin has been proposed as a negative allosteric modulator in this study. The behaviour of this ligand strongly limits the possible choices about the approach to use to determine the binding mechanism to hCXCR4, because its binding site probably does not match with the CXCL12 binding site with a certain extent.

Indeed, this would help in synthetize new ligands and playing with structure-activity relationship studies to find new effective therapeutics which may take advantage of

the biased antagonism of adrenomedullin, which would be particularly helpful from patients' perspective.

Lastly, it could be helpful to get insights about the effects of adrenomedullin on hACKR3-hCXCR4 hetero-dimer, to see whether the behaviour of this ligand may change in case of co-expression and co-localization of both receptors, but also with hetero-dimers formed between these chemokine receptors and other GPCRs that have been reported to be able to bind adrenomedullin, such as hCLR and hRAMPs receptors.

Then, all these experimental approaches could potentiate our knowledge on these chemokine receptors, and this would help also to translate these findings to an *in vivo* approach, and, to follow, to human-based studies, with a great potential for improving patients' health and our understanding of a huge spectrum of diseases in which adrenomedullin might play a crucial role.

CHAPTER 7 - REFERENCES

Adlere, I., Caspar, B., Arimont, M., Dekkers, S., Visser, K., Stuijt, J., de Graaf, C., Stocks, M., Kellam, B., Briddon, S., Wijtmans, M., de Esch, I., Hill, S., & Leurs, R. (2019). Modulators of CXCR4 and CXCR7/ACKR3 Function. *Molecular pharmacology*, *96*(6), 737–752. https://doi.org/10.1124/mol.119.117663

Adlere, I., Sun, S., Zarca, A., Roumen, L., Gozelle, M., Viciano, C. P., Caspar, B., Arimont, M., Bebelman, J. P., Briddon, S. J., Hoffmann, C., Hill, S. J., Smit, M. J., Vischer, H. F., Wijtmans, M., de Graaf, C., de Esch, I. J. P., & Leurs, R. (2019). Structure-based exploration and pharmacological evaluation of N-substituted piperidin-4-yl-methanamine CXCR4 chemokine receptor antagonists. *European journal of medicinal chemistry*, *162*, 631–649. https://doi.org/10.1016/j.ejmech.2018.10.060

Ahn D., Ham D., Chung K. Y., The conformational transition during G protein-coupled receptor (GPCR) and G protein interaction. *Curr. Opin. Struct. Biol.* 69, 117–123 (2021).

Alampour-Rajabi, S., El Bounkari, O., Rot, A., Müller-Newen, G., Bachelerie, F., Gawaz, M., Weber, C., Schober, A., & Bernhagen, J. (2015). MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 29(11), 4497–4511. https://doi.org/10.1096/fj.15-273904

Albee LJ, Eby JM, Tripathi A, LaPorte HM, Gao X, Volkman BF, Gaponenko V, and Majetschak M (2017) a1-Adrenergic receptors function within hetero-oligomeric complexes with atypical chemokine receptor 3 and chemokine (C-X-C motif) receptor 4 in vascular smooth muscle cells. J Am Heart Assoc 6:e006575 DOI: 10.1161/JAHA.117.006575.

Alekhina O, Marchese A. β-Arrestin1 and Signal-transducing Adaptor Molecule 1 (STAM1) Cooperate to Promote Focal Adhesion Kinase Autophosphorylation and Chemotaxis via the Chemokine Receptor CXCR4. J Biol Chem. 2016 Dec 9;291(50):26083-26097. doi: 10.1074/jbc.M116.757138. Epub 2016 Oct 27. PMID: 27789711; PMCID: PMC5207078.

Allen, S. J., Crown, S. E. and Handel, T. M. (2007), 'Chemokine:Receptor Structure, Interactions, and Antagonism', *Annual Review of Immunology* **25**(1), 787–820. **URL:** *http://www.annualreviews.org/doi/10.1146/annurev.immunol.24.021605.090529*

Allen, J. A., Yost, J. M., Setola, V., Chen, X., Sassano, M. F., Chen, M., et al. (2011). Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18488–18493. doi: 10.1073/pnas.1104807108

Andresen BT. A pharmacological primer of biased agonism. *Endocr Metab Immune Disord DrugTargets*. 2011;11(2):92-98. doi:10.2174/187153011795564179

Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014 Oct 7;5:491. doi: 10.3389/fimmu.2014.00491. PMID: 25339958; PMCID: PMC4188125.

Arimont, M., Sun, S. L., Leurs, R., Smit, M., de Esch, I. J. P., & de Graaf, C. (2017). Structural Analysis of Chemokine Receptor-Ligand Interactions. *Journal of medicinal chemistry*, *60*(12), 4735–4779. https://doi.org/10.1021/acs.jmedchem.6b01309

Ashizuka S, Kita T, Inatsu H, Kitamura K. Adrenomedullin: A Novel Therapeutic for the Treatment of Inflammatory Bowel Disease. Biomedicines. 2021 Aug 23;9(8):1068. doi: 10.3390/biomedicines9081068. PMID: 34440272; PMCID: PMC8391925.

Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics. 2011 Jan 7;12:14. doi: 10.1186/1471-2164-12-14. PMID: 21214938; PMCID: PMC3024950.

Attwood, T. K. and Findlay, J. B. (1994), 'Fingerprinting g-protein-coupled receptors', *Protein Engineering, Design and Selection* **7**(2), 195–203.

Babcock, G. J., Farzan, M., & Sodroski, J. (2003). Ligand-independent dimerization of CXCR4, a principal HIV-1 coreceptor. *The Journal of biological chemistry*, *278*(5), 3378–3385. https://doi.org/10.1074/jbc.M210140200

Bachelerie, F., Ben-baruch, A., Burkhardt, A. M., Combadiere, C., Farber, J. M., Graham, G. J., Horuk, R., Sparre-ulrich, A. H., Locati, M., Luster, A. D., Mantovani, A., Matsushima, K., Murphy, P. M., Nibbs, R., Nomiyama, H., Power, C. A., Proudfoot, A. E. I., Rosenkilde, M. M. and Rot, A. (2014), 'International Union of Basic and Clinical Pharmacology. LXXXIX . Update on the Extended Family of Chemokine Receptors and Introducing a New Nomenclature for Atypical Chemokine Receptors', Pharmacological reviews 66(January 2014), 1–79.

Baek K. H. (2003). Conjugation and deconjugation of ubiquitin regulating the destiny of proteins. *Experimental & molecular medicine*, *35*(1), 1–7. https://doi.org/10.1038/emm.2003.1

Baggiolini, M. (2001), 'Chemokines in pathology and medicine', *Journal of Internal Medicine* **250**, 91–104.

Bailey, S., Harris, M., Barkan, K., Winfield, I., Harper, M. T., Simms, J., Ladds, G., Wheatley, M., & Poyner, D. (2019). Interactions between RAMP2 and CRF receptors: The effect of receptor subtypes, splice variants and cell context. *Biochimica et biophysica acta. Biomembranes*, *1861*(5), 997–1003. https://doi.org/10.1016/j.bbamem.2019.02.008

Balabanian, K., Harriague, J., Decrion, C., Lagane, B., Shorte, S., Baleux, F., Virelizier, J.-L., Arenzana-Seisdedos, F. and Chakrabarti, L. A. (2004), 'CXCR4-Tropic HIV-1 Envelope Glycoprotein Functions as a Viral Chemokine in Unstimulated Primary CD4+ T Lymphocytes', *The Journal of Immunology* **173**(12), 7150–7160. **URL:** *http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.173.12.7150*

Balabanian, K., Lagane, B., Infantino, S., Chow, K. Y., Harriague, J., Moepps, B., Arenzana-Seisdedos, F., Thelen, M. and Bachelerie, F. (2005), 'The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes', *Journal of Biological Chemistry* **280**(42), 35760–35766.

Balabanian, K., Lagane, B., Pablos, J. L., Laurent, L., Planchenault, T., Verola, O., Lebbe, C., Kerob, D., Dupuy, A., Hermine, O., Nicolas, J. F., Latger-Cannard, V., Bensoussan, D., Bordigoni, P., Baleux, F., Le Deist, F., Virelizier, J. L., Arenzana-Seisdedos, F., & Bachelerie, F. (2005). WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood*, *105*(6), 2449–2457. https://doi.org/10.1182/blood-2004-06-2289

Balkwill, F. (2004a), 'Cancer and the chemokine network', Nature Reviews Cancer 4(7), 540–550. URL: http://www.nature.com/doifinder/10.1038/nrc1388

Balkwill, F. (2004b), 'The significance of cancer cell expression of the chemokine receptor CXCR4', Seminars in Cancer Biology 14(3), 171–179.

Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods in neurosciences.* 1995;25:366–428

Bao Y, Wang Z, Liu B, Lu X, Xiong Y, Shi J, Li P, Chen J, Zhang Z, Chen M, Wang L, Wu Z. A feed-forward loop between nuclear translocation of CXCR4 and HIF-1α promotes renal cell carcinoma metastasis. Oncogene. 2019 Feb;38(6):881-895. doi: 10.1038/s41388-018-0452-4. Epub 2018 Sep 3. PMID: 30177838; PMCID: PMC6367212.

Barki N, Jenkins L, Marsango S, Dedeo D, Bolognini D, Dwomoh L, Abdelmalik AM, Nilsen M, Stoffels M, Nagel F, Schulz S, Tobin AB, Milligan G. Phosphorylation bar-coding of free fatty acid receptor 2 is generated in a tissue-specific manner. Elife. 2023 Dec 12;12:RP91861. doi: 10.7554/eLife.91861. PMID: 38085667; PMCID: PMC10715726.

Beautrait, A., Paradis, J. S., Zimmerman, B., Giubilaro, J., Nikolajev, L., Armando, S., Kobayashi, H., Yamani, L., Namkung, Y., Heydenreich, F. M., Khoury, E., Audet, M., Roux, P. P., Veprintsev, D. B., Laporte, S. A., & Bouvier, M. (2017). A new inhibitor of the β-arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. *Nature communications*, *8*, 15054. https://doi.org/10.1038/ncomms15054

Berg KA, Clarke WP. Making Sense of Pharmacology: Inverse Agonism and Functional Selectivity. *Int J Neuropsychopharmacol*. 2018;21(10):962-977. doi:10.1093/ijnp/pyy071

Bianchi, M. E., & Mezzapelle, R. (2020). The Chemokine Receptor CXCR4 in Cell Proliferation and Tissue Regeneration. *Frontiers in immunology*, *11*, 2109. https://doi.org/10.3389/fimmu.2020.02109

Bikfalvi, A., & Billottet, C. (2020). The CC and CXC chemokines: major regulators of tumor progression and the tumor microenvironment. *American journal of physiology. Cell physiology*, *318*(3), C542–C554. https://doi.org/10.1152/ajpcell.00378.2019

Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A. and Mackay, C. R. (1997), 'The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes', *Proceedings of the National Academy of Sciences* **94**(5), 1925–1930. **URL:** *http://www.pnas.org/cgi/doi/10.1073/pnas.94.5.1925*

Blum NK, Schaffner A, Drube J, Nagel F, Reinscheid RK, Hoffmann C, Schulz S. Rapid elucidation of agonist-driven regulation of the neurokinin 1 receptor using a GPCR phosphorylation immunoassay. Eur J Pharmacol. 2024 Apr 19;973:176587. doi: 10.1016/j.ejphar.2024.176587. Epub ahead of print. PMID: 38642667.

Bohn, L. M., Dykstra, L. A., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (2004). Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol. Pharmacol.* 66, 106–112. doi: 10.1124/mol.66.1.106

Boitano S, Flynn AN, Schulz SM, Hoffman J, Price TJ, Vagner J. Potent agonists of the protease activated receptor 2 (PAR2). J Med Chem. 2011 Mar 10;54(5):1308-13. doi: 10.1021/jm1013049. Epub 2011 Feb 4. PMID: 21294569; PMCID: PMC3069554.

Boldajipour, B., Mahabaleshwar, H., Kardash, E., Reichman-Fried, M., Blaser, H., Minina, S., Wilson, D., Xu, Q. and Raz, E. (2008), 'Control of Chemokine-Guided Cell Migration by Ligand Sequestration', *Cell* **132**(3), 463–473.

Bongers, G., Maussang, D., Muniz, L. R., Noriega, V. M., Fraile-Ramos, A., Barker, N., Marchesi, F., Thirunarayanan, N., Vischer, H. F., Qin, L., Mayer, L., Harpaz, N., Leurs, R., Furtado, G. C., Clevers, H., Tortorella, D., Smit, M. J., & Lira, S. A. (2010). The cytomegalovirus-encoded chemokine receptor US28 promotes intestinal neoplasia in transgenic mice. The Journal of clinical investigation, 120(11), 3969–3978. https://doi.org/10.1172/JCI42563

Bradley CA. CXCR4-HIF-1α interaction drives metastasis. Nat Rev Urol. 2018 Dec;15(12):726. doi: 10.1038/s41585-018-0097-7. PMID: 30224776.

Bräuner-Osborne H, Wellendorph P, Jensen AA. Structure, pharmacology and therapeutic prospects of family C G-protein coupled receptors. *Curr Drug Targets*. 2007;8(1):169-184. doi:10.2174/138945007779315614

Brown MS, Goldstein JL. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc Natl Acad Sci USA.* (1979) 76:3330–7. doi: 10.1073/pnas.76.7.3330

Brumbaugh K, Liao WC, Houchins JP, Cooper J, Stoesz S. Phosphosite-Specific Antibodies: A Brief Update on Generation and Applications. Methods Mol Biol. 2017;1554:1-40. doi: 10.1007/978-1-4939-6759-9_1. PMID: 28185181.

Burns, J. M., Summers, B. C., Wang, Y., Melikian, A., Berahovich, R., Miao, Z., Penfold, M. E., Sunshine, M. J., Littman, D. R., Kuo, C. J., Wei, K., McMaster, B. E., Wright, K., Howard, M. C., & Schall, T. J. (2006). A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. The Journal of experimental medicine, 203(9), 2201–2213. https://doi.org/10.1084/jem.20052144

Busillo, J. M., Armando, S., Sengupta, R., Meucci, O., Bouvier, M., & Benovic, J. L. (2010). Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *The Journal of biological chemistry*, *285*(10), 7805–7817. https://doi.org/10.1074/jbc.M109.091173

Busillo, J. M., & Benovic, J. L. (2007). Regulation of CXCR4 signaling. *Biochimica et biophysica acta*, *1768*(4), 952–963. https://doi.org/10.1016/j.bbamem.2006.11.002

Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, Mistry S, and Tobin AB (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. J Biol Chem 286:11506–11518.

Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S. R., Neve, K., Fuxe, K., Agnati, L. F., Woods, A. S., Ferré, S., Lluis, C., Bouvier, M., & Franco, R. (2003). Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *The Journal of biological chemistry*, *278*(47), 46741–46749. https://doi.org/10.1074/jbc.M306451200

Canals, M., Scholten, D. J., de Munnik, S., Han, M. K., Smit, M. J., & Leurs, R. (2012). Ubiquitination of CXCR7 controls receptor trafficking. *PloS one*, *7*(3), e34192. https://doi.org/10.1371/journal.pone.0034192

Cao, J., Huang, S., Qian, J., Huang, J., Jin, L., Su, Z., Yang, J. and Liu, J. (2009), 'Evolution of the class C GPCR Venus flytrap modules involved positive selected functional divergence', *BMC Evolutionary Biology* **9**(1), 1–12.

Capoferri, Davide (2020) *In vitro pharmacological characterisation of the role of ACKR3 on CXCR4-dependent and -independent function.* PhD thesis, University of Glasgow

Caspar, B., Cocchiara, P., Melet, A., Van Emelen, K., Van der Aa, A., Milligan, G., & Herbeuval, J. P. (2022). CXCR4 as a novel target in immunology: moving away from typical antagonists. *Future drug discovery*, *4*(2), FDD77. https://doi.org/10.4155/fdd-2022-0007

Castillo JJ, Ghobrial IM, Treon SP. Biology, prognosis, and therapy of Waldenström Macroglobulinemia. Cancer Treat Res. 2015;165:177-95. doi: 10.1007/978-3-319-13150-4_7. PMID: 25655610.

Charo, I. F. and Ransohoff, R. M. (2006), 'The Many Roles of Chemokines and Chemokine Receptors in Inflammation', *New England Journal of Medicine* **354**(6), 610–621. **URL:** *http://www.nejm.org/doi/abs/10.1056/NEJMra052723*

Chatterjee S, Behnam Azad B, Nimmagadda S. The intricate role of CXCR4 in cancer. Adv Cancer Res. 2014;124:31-82. doi: 10.1016/B978-0-12-411638-2.00002-1. PMID: 25287686; PMCID: PMC4322894.

Chatterjee, M., Borst, O., Walker, B., Fotinos, A., Vogel, S., Seizer, P., Mack, A., Alampour-Rajabi, S., Rath, D., Geisler, T., Lang, F., Langer, H. F., Bernhagen, J. and Gawaz, M. (2014), 'Macrophage migration inhibitory factor limits activation-induced apoptosis of platelets via CXCR7-dependent Akt signaling', Circulation Research 115(11), 939–949.

Chatterjee M, von Ungern-Sternberg SN, Seizer P, Schlegel F, Büttcher M, Sindhu NA, Müller S, Mack A, and Gawaz M (2015) Platelet-derived CXCL12 regulates monocyte function, survival, differentiation into macrophages and foam cells through differential involvement of CXCR4-CXCR7. Cell Death Dis 6:e1989 DOI: 10.1038/cddis.2015.233.

Chen, K., Bao, Z., Tang, P., Gong, W., Yoshimura, T. and Wang, J. M. (2018), 'Chemokines in homeostasis and diseases', *Cellular and Molecular Immunology* **15**(July 2017), 324–334.

Chen, Q., Schafer, C. T., Mukherjee, S., Gustavsson, M., Agrawal, P., Yao, X. Q., Kossiakoff, A. A., Handel, T. M., & Tesmer, J. J. G. (2023). ACKR3-arrestin2/3 complexes reveal molecular consequences of GRK-dependent barcoding. bioRxiv : the preprint server for biology, 2023.07.18.549504. https://doi.org/10.1101/2023.07.18.549504

Cheng, Z.-J., Zhao, J., Sun, Y., Hu, W., Wu, Y.-L., Cen, B., Wu, G.-X. and Pei, G. (2000), 'b-Arrestin Differentially Regulates the Chemokine Receptor CXCR4-mediated Signaling and Receptor Internalization, and This Implicates Multiple Interaction Sites between b-Arrestin and CXCR4', *Journal of Biological Chemistry* **4**(January 28), 2479–2485.

Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science. 1995 Dec 15;270(5243):1811-5. doi: 10.1126/science.270.5243.1811. PMID: 8525373.

Coke, C. J., Scarlett, K. A., Chetram, M. A., Jones, K. J., Sandifer, B. J., Davis, A. S., Marcus, A. I., & Hinton, C. V. (2016). Simultaneous Activation of Induced Heterodimerization between CXCR4 Chemokine Receptor and Cannabinoid Receptor 2 (CB2) Reveals a Mechanism for Regulation of Tumor Progression. *The Journal of biological chemistry*, *291*(19), 9991–10005. https://doi.org/10.1074/jbc.M115.712661

Contento RL, Molon B, Boularan C, Pozzan T, Manes S, Marullo S, Viola A. CXCR4-CCR5: a couple modulating T cell functions. Proc Natl Acad Sci U S A. 2008;105:10101–6.

Costa T, Herz A, (1989), Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc Natl Acad Sci USA 86:7321–7325

Costantini, S., Raucci, R., De Vero, T., Castello, G., & Colonna, G. (2013). Common structural interactions between the receptors CXCR3, CXCR4 and CXCR7 complexed with their natural ligands, CXCL11 and CXCL12, by a modeling approach. Cytokine, 64(1), 316–321. https://doi.org/10.1016/j.cyto.2013.05.024

Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., & Clark-Lewis, I. (1997). Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *The EMBO journal*, *16*(23), 6996–7007. https://doi.org/10.1093/emboj/16.23.6996

Daaka, Y., Pitcher, J. A., Richardson, M., Stoffel, R. H., Robishaw, J. D., & Lefkowitz, R. J. (1997). Receptor and G betagamma isoform-specific interactions with G protein-coupled receptor kinases. Proceedings of the National Academy of Sciences of the United States of America, 94(6), 2180–2185. https://doi.org/10.1073/pnas.94.6.2180

Das S, Sarrou E, Podgrabinska S, Cassella M, Mungamuri SK, Feirt N, Gordon R, Nagi CS, Wang Y, Entenberg D, Condeelis J, Skobe M. Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. J Exp Med. 2013;210:1509–28

Davoine, F., & Lacy, P. (2014). Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Frontiers in immunology*, *5*, 570. https://doi.org/10.3389/fimmu.2014.00570

De Ceuninck, F., Duguet, F., Aussy, A., Laigle, L., & Moingeon, P. (2021). IFN-α: A key therapeutic target for multiple autoimmune rheumatic diseases. Drug discovery today, 26(10), 2465–2473. https://doi.org/10.1016/j.drudis.2021.06.010

De Clercq E. (2005). Potential clinical applications of the CXCR4 antagonist bicyclam AMD3100. *Mini reviews in medicinal chemistry*, *5*(9), 805–824. https://doi.org/10.2174/1389557054867075

De Clercq E. Mozobil® (Plerixafor, AMD3100), 10 years after its approval by the US Food and Drug Administration. *Antivir Chem Chemother*. 2019;27:2040206619829382. doi:10.1177/2040206619829382

Décaillot, F. M., Kazmi, M. A., Lin, Y., Ray-Saha, S., Sakmar, T. P., & Sachdev, P. (2011). CXCR7/CXCR4 heterodimer constitutively recruits beta-arrestin to enhance cell

migration. *The Journal of biological chemistry*, *286*(37), 32188–32197. https://doi.org/10.1074/jbc.M111.277038

Del Castillo J, Katz B (1957), Interaction at end-plate receptors between different choline derivatives. Proc R Soc Lond B Biol Sci 146:369–381

Digby GJ, Conn PJ, Lindsley CW. Orthosteric- and allosteric induced ligand-directed trafficking at GPCRs. *Current Opinion in Drug Discovery & Development* 2010; 13: 587–94

Divorty, Nina (2017) *Investigating G protein-coupled receptor 35 signalling and its role in cardiovascular disease.* PhD thesis, University of Glasgow

Divorty, N., Jenkins, L., Ganguly, A., Butcher, A. J., Hudson, B. D., Schulz, S., Tobin, A. B., Nicklin, S. A., & Milligan, G. (2022). Agonist-induced phosphorylation of orthologues of the orphan receptor GPR35 functions as an activation sensor. *The Journal of biological chemistry*, *298*(3), 101655. https://doi.org/10.1016/j.jbc.2022.101655

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

Drake, M. T., Shenoy, S. K., & Lefkowitz, R. J. (2006). Trafficking of G protein-coupled receptors. *Circulation research*, *99*(6), 570–582. https://doi.org/10.1161/01.RES.0000242563.47507.ce

Drandi, D., Decruyenaere, P., Ferrante, M., Offner, F., Vandesompele, J., & Ferrero, S. (2022). Nucleic Acid Biomarkers in Waldenström Macroglobulinemia and IgM-MGUS: Current Insights and Clinical Relevance. *Diagnostics (Basel, Switzerland)*, *12*(4), 969. https://doi.org/10.3390/diagnostics12040969

Drube J, Haider RS, Matthees ESF, Reichel M, Zeiner J, Fritzwanker S, Ziegler C, Barz S, Klement L, Filor J, Weitzel V, Kliewer A, Miess-Tanneberg E, Kostenis E, Schulz S, Hoffmann C. GPCR kinase knockout cells reveal the impact of individual GRKs on arrestin binding and GPCR regulation. Nat Commun. 2022 Jan 27;13(1):540. doi: 10.1038/s41467-022-28152-8. PMID: 35087057; PMCID: PMC8795447.

Dupuis J, Caron A, Ruël N: Biodistribution, plasma kinetics and quantification of single-pass pulmonary clearance of adrenomedullin. Clin Sci (Lond). 2005, 109: 97-102.

Eason, M. G., Jacinto, M. T., & Liggett, S. B. (1994). Contribution of ligand structure to activation of alpha 2-adrenergic receptor subtype coupling to Gs. *Molecular pharmacology*, *45*(4), 696–702.

Eby JM, Abdelkarim H, Albee LJ, Tripathi A, Gao X, Volkman BF, Gaponenko V, Majetschak M. Functional and structural consequences of chemokine (C-X-C motif) receptor 4 activation with cognate and non-cognate agonists. Mol Cell Biochem. 2017 Oct;434(1-2):143-151. doi: 10.1007/s11010-017-3044-7. Epub 2017 Apr 28. PMID: 28455789; PMCID: PMC5660673.

Eichmann, T., Lorenz, K., Hoffmann, M., Brockmann, J., Krasel, C., Lohse, M. J., & Quitterer, U. (2003). The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site. *The Journal of biological chemistry*, *278*(10), 8052–8057. https://doi.org/10.1074/jbc.M204795200

Endres, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Turner, J. D., McKnight, A., Thomas, J. F., Stoebenau-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N., Power, C. A., Sutterwala, S. S., Doms, R. W., Landau, N. R. and Hoxie, J. A. (1996), 'CD4-independent infection by HIV-2 is mediated by Fusin/CXCR4', *Cell* **87**(4), 745–756.

Eto T., Kitamura K. Adrenomedullin and Its Role in Renal Diseases. Nephron. 2001;89:121–134. doi: 10.1159/000046059

Feinstein, T. N., Yui, N., Webber, M. J., Wehbi, V. L., Stevenson, H. P., King, J. D., Hallows, K. R., Brown, D., Bouley, R. and Vilardaga, J. P. (2013), 'Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin', *Journal of Biological Chemistry* **288**(39), 27849–27860.

Feng, Z., Dubyak, G. R., Lederman, M. M., & Weinberg, A. (2006). Cutting edge: human beta defensin 3--a novel antagonist of the HIV-1 coreceptor CXCR4. Journal of immunology (Baltimore, Md. : 1950), 177(2), 782–786. https://doi.org/10.4049/jimmunol.177.2.782

Feng, Z., Dubyak, G. R., Jia, X., Lubkowski, J. T., & Weinberg, A. (2013). Human β-defensin-3 structure motifs that are important in CXCR4 antagonism. The FEBS journal, 280(14), 3365–3375. https://doi.org/10.1111/febs.12328

Ferguson, S. S. G. (2001), 'Evolving concepts in G protein-coupled receptor endocytosis: the tole in receptor desensitization and signaling', *Pharmacological Reviews* **53**(1), 1–24.

Ferguson SS, Zhang J, Barak LS, Caron MG. 1998. Molecular mechanisms of G protein coupled receptor desensitization and resensitization. Life Sci. 62:1561–65

Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., & Patel, D. D. (2002). Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(11), 7478–7483. https://doi.org/10.1073/pnas.112198299

Fotiadis, D., Jastrzebska, B., Philippsen, A., M¨uller, D. J., Palczewski, K. and Engel, A. (2006), 'Structure of the rhodopsin dimer: a working model for G-protein-coupled receptors', *Current Opinion in Structural Biology* **16**(2), 252–259.

Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., & Palczewski, K. (2004). The G protein-coupled receptor rhodopsin in the native membrane. *FEBS letters*, *564*(3), 281–288. https://doi.org/10.1016/S0014-5793(04)00194-2

Fourgeaud, L., Bessis, A. S., Rossignol, F., Pin, J. P., Olivo-Marin, J. C., & Hémar, A. (2003). The metabotropic glutamate receptor mGluR5 is endocytosed by a clathrin-independent pathway. *The Journal of biological chemistry*, *278*(14), 12222–12230. https://doi.org/10.1074/jbc.M205663200

Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*. 2003;63(6):1256-1272. doi:10.1124/mol.63.6.1256

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

Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prézeau, L., & Pin, J. P. (2001). Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *The EMBO journal*, *20*(9), 2152–2159. https://doi.org/10.1093/emboj/20.9.2152

Ganguly A, Quon T, Jenkins L, Joseph B, Al-Awar R, Chevigne A, Tobin AB, Uehling DE, Hoffmann C, Drube J, Milligan G. G protein-receptor kinases 5/6 are the key regulators of G protein-coupled receptor 35-arrestin interactions. J Biol Chem. 2023 Oct;299(10):105218. doi: 10.1016/j.jbc.2023.105218. Epub 2023 Sep 1. PMID: 37660910; PMCID: PMC10520886.

Garayoa M, Martinez A, Lee S, Pio R, An WG, Neckers L, Trepel J, Montuenga LM, Ryan H, Johnson R, Gassmann M, Cuttitta F: Hypoxia-inducible factor-1 (HIF-1) up-regulates

adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol Endocrinol. 2000, 14: 848-862.

Ge, B., Lao, J., Li, J., Chen, Y., Song, Y., & Huang, F. (2017). Single-molecule imaging reveals dimerization/oligomerization of CXCR4 on plasma membrane closely related to its function. *Scientific reports*, *7*(1), 16873. https://doi.org/10.1038/s41598-017-16802-7

Ge, Y., Huang, M., & Yao, Y. M. (2021). The Effect and Regulatory Mechanism of High Mobility Group Box-1 Protein on Immune Cells in Inflammatory Diseases. *Cells*, *10*(5), 1044. https://doi.org/10.3390/cells10051044

Gerrits, H., van Ingen Schenau, D. S., Bakker, N. E., van Disseldorp, A. J., Strik, A., Hermens, L. S., Koenen, T. B., Krajnc-Franken, M. A., & Gossen, J. A. (2008). Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. Genesis (New York, N.Y.: 2000), 46(5), 235–245. https://doi.org/10.1002/dvg.20387

Gilman A. G. (1987). G proteins: transducers of receptor-generated signals. *Annual review of biochemistry*, *56*, 615–649. https://doi.org/10.1146/annurev.bi.56.070187.003151

Goo, R. S. (2001), 'Regulation of Phosphoinositide-Specific Phospholipase C', *Annu. Rev. Biochem* **70**, 281–312.

Goodman OB Jr, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL: Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature 1996, 383:447-450.

Gorbachev AV, Fairchild RL. Regulation of chemokine expression in the tumor microenvironment. Crit Rev Immunol. 2014;34(2):103-20. doi: 10.1615/critrevimmunol.2014010062. PMID: 24940911; PMCID: PMC7191635.

Grozdanovic M, Laffey KG, Abdelkarim H, Hitchinson B, Harijith A, Moon HG, Park GY, Rousslang LK, Masterson JC, Furuta GT, Tarasova NI, Gaponenko V, Ackerman SJ. Novel peptide nanoparticle-biased antagonist of CCR3 blocks eosinophil recruitment and airway hyperresponsiveness. J Allergy Clin Immunol. 2019 Feb;143(2):669-680.e12. doi: 10.1016/j.jaci.2018.05.003. Epub 2018 May 17. PMID: 29778505; PMCID: PMC6240402.

Gurevich, E. V., & Gurevich, V. V. (2006). Arrestins: ubiquitous regulators of cellular signaling pathways. *Genome biology*, *7*(9), 236. https://doi.org/10.1186/gb-2006-7-9-236

Gurevich, E. V., Tesmer, J. J., Mushegian, A., and Gurevich, V. V. (2012). G protein coupled receptor kinases: more than just kinases and not only for GPCRs. Pharmacol. Ther. 133, 40–46. doi: 10.1016/j.pharmthera.2011.08.001

Gurevich, V. V., & Gurevich, E. V. (2006). The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacology & therapeutics*, *110*(3), 465–502. https://doi.org/10.1016/j.pharmthera.2005.09.008

Gurevich, V. V., & Gurevich, E. V. (2019). GPCR Signaling Regulation: The Role of GRKs and Arrestins. *Frontiers in pharmacology*, *10*, 125. https://doi.org/10.3389/fphar.2019.00125

Gustavsson M, Dyer DP, Zhao C, Handel TM. Kinetics of CXCL12 binding to atypical chemokine receptor 3 reveal a role for the receptor N terminus in chemokine binding. *Sci Signal*. 2019;12(598):eaaw3657. Published 2019 Sep 10. doi:10.1126/scisignal.aaw3657

Gustavsson, M., Wang, L., Van Gils, N., Stephens, B. S., Zhang, P., Schall, T. J., Yang, S., Abagyan, R., Chance, M. R., Kufareva, I. and Handel, T. M. (2017), 'Structural basis of ligand interaction with atypical chemokine receptor 3', *Nature Communications* **8**, 1–14.

Haga, K., and Haga, T. (1992). Activation by G protein beta gamma subunits of agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. J. Biol. Chem. 267, 2222–2227.

Hamatake M, Aoki T, Futahashi Y, Urano E, Yamamoto N, and Komano J (2009) Ligand-independent higher-order multimerization of CXCR4, a G-protein-coupled chemokine receptor involved in targeted metastasis. Cancer Sci 100:95–102.

Hamidi H, Lilja J, Ivaska J. Using xCELLigence RTCA Instrument to Measure Cell Adhesion. Bio Protoc. 2017 Dec 20;7(24):e2646. doi: 10.21769/BioProtoc.2646. PMID: 29367941; PMCID: PMC5777632.

Hanaoka, H., Okazaki, Y., Hashiguchi, A., Yasuoka, H., Takeuchi, T., & Kuwana, M. (2015). Overexpression of CXCR4 on circulating B cells in patients with active systemic lupus erythematosus. *Clinical and experimental rheumatology*, *33*(6), 863–870.

Hanes, M. S., Salanga, C. L., Chowdry, A. B., Comerford, I., McColl, S. R., Kufareva, I., & Handel, T. M. (2015). Dual targeting of the chemokine receptors CXCR4 and ACKR3 with novel engineered chemokines. *The Journal of biological chemistry*, *290*(37), 22385–22397. https://doi.org/10.1074/jbc.M115.675108

Hao, H., Hu, S., Chen, H., Bu, D., Zhu, L., Xu, C., Chu, F., Huo, X., Tang, Y., Sun, X., Ding, B. S., Liu, D. P., Hu, S., & Wang, M. (2017). Loss of Endothelial CXCR7 Impairs Vascular Homeostasis and Cardiac Remodeling After Myocardial Infarction: Implications for Cardiovascular Drug Discovery. Circulation, 135(13), 1253–1264. https://doi.org/10.1161/CIRCULATIONAHA.116.023027

Haraguchi, T., Uchida, T., Yoshida, M., Kojima, H., Habara, M. and Ikezaki, H. (2018), 'The Utility of the Artificial Taste Sensor in Evaluating the Bitterness of Drugs: Correlation with Responses of Human TASTE2 Receptors (hTAS2Rs)', Chemical & Pharmaceutical Bulletin 66(1), 71–77. URL: https://www.jstage.jst.go.jp/article/cpb/66/1/66 c17-00619/ article

Hartmann TN, Grabovsky V, Pasvolsky R, Shulman Z, Buss EC, Spiegel A, Nagler A, Lapidot T, Thelen M, and Alon R (2008) A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. J Leukoc Biol 84:1130–1140 DOI: 10.1189/jlb.0208088.

Hattermann, K., Held-Feindt, J., Lucius, R., Muerkoester, S. S., Penfold, M. E. T., Schall, T. J. and Mentlein, R. (2010), 'The chemokine receptor CXCR7 is highly expressed in human glioma cells and mediates antiapoptotic effects', *Cancer Research* **70**(8), 3299–3308.

Hausdorff WP, Caron MG, and Lefkowitz RJ (1990) Turning off the signal: desensitization of beta-adrenergic receptor function. FASEB J 4:2881–2889

Heinrich, E. L., Lee, W., Lu, J., Lowy, A. M. and Kim, J. (2012), 'Chemokine CXCL12 activates dual CXCR4 and CXCR7-mediated signaling pathways in pancreatic cancer cells', *Journal of Translational Medicine* **10**(68), 1–9. **URL:** *http://www.translational-medicine.com/content/10/1/68*

Hendrix, C. W., Collier, A. C., Lederman, M. M., Schols, D., Pollard, R. B., Brown, S., Jackson, J. B., Coombs, R. W., Glesby, M. J., Flexner, C. W., Bridger, G. J., Badel, K., MacFarland, R. T., Henson, G. W. and Calandra, G. (2004), 'Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection', *Journal of Acquired Immune Deficiency Syndromes* **37**(2), 1253–1262.

Hernandez PA, Gorlin RJ, Lukens JN, Taniuchi S, Bohinjec J, Francois F, Klotman ME, Diaz GA. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM

syndrome, a combined immunodeficiency disease. Nat Genet. 2003 May;34(1):70-4. doi: 10.1038/ng1149. PMID: 12692554.

Heuninck, J., Perpiñá Viciano, C., Işbilir, A., Caspar, B., Capoferri, D., Briddon, S. J., Durroux, T., Hill, S. J., Lohse, M. J., Milligan, G., Pin, J. P., & Hoffmann, C. (2019). Context-Dependent Signaling of CXC Chemokine Receptor 4 and Atypical Chemokine Receptor 3. *Molecular pharmacology*, *96*(6), 778–793. https://doi.org/10.1124/mol.118.115477

Heusinkveld LE, Majumdar S, Gao JL, McDermott DH, Murphy PM. WHIM Syndrome: from Pathogenesis Towards Personalized Medicine and Cure. J Clin Immunol. 2019 Aug;39(6):532-556. doi: 10.1007/s10875-019-00665-w. Epub 2019 Jul 16. PMID: 31313072; PMCID: PMC6698215.

Hitchinson, B., Eby, J. M., Gao, X., Guite-Vinet, F., Ziarek, J. J., Abdelkarim, H., Lee, Y., Okamoto, Y., Shikano, S., Majetschak, M., Heveker, N., Volkman, B. F., Tarasova, N. I., & Gaponenko, V. (2018). Biased antagonism of CXCR4 avoids antagonist tolerance. Science signaling, 11(552), eaat2214. https://doi.org/10.1126/scisignal.aat2214

Hoffmann, F., Müller, W., Schütz, D., Penfold, M. E., Wong, Y. H., Schulz, S., & Stumm, R. (2012). Rapid uptake and degradation of CXCL12 depend on CXCR7 carboxyl-terminal serine/threonine residues. *The Journal of biological chemistry*, *287*(34), 28362–28377. https://doi.org/10.1074/jbc.M111.335679

Holz GG, Turner TJ. Pertussis toxin-sensitive GTP-binding proteins characterized in synaptosomal fractions of embryonic avian cerebral cortex. Comp Biochem Physiol B Biochem Mol Biol. 1998 Jan;119(1):201-11. doi: 10.1016/s0305-0491(97)00308-8. PMID: 9530821; PMCID: PMC2924613.

Hosey M. M. (1999). What molecular events underlie heterologous desensitization? Focus on "receptor phosphorylation does not mediate cross talk between muscarinic M(3) and bradykinin B(2) receptors". *The American journal of physiology*, *277*(5), C856–C858. https://doi.org/10.1152/ajpcell.1999.277.5.C856

Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018 Aug;285(16):2944-2971. doi: 10.1111/febs.14466. Epub 2018 Apr 24. PMID: 29637711; PMCID: PMC6120486.

Infantino, S., Moepps, B., & Thelen, M. (2006). Expression and regulation of the orphan receptor RDC1 and its putative ligand in human dendritic and B cells. Journal of

immunology (Baltimore, Md. : 1950), 176(4), 2197–2207. https://doi.org/10.4049/jimmunol.176.4.2197

Ikeda S, Keneko M, and Fujiwara S (2007) inventors, Takeda Pharmaceutical Co. Ltd., Ikeda S, Keneko M, Fujiwara S, assignees. Cardiotonic agent comprising GRK inhibitor. International patent WO 2007034846. 2007 Mar 29

Ikeda, Y., Kumagai, H., Skach, A., Sato, M. and Yanagisawa, M. (2013), 'Modulation of circadian glucocorticoid oscillation via adrenal Opioid-CXCR7 Signaling alters emotional behavior', *Cell* **155**(6), 1323–1336. **URL:** *http://dx.doi.org/10.1016/j.cell.2013.10.052*

Işbilir, A., Möller, J., Arimont, M., Bobkov, V., Perpiñá-Viciano, C., Hoffmann, C., Inoue, A., Heukers, R., de Graaf, C., Smit, M. J., Annibale, P., & Lohse, M. J. (2020). Advanced fluorescence microscopy reveals disruption of dynamic CXCR4 dimerization by subpocket-specific inverse agonists. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(46), 29144–29154. https://doi.org/10.1073/pnas.2013319117

Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K. and Nagata, S. (1991), 'Molecular cloning and expression of a cDNA encoding the secretin receptor', The EMBO Journal 10(7), 1635–1641

Ishimoto, N., Park, J. H., Kawakami, K., Tajiri, M., Mizutani, K., Akashi, S., Tame, J. R. H., Inoue, A., & Park, S. Y. (2023). Structural basis of CXC chemokine receptor 1 ligand binding and activation. *Nature communications*, *14*(1), 4107. https://doi.org/10.1038/s41467-023-39799-2

Ivins, S., Chappell, J., Vernay, B., Suntharalingham, J., Martineau, A., Mohun, T. J. and Scambler, P. J. (2015), 'The CXCL12/CXCR4 Axis Plays a Critical Role in Coronary Artery Development', *Developmental Cell* **33**(4), 455–468. **URL:** *http://dx.doi.org/10.1016/j.devcel.2015.03.026*

Iwasa, S., Yanagawa, T., Fan, J. and Katoh, R. (2009), 'Expression of CXCR4 and its ligand SDF-1 in intestinal-type gastric cancer is associated with lymph node and liver metastasis', *Anticancer Research* **29**(11), 4751–4758.

Jankauskas, S. S., Wong, D. W. L., Bucala, R., Djudjaj, S., & Boor, P. (2019). Evolving complexity of MIF signaling. *Cellular signalling*, *57*, 76–88. https://doi.org/10.1016/j.cellsig.2019.01.006

Janssens, R., Struyf, S. and Proost, P. (2018), 'The unique structural and functional features of CXCL12', *Cellular and Molecular Immunology* **15**(4), 299–311.

Jørgensen, A.S., Daugvilaite, V., De Filippo, K. *et al.* Biased action of the CXCR4-targeting drug plerixafor is essential for its superior hematopoietic stem cell mobilization. *Commun Biol* **4**, 569 (2021). https://doi.org/10.1038/s42003-021-02070-9

Kalinina OV, Pfeifer N, Lengauer T. Modelling binding between CCR5 and CXCR4 receptors and their ligands suggests the surface electrostatic potential of the co-receptor to be a key player in the HIV-1 tropism. Retrovirology. 2013 Nov 11;10:130. doi: 10.1186/1742-4690-10-130. PMID: 24215935; PMCID: PMC3833284.

Kallifatidis, G., Munoz, D., Singh, R. K., Salazar, N., James, J. and Lokeshwar, B. L. (2016), 'β-Arrestin-2 Counters CXCR7-mediated EGFR Transactivation and Proliferation', Molecular Cancer Research 14(5), 493–503.

Katada, T., & Ui, M. (1979). Effect of in vivo pretreatment of rats with a new protein purified from Bordetella pertussis on in vitro secretion of insulin: role of calcium. *Endocrinology*, *104*(6), 1822–1827. https://doi.org/10.1210/endo-104-6-1822

Katritch V, Cherezov V, Stevens RC. Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol*. 2013;53:531-556. doi:10.1146/annurev-pharmtox-032112-135923

Kaul M, Ma Q, Medders KE, Desai MK, Lipton SA. HIV-1 coreceptors CCR5 and CXCR4 both mediate neuronal cell death but CCR5 paradoxically can also contribute to protection. Cell Death Differ. 2007;14:296–305.

Kaya AI, Perry NA, Gurevich VV, Iverson TM. Phosphorylation barcode-dependent signal bias of the dopamine D1 receptor. Proc Natl Acad Sci U S A. 2020 Jun 23;117(25):14139-14149. doi: 10.1073/pnas.1918736117. Epub 2020 Jun 5. PMID: 32503917; PMCID: PMC7321966.

Kelly E, Bailey CP, Henderson G. Agonist-selective mechanisms of GPCR desensitization. Br J Pharmacol. 2008 Mar;153 Suppl 1(Suppl 1):S379-88. doi: 10.1038/sj.bjp.0707604. Epub 2007 Dec 3. PMID: 18059321; PMCID: PMC2268061.

Kenakin T. Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. Trends Pharmacol Sci. 1995 Jul;16(7):232-8. doi: 10.1016/s0165-6147(00)89032-x. PMID: 7667897.

Kennedy, J. E. and Marchese, A. (2015), Regulation of GPCR trafficking by ubiquitin, *in* 'Progress in Molecular Biology and Translational Science', 1 edn, Vol. 132, Elsevier Inc., pp. 15–38. **URL:** *http://dx.doi.org/10.1016/bs.pmbts.2015.02.005*

Khan, S. M., Sleno, R., Gora, S., Zylbergold, P., Laverdure, J.-P., Labbe, J.-C., Miller, G. J. and Hebert, T. E. (2013), 'The Expanding Roles of Gbg Subunits in G Protein-Coupled Receptor Signaling and Drug Action', *Pharmacological Reviews* **65**(2), 545–577. **URL:** *http://pharmrev.aspetjournals.org/cgi/doi/10.1124/pr.111.005603*

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc Natl Acad Sci U S A. 2005 Feb 1;102(5):1442-7. doi: 10.1073/pnas.0409532102. Epub 2005 Jan 25. PMID: 15671181; PMCID: PMC547874.

Kim OJ, Gardner BR, Williams DB, Marinec PS, Cabrera DM, Peters JD, Mak CC, Kim KM, and Sibley DR (2004) The role of phosphorylation in D1 dopamine receptor desensitization: evidence for a novel mechanism of arrestin association. J Biol Chem 279:7999–8010.

Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics. 2006 Apr;5(4):749-57. doi: 10.1074/mcp.T500024-MCP200. Epub 2005 Dec 11. PMID: 16340016.

Kirkwood KJ, Ahmad Y, Larance M, Lamond AI. Characterization of native protein complexes and protein isoform variation using size-fractionation-based quantitative proteomics. Mol Cell Proteomics. 2013 Dec;12(12):3851-73. doi: 10.1074/mcp.M113.032367. Epub 2013 Sep 16. PMID: 24043423; PMCID: PMC3861729.

Kitamura K., Kangawa K., Kawamoto M., Ichiki Y., Nakamura S., Matsuo H., Eto T. Adrenomedullin: A novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. 1993;192:553–560. doi: 10.1006/bbrc.1993.1451.

Klaasse EC, Ijzerman AP, de Grip WJ, Beukers MW. Internalization and desensitization of adenosine receptors. Purinergic Signal. 2008 Mar;4(1):21-37. doi: 10.1007/s11302-007-9086-7. Epub 2007 Nov 13. PMID: 18368531; PMCID: PMC2245999.

Klein, K. R., Karpinich, N. O., Espenschied, S. T., Willcockson, H. H., Dunworth, W. P., Hoopes, S. L., Kushner, E. J., Bautch, V. L. and Caron, K. M. (2014), 'Decoy Receptor CXCR7 Modulates Adrenomedullin-Mediated Cardiac and Lymphatic Vascular Development', *Developmental Cell* **30**(5), 528–540. **URL:** *http://dx.doi.org/10.1016/j.devcel.2014.07.012*

Kleist AB, Getschman AE, Ziarek JJ, Nevins AM, Gauthier PA, Chevigné A, Szpakowska M, Volkman BF. New paradigms in chemokine receptor signal transduction: Moving

beyond the two-site model. Biochem Pharmacol. 2016 Aug 15;114:53-68. doi: 10.1016/j.bcp.2016.04.007. Epub 2016 Apr 19. PMID: 27106080; PMCID: PMC5145291.

Kmiecik S, Jamroz M, Kolinski M. Structure prediction of the second extracellular loop in G-protein-coupled receptors. *Biophys J.* 2014;106(11):2408-2416. doi: 10.1016/j.bpj.2014.04.022

Kobilka BK. G protein coupled receptor structure and activation. Biochim Biophys Acta. 2007 Apr;1768(4):794-807. doi: 10.1016/j.bbamem.2006.10.021. Epub 2006 Nov 15. PMID: 17188232; PMCID: PMC1876727.

Koch, C., & Engele, J. (2020). Functions of the CXCL12 Receptor ACKR3/CXCR7-What Has Been Perceived and What Has Been Overlooked. *Molecular pharmacology*, *98*(5), 577–585. https://doi.org/10.1124/molpharm.120.000056

Koenig, J. A., & Edwardson, J. M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends in pharmacological sciences*, *18*(8), 276–287. https://doi.org/10.1016/s0165-6147(97)01091-2

Kohout, T. A., & Lefkowitz, R. J. (2003). Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Molecular pharmacology*, *63*(1), 9–18. https://doi.org/10.1124/mol.63.1.9

Komolov, K. E., & Benovic, J. L. (2018). G protein-coupled receptor kinases: Past, present and future. Cellular signalling, 41, 17–24. https://doi.org/10.1016/j.cellsig.2017.07.004

Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G. and Sternweis, P. C. (1998), 'p115 RhoGEF, a GTPase Activating Protein for Ga12 and Ga13', **283**(June), 13–15.

Kroeger KM, Hanyaloglu AC, Seeber RM, Miles LE, Eidne KA (2001) Constitutive and agonist-dependent homo-oligomerization of thethyrotropin-releasing hormone receptor. Detection in living cells usingbioluminescence resonance energy transfer. J Biol Chem276:12736–12743

Krasnoperov, V., Bittner, M. A., Holz, R. W., Chepurny, O., & Petrenko, A. G. (1999). Structural requirements for alpha-latrotoxin binding and alpha-latrotoxin-stimulated secretion. A study with calcium-independent receptor of alpha-latrotoxin (CIRL) deletion mutants. The Journal of biological chemistry, 274(6), 3590–3596. https://doi.org/10.1074/jbc.274.6.3590

Krueger, K. M., Daaka, Y., Pitcher, J. A., & Lefkowitz, R. J. (1997). The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *The Journal of biological chemistry*, *272*(1), 5–8. https://doi.org/10.1074/jbc.272.1.5

Krupnick, J. G. and Benovic, J. L. (1998), 'the Role of Receptor Kinases and Arrestins in G Protein–Coupled Receptor Regulation', *Annual Review of Pharmacology and Toxicology* **38**(1), 289–319. **URL:** *http://www.annualreviews.org/doi/10.1146/annurev.pharmtox.38.1.289*

Kubo A., Minamino N., Isumi Y., Katafuchi T., Kangawa K., Dohi K., Matsuo H. Production of adrenomedullin in macrophage cell line and peritoneal macrophage. *J. Biol. Chem.* 1998;273:16730–16738. doi: 10.1074/jbc.273.27.16730

Kufareva, I., Salanga, C. L. and Handel, T. M. (2015), 'Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies', *Immunol Cell Biol* **93(4)**, 372–383.

Kumar, A., Kremer, K. N., Dominguez, D., Tadi, M. and Hedin, K. E. (2011), 'G 13 and Rho Mediate Endosomal Trafficking of CXCR4 into Rab11+ Vesicles upon Stromal Cell-Derived Factor-1 Stimulation', *The Journal of Immunology* **186**(2), 951–958. **URL:** *http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002019*

Kumari, P., Srivastava, A., Ghosh, E., Ranjan, R., Dogra, S., Yadav, P. N. and Shukla, A. K. (2017), 'Core engagement with *β*-arrestin is dispensable for agonist-induced vasopressin receptor endocytosis and ERK activation', *Molecular Biology of the Cell* **28**(8), 1003–1010. **URL:** *http://www.molbiolcell.org/lookup/doi/10.1091/mbc.E16-12-0818*

Kwag, R., Lee, J., Kim, D., Lee, H., Yeom, M., Woo, J., Cho, Y., Kim, H. J., Kim, J., Keum, G., Jeon, B., & Choo, H. (2021). Discovery of G Protein-Biased Antagonists against 5-HT_7R. *Journal of medicinal chemistry*, *64*(18), 13766–13779. https://doi.org/10.1021/acs.jmedchem.1c01093

Lacy P. Editorial: secretion of cytokines and chemokines by innate immune cells. Front Immunol. 2015 Apr 22;6:190. doi: 10.3389/fimmu.2015.00190. PMID: 25954279; PMCID: PMC4406090.

Lacy, M., Kontos, C., Brandhofer, M., Hille, K., Gröning, S., Sinitski, D., Bourilhon, P., Rosenberg, E., Krammer, C., Thavayogarajah, T., Pantouris, G., Bakou, M., Weber, C., Lolis, E., Bernhagen, J., & Kapurniotu, A. (2018). Identification of an Arg-Leu-Arg

tripeptide that contributes to the binding interface between the cytokine MIF and the chemokine receptor CXCR4. *Scientific reports*, *8*(1), 5171. https://doi.org/10.1038/s41598-018-23554-5

Laganà M, Schlecht-Louf G, Bachelerie F. The G Protein-Coupled Receptor Kinases (GRKs) in Chemokine Receptor-Mediated Immune Cell Migration: From Molecular Cues to Physiopathology. Cells. 2021 Jan 5;10(1):75. doi: 10.3390/cells10010075. PMID: 33466410; PMCID: PMC7824814.

Lagane, B., Chow, K. Y., Balabanian, K., Levoye, A., Harriague, J., Planchenault, T., Baleux, F., Gunera-Saad, N., Arenzana-Seisdedos, F., & Bachelerie, F. (2008). CXCR4 dimerization and beta-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. Blood, 112(1), 34–44. https://doi.org/10.1182/blood-2007-07-102103

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

Langenhan, T., Aust, G. and Hamann, J. (2013), 'Sticky Signaling–Adhesion Class G ProteinCoupled Receptors Take the Stage', Science Signaling 6(276), re3–re3. URL: http://stke.sciencemag.org/cgi/doi/10.1126/scisignal.2003825

Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS: The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* 1999, 96:3712-3717.

LaRocca TJ, Altman P, Jarrah AA, Gordon R, Wang E, Hadri L, Burke MW, Haddad GE, Hajjar RJ, Tarzami ST. CXCR4 Cardiac Specific Knockout Mice Develop a Progressive Cardiomyopathy. Int J Mol Sci. 2019 May 8;20(9):2267. doi: 10.3390/ijms20092267. PMID: 31071921; PMCID: PMC6539363.

Larráyoz IM, Martínez-Herrero S, García-Sanmartín J, Ochoa-Callejero L, Martínez A. Adrenomedullin and tumour microenvironment. J Transl Med. 2014 Dec 5;12:339. doi: 10.1186/s12967-014-0339-2. PMID: 25475159; PMCID: PMC4272513.

Latif R, Graves P, Davies TF (2002) Ligand-dependent inhibition ofoligomerization at the human thyrotropin receptor. J Biol Chem277:45059–45067

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem 273: 18677–18680.

Levoye, A., Balabanian, K., Baleux, F., Bachelerie, F., & Lagane, B. (2009). CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood*, *113*(24), 6085–6093. https://doi.org/10.1182/blood-2008-12-196618

Liebscher, I., Schöneberg, T. and Prömel, S. (2013), 'Progress in demystification of adhesion G protein-coupled receptors', Biological Chemistry 394(8), 937–950.

Lin, L. C., Quon, T., Engberg, S., Mackenzie, A. E., Tobin, A. B., & Milligan, G. (2021). G Protein-Coupled Receptor GPR35 Suppresses Lipid Accumulation in Hepatocytes. *ACS pharmacology & translational science*, *4*(6), 1835–1848. https://doi.org/10.1021/acsptsci.1c00224

Lipfert J, Ödemis V, and Engele J (2013) Grk2 is an essential regulator of CXCR7 signalling in astrocytes. Cell Mol Neurobiol 33:111–118.

Liu, C., Pham, K., Luo, D., Reynolds, B. A., Hothi, P., Foltz, G. and Harrison, J. K. (2013), 'Expression and Functional Heterogeneity of Chemokine Receptors CXCR4 and CXCR7 in Primary Patient-Derived Glioblastoma Cells', PLoS ONE 8(3), 1–12.

Liu, F., Lang, R., Wei, J., Fan, Y., Cui, L., Gu, F., Guo, X., Pringle, G. A., Zhang, X. and Fu, L. (2009), 'Increased expression of SDF-1/CXCR4 is associated with lymph node metastasis of invasive micropapillary carcinoma of the breast', *Histopathology* **54**(6), 741–750.

Löhse, M. J., Benovic, J. L., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1990), 'beta-Arrestin : A Protein That Regulates beta-Adrenergic Receptor Function', *Science* **248**(4962), 1547–1550.

Lokeshwar BL, Kallifatidis G, Hoy JJ. Atypical chemokine receptors in tumor cell growth and metastasis. Adv Cancer Res. 2020;145:1-27. doi: 10.1016/bs.acr.2019.12.002. Epub 2020 Jan 27. PMID: 32089162.

Lowe JD, Sanderson HS, Cooke AE, Ostovar M, Tsisanova E, Withey SL, Chavkin C, Husbands SM, Kelly E, Henderson G, Bailey CP. Role of G Protein-Coupled Receptor Kinases 2 and 3 in μ-Opioid Receptor Desensitization and Internalization. Mol Pharmacol. 2015 Aug;88(2):347-56. doi: 10.1124/mol.115.098293. Epub 2015 May 26. PMID: 26013542; PMCID: PMC4518089.

Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. Clin Dev Immunol. 2012;2012:925135. doi: 10.1155/2012/925135. Epub 2012 Mar 14. PMID: 22474485; PMCID: PMC3312336.

Luker KE, Gupta M, Luker GD. Imaging chemokine receptor dimerization with firefly luciferase complementation. *FASEB J*. 2009;23(3):823-834. doi:10.1096/fj.08-116749

Luttrell, L. M. and Lefkowitz, R. J. (2002), 'The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals.', *Journal of cell science* **115**(Pt 3), 455–65. **URL:** *http://www.ncbi.nlm.nih.gov/pubmed/11861753*

Ma Q., Jones D., Borghesani P.R., Segal R.A., Nagasawa T., Kishimoto T., Bronson R.T., Springer T.A. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc. Natl. Acad. Sci. USA. 1998;95:9448–9453. doi: 10.1073/pnas.95.16.9448

Magalhaes AC, Dunn H, Ferguson SS. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. Br J Pharmacol. 2012 Mar;165(6):1717-1736. doi: 10.1111/j.1476-5381.2011.01552.x. PMID: 21699508; PMCID: PMC3372825.

Mahmud, Z. A., Jenkins, L., Ulven, T., Labéguère, F., Gosmini, R., De Vos, S., Hudson, B. D., Tikhonova, I. G., & Milligan, G. (2017). Three classes of ligands each bind to distinct sites on the orphan G protein-coupled receptor GPR84. *Scientific reports*, *7*(1), 17953. https://doi.org/10.1038/s41598-017-18159-3

Malik R, Marchese A. Arrestin-2 interacts with the endosomal sorting complex required for transport machinery to modulate endosomal sorting of CXCR4. Mol Biol Cell. 2010 Jul 15;21(14):2529-41. doi: 10.1091/mbc.e10-02-0169. Epub 2010 May 26. PMID: 20505072; PMCID: PMC2903679.

Marchese, A., & Benovic, J. L. (2001). Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. The Journal of biological chemistry, 276(49), 45509–45512. https://doi.org/10.1074/jbc.C100527200

Marsango, S., Varela, M. J., & Milligan, G. (2015). Approaches to Characterize and Quantify Oligomerization of GPCRs. *Methods in molecular biology (Clifton, N.J.)*, *1335*, 95–105. https://doi.org/10.1007/978-1-4939-2914-6_7

Marsango, S., Ward, R. J., Jenkins, L., Butcher, A. J., Al Mahmud, Z., Dwomoh, L., Nagel, F., Schulz, S., Tikhonova, I. G., Tobin, A. B., & Milligan, G. (2022). Selective phosphorylation of threonine residues defines GPR84-arrestin interactions of biased ligands. *The Journal of biological chemistry*, *298*(5), 101932. https://doi.org/10.1016/j.jbc.2022.101932

Martínez-Herrero S., Martínez A. Adrenomedullin Regulates Intestinal Physiology and Pathophysiology. *Domest. Anim. Endocrinol.* 2016;56:S66–S83. doi: 10.1016/j.domaniend.2016.02.004.

Martínez-Muñoz, L., Villares, R., Rodríguez-Fernández, J. L., Rodríguez-Frade, J. M., & Mellado, M. (2018). Remodeling our concept of chemokine receptor function: From monomers to oligomers. Journal of leukocyte biology, 104(2), 323–331. https://doi.org/10.1002/JLB.2MR1217-503R

Marutsuka K., Hatakeyama K., Sato Y., Yamashita A., Sumiyoshi A., Asada Y. Immunohistological Localization and Possible Functions of Adrenomedullin. *Hypertens. Res.* 2003;26:S33–S40. doi: 10.1291/hypres.26.S33.

McHugh J. (2019). Hitting the right spot on CXCR4. *Nature reviews. Rheumatology*, *15*(9), 514. https://doi.org/10.1038/s41584-019-0287-6

Meyrath M, Palmer CB, Reynders N, et al. Proadrenomedullin N-Terminal 20 Peptides (PAMPs) Are Agonists of the Chemokine Scavenger Receptor ACKR3/CXCR7. ACS Pharmacol Transl Sci. 2021;4(2):813-823. Published 2021 Mar 22. doi:10.1021/acsptsci.1c00006

Meyrath, M., Szpakowska, M., Zeiner, J., Massotte, L., Merz, M. P., Benkel, T., Simon, K., Ohnmacht, J., Turner, J. D., Krüger, R., Seutin, V., Ollert, M., Kostenis, E., & Chevigné, A. (2020). The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides. *Nature communications*, *11*(1), 3033. https://doi.org/10.1038/s41467-020-16664-0

Milligan G. (2007). G protein-coupled receptor dimerisation: molecular basis and relevance to function. *Biochimica et biophysica acta*, *1768*(4), 825–835. https://doi.org/10.1016/j.bbamem.2006.09.021

Milligan, G. and Kostenis, E. (2006), 'Heterotrimeric G-proteins: A short history', *British Journal of Pharmacology* **147**(SUPPL. 1), S46–S55. **URL:** *http://doi.wiley.com/10.1038/sj.bjp.0706405*

Mohr K, Trankle C, Kostenis E, Barocelli E, De Amici M, Holzgrabe U. Rational design of dualsteric GPCR ligands: quests and promise. British Journal of Pharmacology 2010; 159: 9971008

Monk, K. R., Hamann, J., Langenhan, T., Nijmeijer, S., Schoneberg, T. and Liebscher, I. (2015), 'Adhesion G Protein-Coupled Receptors: From In Vitro Pharmacology to In Vivo

Mechanisms', Molecular Pharmacology 88(3), 617–623. URL: http://molpharm.aspetjournals.org/cgi/doi/10.1124/mol.115.098749

Moon, T. C., Befus, A. D., & Kulka, M. (2014). Mast cell mediators: their differential release and the secretory pathways involved. *Frontiers in immunology*, *5*, 569. https://doi.org/10.3389/fimmu.2014.00569

Mueller W, Schütz D, Nagel F, Schulz S, and Stumm R (2013) Hierarchical organization of multi-site phosphorylation at the CXCR4 C terminus. PLoS One 8:e64975

Murphy, P. M., & Heusinkveld, L. (2018). Multisystem multitasking by CXCL12 and its receptors CXCR4 and ACKR3. Cytokine, 109, 2–10. https://doi.org/10.1016/j.cyto.2017.12.022

Mysinger MM, Weiss DR, Ziarek JJ, Gravel S, Doak AK, Karpiak J, Heveker N, Shoichet BK, Volkman BF. Structure-based ligand discovery for the protein-protein interface of chemokine receptor CXCR4. Proc Natl Acad Sci U S A. 2012 Apr 3;109(14):5517-22. doi: 10.1073/pnas.1120431109. Epub 2012 Mar 19. PMID: 22431600; PMCID: PMC3325704.

Naderi N, Yang DT. Lymphoplasmacytic lymphoma and Waldenström macroglobulinemia. Arch Pathol Lab Med. 2013 Apr;137(4):580-5. doi: 10.5858/arpa.2012-0034-RS. PMID: 23544948.

Namkung, Y., Le Gouill, C., Lukashova, V., Kobayashi, H., Hogue, M., Khoury, E., Song, M., Bouvier, M., & Laporte, S. A. (2016). 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

Naumann, U., Cameroni, E., Pruenster, M., Mahabaleshwar, H., Raz, E., Zerwes, H. G., Rot, A. and Thelen, M. (2010), 'CXCR7 functions as a scavenger for CXCL12 and CXCL11', *PLoS ONE* **5**(2).

Neubig, R. R., Spedding, M., Kenakin, T. and Christopoulos, A. (2003), 'International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on Terms and Symbols in Quantitative Pharmacology', *Pharmacological Reviews* **55**(4), 597–606.

Neves, M., Marolda, V., Mayor, F., Jr, & Penela, P. (2022). Crosstalk between CXCR4/ACKR3 and EGFR Signaling in Breast Cancer Cells. *International journal of molecular sciences*, *23*(19), 11887. https://doi.org/10.3390/ijms231911887

Nibbs, R. J., & Graham, G. J. (2013). Immune regulation by atypical chemokine receptors. *Nature reviews. Immunology*, *13*(11), 815–829. https://doi.org/10.1038/nri3544

Nicholas, J. (2010), 'Human herpesvirus 8-encoded cytokines', *Future Virology* **5(2)**(March), 197–206.

Nichols, B. J., & Lippincott-Schwartz, J. (2001). Endocytosis without clathrin coats. *Trends in cell biology*, *11*(10), 406–412. https://doi.org/10.1016/s0962-8924(01)02107-9

Nicoli, A., Dunkel, A., Giorgino, T., de Graaf, C., & Di Pizio, A. (2022). Classification Model for the Second Extracellular Loop of Class A GPCRs. Journal of chemical information and modeling, 62(3), 511–522. https://doi.org/10.1021/acs.jcim.1c01056

Nishikimi, T., Saito, Y., Kitamura, K., Ishimitsu, T., Eto, T., Kangawa, K., Matsuo, H., Omae, T., & Matsuoka, H. (1995). Increased plasma levels of adrenomedullin in patients with heart failure. Journal of the American College of Cardiology, 26(6), 1424–1431. https://doi.org/10.1016/0735-1097(95)00338-X

Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ. Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. Sci Signal. 2011 Aug 9;4(185):ra51. doi: 10.1126/scisignal.2001707. PMID: 21868357; PMCID: PMC3415961.

Nussinov R, Tsai CJ. The different ways through which specificity works in orthosteric and allosteric drugs. Curr Pharm Des. 2012;18(9):1311-6. doi: 10.2174/138161212799436377. PMID: 22316155; PMCID: PMC7458136.

Nygaard, R., Frimurer, T. M., Holst, B., Rosenkilde, M. M. and Schwartz, T. W. (2009), 'Ligand binding and micro-switches in 7TM receptor structures', Trends in Pharmacological Sciences 30(5), 249–259

Odemis, V., Lipfert, J., Kraft, R., Hajek, P., Abraham, G., Hattermann, K., Mentlein, R., & Engele, J. (2012). The presumed atypical chemokine receptor CXCR7 signals through G(i/o) proteins in primary rodent astrocytes and human glioma cells. *Glia*, *60*(3), 372–381. https://doi.org/10.1002/glia.22271

Orsini, M. J., Parent, J. L., Mundell, S. J., Marchese, A., & Benovic, J. L. (1999). Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. *The Journal of biological chemistry*, *274*(43), 31076–31086. https://doi.org/10.1074/jbc.274.43.31076

Ostrowski, M. A., Justement, S. J., Catanzaro, A., Hallahan, C. A., Ehler, L. A., Mizell, S. B., Kumar, P. N., Mican, J. A., Chun, T. W., & Fauci, A. S. (1998). Expression of chemokine receptors CXCR4 and CCR5 in HIV-1-infected and uninfected individuals. *Journal of immunology (Baltimore, Md. : 1950)*, *161*(6), 3195–3201.

Pal, A., & Donato, N. J. (2014). Ubiquitin-specific proteases as therapeutic targets for the treatment of breast cancer. *Breast cancer research : BCR*, *16*(5), 461. https://doi.org/10.1186/s13058-014-0461-3

Paradis, J. S., Feng, X., Murat, B., Jefferson, R. E., Sokrat, B., Szpakowska, M., Hogue, M., Bergkamp, N. D., Heydenreich, F. M., Smit, M. J., Chevigné, A., Bouvier, M., & Barth, P. (2022). Computationally designed GPCR quaternary structures bias signaling pathway activation. *Nature communications*, *13*(1), 6826. https://doi.org/10.1038/s41467-022-34382-7

Patwardhan A, Cheng N, Trejo J. Post-Translational Modifications of G Protein-Coupled Receptors Control Cellular Signaling Dynamics in Space and Time. Pharmacol Rev. 2021 Jan;73(1):120-151. doi: 10.1124/pharmrev.120.000082. PMID: 33268549; PMCID: PMC7736832.

Patten DA, Shetty S. More Than Just a Removal Service: Scavenger Receptors in Leukocyte Trafficking. Front Immunol. 2018 Dec 12;9:2904. doi: 10.3389/fimmu.2018.02904. PMID: 30631321; PMCID: PMC6315190.

Percherancier, Y., Berchiche, Y. A., Slight, I., Volkmer-Engert, R., Tamamura, H., Fujii, N., Bouvier, M., & Heveker, N. (2005). Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *The Journal of biological chemistry*, *280*(11), 9895–9903. https://doi.org/10.1074/jbc.M411151200

Perpiñá-Viciano, C., Işbilir, A., Zarca, A., Caspar, B., Kilpatrick, L. E., Hill, S. J., Smit, M. J., Lohse, M. J., & Hoffmann, C. (2020). Kinetic Analysis of the Early Signaling Steps of the Human Chemokine Receptor CXCR4. Molecular pharmacology, 98(2), 72–87. https://doi.org/10.1124/mol.119.118448

Phillips, R. J., Mestas, J., Gharaee-Kermani, M., Burdick, M. D., Sica, A., Belperio, J. A., Keane, M. P. and Strieter, R. M. (2005), 'Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling

pathway and activation of hypoxia ind', *Journal of Biological Chemistry* **280**(23), 22473–22481.

Pitcher, J. A., Freedman, N. J. and Lefkowitz, R. J. (1998), 'G Protein – Coupled Receptor Kinases', *Annual Review of Biochemistry* **67**, 653–692.

Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., et al. (1992). Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. Science 257, 1264–1267. doi: 10.1126/science.1325672

Pitcher, J. A., Touhara, K., Payne, E. S., and Lefkowitz, R. J. (1995). Pleckstrin homology domain-mediated membrane association and activation of the betaadrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. J. Biol. Chem. 270, 11707–11710. doi: 10.1074/jbc.270.20. 11707

Poltavets V, Faulkner JW, Dhatrak D, Whitfield RJ, McColl SR, Kochetkova M. CXCR4-CCR7 Heterodimerization Is a Driver of Breast Cancer Progression. Life (Basel). 2021 Oct 7;11(10):1049. doi: 10.3390/life11101049. PMID: 34685420; PMCID: PMC8538406.

Qiao N, Wang L, Wang T, and Li H (2016) Inflammatory CXCL12-CXCR4/CXCR7 axis mediates G-protein signaling pathway to influence the invasion and migration of nasopharyngeal carcinoma cells. Tumour Biol 37:8169–8179 DOI: 10.1007/s13277-015-4686-2.

Qing, M., Jones, D. and Springer, T. A. (1999), 'The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment', *Immunity* **10**(4), 463–471.

Quinn, K. E., Mackie, D. I., & Caron, K. M. (2018). Emerging roles of atypical chemokine receptor 3 (ACKR3) in normal development and physiology. *Cytokine*, *109*, 17–23. https://doi.org/10.1016/j.cyto.2018.02.024

Rajagopal, S., Kim, J., Ahn, S., Craig, S., Lam, C. M., Gerard, N. P., Gerard, C., & Lefkowitz, R. J. (2010). Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(2), 628–632. https://doi.org/10.1073/pnas.0912852107

Rajagopal, S. and Shenoy, S. K. (2018), 'GPCR desensitization: Acute and prolonged phases', *Cellular Signalling* **41**, 9–16. **URL:** *https://doi.org/10.1016/j.cellsig.2017.01.024*

Rajasekaran, D., Gröning, S., Schmitz, C., Zierow, S., Drucker, N., Bakou, M., Kohl, K., Mertens, A., Lue, H., Weber, C., Xiao, A., Luker, G., Kapurniotu, A., Lolis, E., & Bernhagen, J. (2016). Macrophage Migration Inhibitory Factor-CXCR4 Receptor Interactions: EVIDENCE FOR PARTIAL ALLOSTERIC AGONISM IN COMPARISON WITH CXCL12 CHEMOKINE. *The Journal of biological chemistry*, *291*(30), 15881–15895. https://doi.org/10.1074/jbc.M116.717751

Rajarathnam, K., Clark-Lewis, I., & Sykes, B. D. (1995). 1H NMR solution structure of an active monomeric interleukin-8. Biochemistry, 34(40), 12983–12990. https://doi.org/10.1021/bi00040a008

Ray P, Lewin SA, Mihalko LA, Lesher-Perez SC, Takayama S, Luker KE, Luker GD. Secreted CXCL12 (SDF-1) forms dimers under physiological conditions. Biochem J. 2012 Mar 1;442(2):433-42. doi: 10.1042/BJ20111341. PMID: 22142194; PMCID: PMC4419379.

Ray, P., Mihalko, L. A., Coggins, N. L., Moudgil, P., Ehrlich, A., Luker, K. E. and Luker, G. D. (2012), 'Carboxy-terminus of CXCR7 Regulates Receptor Localization and Function', *International Journal of Biochemistry and Cell Biology* **44**(4), 669–678.

Reddy, P. S., & Corley, R. B. (1998). Assembly, sorting, and exit of oligomeric proteins from the endoplasmic reticulum. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *20*(7), 546–554. *https://doi.org/10.1002/(SICI)1521-1878(199807)20:7<546::AIDBIES5>3.0.CO;2-I*

Reiter E, Ahn S, Shukla AK, and Lefkowitz RJ (2012) Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. Annu Rev Pharmacol Toxicol 52:179–197

Reiter, E., & Lefkowitz, R. J. (2006). GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology and metabolism: TEM*, *17*(4), 159–165. https://doi.org/10.1016/j.tem.2006.03.008

Romagnani, P., Lasagni, L., Annunziato, F., Serio, M., & Romagnani, S. (2004). CXC chemokines: the regulatory link between inflammation and angiogenesis. Trends in immunology, 25(4), 201–209. https://doi.org/10.1016/j.it.2004.02.006

Romain B, Hachet-Haas M, Rohr S, Brigand C, Galzi JL, Gaub MP, Pencreach E, Guenot D. Hypoxia differentially regulated CXCR4 and CXCR7 signaling in colon cancer. Mol Cancer. 2014 Mar 14;13:58. doi: 10.1186/1476-4598-13-58. PMID: 24629239; PMCID: PMC3975457.

Rosenbaum DM, Rasmussen SG, Kobilka BK. The structure and function of G-protein-coupled receptors. *Nature*. 2009;459(7245):356-363. doi:10.1038/nature08144

Rosenkilde, M. M., Gerlach, L. O., Hatse, S., Skerlj, R. T., Schols, D., Bridger, G. J., & Schwartz, T. W. (2007). Molecular mechanism of action of monocyclam versus bicyclam non-peptide antagonists in the CXCR4 chemokine receptor. *The Journal of biological chemistry*, *282*(37), 27354–27365. https://doi.org/10.1074/jbc.M704739200

Russell, F. A., King, R., Smillie, S. J., Kodji, X., & Brain, S. D. (2014). Calcitonin gene-related peptide: physiology and pathophysiology. *Physiological reviews*, *94*(4), 1099–1142. https://doi.org/10.1152/physrev.00034.2013

Sadee W, Oberdick J, Wang Z. Biased Opioid Antagonists as Modulators of Opioid Dependence: Opportunities to Improve Pain Therapy and Opioid Use Management. Molecules. 2020 Sep 11;25(18):4163. doi: 10.3390/molecules25184163. PMID: 32932935; PMCID: PMC7571197.

Saini, V., Marchese, A., & Majetschak, M. (2010). CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin. *The Journal of biological chemistry*, *285*(20), 15566–15576. https://doi.org/10.1074/jbc.M110.103408

Saini, V., Staren, D. M., Ziarek, J. J., Nashaat, Z. N., Campbell, E. M., Volkman, B. F., Marchese, A., & Majetschak, M. (2011). The CXC chemokine receptor 4 ligands ubiquitin and stromal cell-derived factor-1α function through distinct receptor interactions. The Journal of biological chemistry, 286(38), 33466–33477. https://doi.org/10.1074/jbc.M111.233742

Salanga, C. L., O'Hayre, M., & Handel, T. (2009). Modulation of chemokine receptor activity through dimerization and crosstalk. *Cellular and molecular life sciences : CMLS*, *66*(8), 1370–1386. https://doi.org/10.1007/s00018-008-8666-1

Salazar N, Muñoz D, Kallifatidis G, Singh RK, Jordà M, and Lokeshwar BL (2014) The chemokine receptor CXCR7 interacts with EGFR to promote breast cancer cell proliferation. Mol Cancer 13:198 DOI: 10.1186/1476-4598-13-198.

Sánchez-Alcañiz, J. A., Haege, S., Mueller, W., Pla, R., Mackay, F., Schulz, S., López-Bendito, G., Stumm, R., & Marín, O. (2011). Cxcr7 controls neuronal migration by regulating chemokine responsiveness. *Neuron*, *69*(1), 77–90. https://doi.org/10.1016/j.neuron.2010.12.006

Sarma, P., Banerjee, R., & Shukla, A. K. (2023). Structural snapshot of a β-arrestin-biased receptor. Trends in pharmacological sciences, 44(1), 1–3. https://doi.org/10.1016/j.tips.2022.08.005

Scarpa M, Molloy C, Jenkins L, Strellis B, Budgett RF, Hesse S, Dwomoh L, Marsango S, Tejeda GS, Rossi M, Ahmed Z, Milligan G, Hudson BD, Tobin AB, Bradley SJ. Biased M1 muscarinic receptor mutant mice show accelerated progression of prion neurodegenerative disease. Proc Natl Acad Sci U S A. 2021 Dec 14;118(50):e2107389118. doi: 10.1073/pnas.2107389118. PMID: 34893539; PMCID: PMC8685681.

Schafer, C. T., Pauszek Iii, R. F., Gustavsson, M., Handel, T. M., & Millar, D. P. (2023). Distinct Activation Mechanisms of CXCR4 and ACKR3 Revealed by Single-Molecule Analysis of their Conformational Landscapes. bioRxiv: the preprint server for biology, 2023.10.31.564925. https://doi.org/10.1101/2023.10.31.564925

Schiraldi, M., Raucci, A., Muñoz, L. M., Livoti, E., Celona, B., Venereau, E., Apuzzo, T., De Marchis, F., Pedotti, M., Bachi, A., Thelen, M., Varani, L., Mellado, M., Proudfoot, A., Bianchi, M. E., & Uguccioni, M. (2012). HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. The Journal of experimental medicine, 209(3), 551–563. https://doi.org/10.1084/jem.20111739

Scholten, D. J., Canals, M., Maussang, D., Roumen, L., Smit, M. J., Wijtmans, M., de Graaf, C., Vischer, H. F., & Leurs, R. (2012). Pharmacological modulation of chemokine receptor function. *British journal of pharmacology*, *165*(6), 1617–1643. https://doi.org/10.1111/j.1476-5381.2011.01551.x

Scofield, S. L. C., Daniels, C. R., Dalal, S., Millard, J. A., Singh, M., & Singh, K. (2018). Extracellular ubiquitin modulates cardiac fibroblast phenotype and function via its interaction with CXCR4. *Life sciences*, *211*, 8–16. https://doi.org/10.1016/j.lfs.2018.09.012

Sergeev, E., Hansen, A. H., Bolognini, D., Kawakami, K., Kishi, T., Aoki, J., Ulven, T., Inoue, A., Hudson, B. D., & Milligan, G. (2017). A single extracellular amino acid in Free Fatty Acid Receptor 2 defines antagonist species selectivity and G protein selection bias. Scientific reports, 7(1), 13741. https://doi.org/10.1038/s41598-017-14096-3

Shankaran, H., Wiley, H. S., & Resat, H. (2007). Receptor downregulation and desensitization enhance the information processing ability of signalling receptors. *BMC systems biology*, *1*, 48. https://doi.org/10.1186/1752-0509-1-48

Shetty SK, Walzem RL, Davies BSJ. A novel NanoBiT-based assay monitors the interaction between lipoprotein lipase and GPIHBP1 in real time. J Lipid Res. 2020 Apr;61(4):546-559. doi: 10.1194/jlr.D119000388. Epub 2020 Feb 6. PMID: 32029511; PMCID: PMC7112140.

Shimizu, N., Soda, Y., Kanbe, K., Liu, H. Y., Mukai, R., Kitamura, T., & Hoshino, H. (2000). A putative G protein-coupled receptor, RDC1, is a novel coreceptor for human and simian immunodeficiency viruses. *Journal of virology*, *74*(2), 619–626. https://doi.org/10.1128/jvi.74.2.619-626.2000

Sibley DR, Strasser RH, Benovic JL, Daniel K, Lefkowitz RJ. Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. Proc Natl Acad Sci U S A. 1986 Dec;83(24):9408-12. doi: 10.1073/pnas.83.24.9408. PMID: 3025843; PMCID: PMC387147.

Sierro, F., Biben, C., Martínez-Muñoz, L., Mellado, M., Ransohoff, R. M., Li, M., Woehl, B., Leung, H., Groom, J., Batten, M., Harvey, R. P., Martínez-A, C., Mackay, C. R., & Mackay, F. (2007). Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(37), 14759–14764. https://doi.org/10.1073/pnas.0702229104

Sigmund, E. C., Bauer, A., Jakobs, B. D., Tatliadim, H., Tacconi, C., Thelen, M., Legler, D. F., & Halin, C. (2023). Reassessing the adrenomedullin scavenging function of ACKR3 in lymphatic endothelial cells. *PloS one*, *18*(5), e0285597. https://doi.org/10.1371/journal.pone.0285597

Simon MI, Strathmann MP, Gautam N. Diversity of G proteins in signal transduction. Science. 1991; 252:802–8. [PubMed: 1902986]

Skolnik EY, Lee CH, Batzer A et al (1993) The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Sch: implications for insulin control of ras signaling. EMBO J 12:1929–1936

Smith, J. S. and Rajagopal, S. (2016), 'The *β*-Arrestins: Multifunctional regulators of G protein-coupled receptors', *Journal of Biological Chemistry* **291**(17), 8969–8977.

Smith, N., Pietrancosta, N., Davidson, S., Dutrieux, J., Chauveau, L., Cutolo, P., Dy, M., Scott-Algara, D., Manoury, B., Zirafi, O., McCort-Tranchepain, I., Durroux, T., Bachelerie, F., Schwartz, O., Münch, J., Wack, A., Nisole, S., & Herbeuval, J. P. (2017). Natural amines

inhibit activation of human plasmacytoid dendritic cells through CXCR4 engagement. *Nature communications*, *8*, 14253. https://doi.org/10.1038/ncomms14253

Smith, N., Rodero, M. P., Bekaddour, N., Bondet, V., Ruiz-Blanco, Y. B., Harms, M., Mayer, B., Bader-Meunier, B., Quartier, P., Bodemer, C., Baudouin, V., Dieudonné, Y., Kirchhoff, F., Sanchez Garcia, E., Charbit, B., Leboulanger, N., Jahrsdörfer, B., Richard, Y., Korganow, A. S., Münch, J., … Herbeuval, J. P. (2019). Control of TLR7-mediated type I IFN signaling in pDCs through CXCR4 engagement-A new target for lupus treatment. *Science advances*, *5*(7), eaav9019. https://doi.org/10.1126/sciadv.aav9019

Soede, R. D. M., Zeelenberg, I. S., Wijnands, Y. M., Kamp, M. and Roos, E. (2001), 'Stromal Cell-Derived Factor-1-Induced LFA-1 Activation During In Vivo Migration of T Cell Hybridoma Cells Requires Gq/11, RhoA, and Myosin, as well as Gi and Cdc42', *The Journal of Immunology* **166**(7), 4293–4301. **URL:** *http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.166.7.4293*

Sohy, D., Parmentier, M., & Springael, J. Y. (2007). Allosteric transinhibition by specific antagonists in CCR2/CXCR4 heterodimers. The Journal of biological chemistry, 282(41), 30062–30069. https://doi.org/10.1074/jbc.M705302200

Sonay Kuloglu, E., McCaslin, D. R., Markley, J. L. and Volkman, B. F. (2002), 'Structural rearrangement of human lymphotactin, a C chemokine, under physiological solution conditions', *Journal of Biological Chemistry* **277**(20), 17863–17870.

Springael JY, Urizar E, Parmentier M. Dimerization of chemokine receptors and its functional consequences. Cytokine Growth Factor Rev. 2005;16:611–623.

Sriram K, Insel PA., G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs?. Mol Pharmacol. 2018;93(4):251-258. doi:10.1124/mol.117.111062

Stallaert W, Dorn JF, van der Westhuizen E, Audet M, Bouvier M. Impedance responses reveal β_2-adrenergic receptor signaling pluridimensionality and allow classification of ligands with distinct signaling profiles. PLoS One. 2012;7(1):e29420. doi: 10.1371/journal.pone.0029420. Epub 2012 Jan 5. PMID: 22242170; PMCID: PMC3252315.

Sterne-Marr, R., Tesmer, J. J., Day, P. W., Stracquatanio, R. P., Cilente, J. A., O'Connor, K. E., Pronin, A. N., Benovic, J. L., & Wedegaertner, P. B. (2003). G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signaling

homology domain for binding G alpha subunits. *The Journal of biological chemistry*, *278*(8), 6050–6058. https://doi.org/10.1074/jbc.M208787200

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.

Syrovatkina, V., Alegre, K. O., Dey, R. and Huang, X. Y. (2016), 'Regulation, Signaling, and Physiological Functions of G-Proteins', Journal of Molecular Biology 428(19), 3850–3868. URL: http://dx.doi.org/10.1016/j.jmb.2016.08.002

Szénási, T., Turu, G., & Hunyady, L. (2023). Interactions between β-arrestin proteins and the cytoskeletal system, and their relevance to neurodegenerative disorders. *Frontiers in endocrinology*, *14*, 957981. https://doi.org/10.3389/fendo.2023.957981

Szpakowska, M., & Chevigné, A. (2016). vCCL2/vMIP-II, the viral master KEYmokine. Journal of leukocyte biology, 99(6), 893–900. https://doi.org/10.1189/jlb.2MR0815-383R

Szpakowska, M., Dupuis, N., Baragli, A., Counson, M., Hanson, J., Piette, J. and Chevigné, A. (2016), 'Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7', *Biochemical Pharmacology* **114**, 14–21. **URL:** *http://linkinghub.elsevier.com/retrieve/pii/S0006295216301058*

Szpakowska, M., Meyrath, M., Reynders, N., Counson, M., Hanson, J., Steyaert, J., & Chevigné, A. (2018). Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. *Biochemical pharmacology*, *153*, 299–309. https://doi.org/10.1016/j.bcp.2018.03.007

Szpakowska, M., Nevins, A. M., Meyrath, M., Rhainds, D., D'huys, T., Guité-Vinet, F., Dupuis, N., Gauthier, P. A., Counson, M., Kleist, A., St-Onge, G., Hanson, J., Schols, D., Volkman, B. F., Heveker, N., & Chevigné, A. (2018). Different contributions of chemokine N-terminal features attest to a different ligand binding mode and a bias towards activation of ACKR3/CXCR7 compared with CXCR4 and CXCR3. British journal of pharmacology, 175(9), 1419–1438. https://doi.org/10.1111/bph.14132

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, et al. (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393: 591–594.

Tamamis P, Floudas CA. Elucidating a key component of cancer metastasis: CXCL12 (SDF-1α) binding to CXCR4. J Chem Inf Model. 2014 Apr 28;54(4):1174-88. doi: 10.1021/ci500069y. Epub 2014 Apr 8. PMID: 24660779; PMCID: PMC4004218.

Tan, L., Yan, W., McCorvy, J. D., & Cheng, J. (2018). Biased Ligands of G Protein-Coupled Receptors (GPCRs): Structure-Functional Selectivity Relationships (SFSRs) and Therapeutic Potential. Journal of medicinal chemistry, 61(22), 9841–9878. https://doi.org/10.1021/acs.jmedchem.8b00435

Tan, W., Martin, D. and Gutkind, J. S. (2006), 'The Gα13-Rho signaling axis is required for SDF-1-induced migration through CXCR', Journal of Biological Chemistry 281(51), 39542– 39549.

Tanaka M, Kakihara S, Hirabayashi K, Imai A, Toriyama Y, Iesato Y, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Kawate H, Tanaka M, Cui N, Wei Y, Zhao Y, Aruga K, Yamauchi A, Murata T, Shindo T. Adrenomedullin-Receptor Activity-Modifying Protein 2 System Ameliorates Subretinal Fibrosis by Suppressing Epithelial-Mesenchymal Transition in Age-Related Macular Degeneration. Am J Pathol. 2021 Apr;191(4):652-668. doi: 10.1016/j.ajpath.2020.12.012. Epub 2020 Dec 29. PMID: 33385343.

Tan L, Yan W, McCorvy JD, Cheng J. Biased Ligands of G Protein-Coupled Receptors (GPCRs): Structure-Functional Selectivity Relationships (SFSRs) and Therapeutic Potential. J Med Chem. 2018 Nov 21;61(22):9841-9878. doi: 10.1021/acs.jmedchem.8b00435. Epub 2018 Jul 10. PMID: 29939744.

Tecchio, C., Micheletti, A., & Cassatella, M. A. (2014). Neutrophil-derived cytokines: facts beyond expression. *Frontiers in immunology*, *5*, 508. https://doi.org/10.3389/fimmu.2014.00508

Teller DC, Okada T, Behnke CA, Palczewski K, Stenkamp RE. Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). Biochemistry. 2001 Jul 3;40(26):7761-72. doi: 10.1021/bi0155091. PMID: 11425302; PMCID: PMC1698954.

Terrillon, S., & Bouvier, M. (2004). Roles of G-protein-coupled receptor dimerization. EMBO reports, 5(1), 30–34. https://doi.org/10.1038/sj.embor.7400052

Thomsen, A. R., 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. and Lefkowitz, R. J. (2016), 'GPCR-G

Protein- β-Arrestin Super-Complex Mediates Sustained G Protein Signaling', Cell **166**(4), 907–919. **URL:** http://dx.doi.org/10.1016/j.cell.2016.07.004

Tirone, M., Tran, N. L., Ceriotti, C., Gorzanelli, A., Canepari, M., Bottinelli, R., Raucci, A., Di Maggio, S., Santiago, C., Mellado, M., Saclier, M., François, S., Careccia, G., He, M., De Marchis, F., Conti, V., Ben Larbi, S., Cuvellier, S., Casalgrandi, M., Preti, A., … Vénéreau, E. (2018). High mobility group box 1 orchestrates tissue regeneration via CXCR4. *The Journal of experimental medicine*, *215*(1), 303–318. https://doi.org/10.1084/jem.20160217

Tobin AB, Butcher AJ, and Kong KC (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. Trends Pharmacol Sci 29:413–420.

Thal DM, Yeow RY, Schoenau C, Huber J, Tesmer JJ. Molecular mechanism of selectivity among G protein-coupled receptor kinase 2 inhibitors. Mol Pharmacol. 2011 Aug;80(2):294-303. doi: 10.1124/mol.111.071522. Epub 2011 May 19. PMID: 21596927; PMCID: PMC3141885.

Thoma, G., Streiff, M. B., Kovarik, J., Glickman, F., Wagner, T., Beerli, C., & Zerwes, H. G. (2008). Orally bioavailable isothioureas block function of the chemokine receptor CXCR4 in vitro and in vivo. *Journal of medicinal chemistry*, *51*(24), 7915–7920. https://doi.org/10.1021/jm801065q

Tiwari P, Fanibunda SE, Kapri D, Vasaya S, Pati S, Vaidya VA. GPCR signaling: role in mediating the effects of early adversity in psychiatric disorders. FEBS J. 2021 Apr;288(8):2602-2621. doi: 10.1111/febs.15738. Epub 2021 Feb 16. PMID: 33523596.

Treon, S. P., Cao, Y., Xu, L., Yang, G., Liu, X. and Hunter, Z. R. (2014), 'Somatic mutations in MYD88 and CXCR4 are determinants of clinical presentation and overall survival in Waldenström macroglobulinemia', *Blood* **123**(18), 2791–2796.

Tripathi, A., Saini, V., Marchese, A., Volkman, B. F., Tang, W. J., & Majetschak, M. (2013). Modulation of the CXC chemokine receptor 4 agonist activity of ubiquitin through C-terminal protein modification. *Biochemistry*, *52*(24), 4184–4192. https://doi.org/10.1021/bi400254f

Trousse, F., Jemli, A., Silhol, M., Garrido, E., Crouzier, L., Naert, G., Maurice, T., & Rossel, M. (2019). Knockdown of the CXCL12/CXCR7 chemokine pathway results in learning

deficits and neural progenitor maturation impairment in mice. *Brain, behavior, and immunity*, *80*, 697–710. https://doi.org/10.1016/j.bbi.2019.05.019

Tsuruda T., Kato J., Kuwasako K., Kitamura K. Adrenomedullin: Continuing to Explore Cardioprotection. *Peptides.* 2019;111:47–54. doi: 10.1016/j.peptides.2018.03.012.

Uehling, D. E., Joseph, B., Chung, K. C., Zhang, A. X., Ler, S., Prakesch, M. A., Poda, G., Grouleff, J., Aman, A., Kiyota, T., Leung-Hagesteijn, C., Konda, J. D., Marcellus, R., Griffin, C., Subramaniam, R., Abibi, A., Strathdee, C. A., Isaac, M. B., Al-Awar, R., & Tiedemann, R. E. (2021). Design, Synthesis, and Characterization of 4-Aminoquinazolines as Potent Inhibitors of the G Protein-Coupled Receptor Kinase 6 (GRK6) for the Treatment of Multiple Myeloma. Journal of medicinal chemistry, 64(15), 11129–11147. https://doi.org/10.1021/acs.jmedchem.1c00506

Ulvmar, M. H., Elin, H. and Rot, A. (2011), 'Atypical chemokine receptors', *Experimental Cell Research* **317**, 556–568.

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., Javitch, J. A., Roth, B. L., Christopoulos, A., Sexton, P. M., Miller, K. J., Spedding, M., & Mailman, R. B. (2007). Functional selectivity and classical concepts of quantitative pharmacology. *The Journal of pharmacology and experimental therapeutics*, *320*(1), 1–13. https://doi.org/10.1124/jpet.106.104463

U.S. National Library of Medicine Clinical Trials Database; search term 'CXCR4. (2022) . clinicaltrials.gov

Van Hout A, D'Huys T, Oeyen M, Schols D, Van Loy T. Comparison of cell-based assays for the identification and evaluation of competitive CXCR4 inhibitors. PLoS ONE 12(4), 1–18 (2017).

Van Hout, A., Klarenbeek, A., Bobkov, V., Doijen, J., Arimont, M., Zhao, C., Heukers, R., Rimkunas, R., de Graaf, C., Verrips, T., van der Woning, B., de Haard, H., Rucker, J. B., Vermeire, K., Handel, T., Van Loy, T., Smit, M. J., & Schols, D. (2018). CXCR4-targeting nanobodies differentially inhibit CXCR4 function and HIV entry. *Biochemical pharmacology*, *158*, 402–412. https://doi.org/10.1016/j.bcp.2018.10.015

Vela, M., Aris, M., Llorente, M., Garcia-Sanz, J. A. and Kremer, L. (2015), 'Chemokine receptor-specific antibodies in cancer immunotherapy: Achievements and challenges', *Frontiers in Immunology* **6**(JAN), 1-15.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D. J., Cattaneo, A., De Marchis, F., Liu, J., Antonelli, A., Preti, A., Raeli, L., Shams, S. S., Yang, H., Varani, L., Andersson, U., Tracey, K. J., Bachi, A., Uguccioni, M., & Bianchi, M. E. (2012). Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. *The Journal of experimental medicine*, *209*(9), 1519–1528. https://doi.org/10.1084/jem.20120189

Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Molecular signatures of G-protein-coupled receptors. Nature. 2013;494(7436):185-194. doi:10.1038/nature11896

Verweij EWE, Al Araaj B, Prabhata WR, Prihandoko R, Nijmeijer S, Tobin AB, Leurs R, Vischer HF. Differential Role of Serines and Threonines in Intracellular Loop 3 and C-Terminal Tail of the Histamine H_4 Receptor in β-Arrestin and G Protein-Coupled Receptor Kinase Interaction, Internalization, and Signaling. ACS Pharmacol Transl Sci. 2020 Mar 16;3(2):321-333. doi: 10.1021/acsptsci.0c00008. PMID: 32296771; PMCID: PMC7155198.

Vila-Coro, A. J., Rodríguez-Frade, J. M., Martín De Ana, A., Moreno-Ortíz, M. C., Martínez-A, C., & Mellado, M. (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *13*(13), 1699–1710.

Viskaitis, P., Irvine, E. E., Smith, M. A., Choudhury, A. I., Alvarez-Curto, E., Glegola, J. A., Hardy, D. G., Pedroni, S. M. A., Paiva Pessoa, M. R., Fernando, A. B. P., Katsouri, L., Sardini, A., Ungless, M. A., Milligan, G., & Withers, D. J. (2017). Modulation of SF1 Neuron Activity Coordinately Regulates Both Feeding Behavior and Associated Emotional States. *Cell reports*, *21*(12), 3559–3572. https://doi.org/10.1016/j.celrep.2017.11.089

Wang, A., Fairhurst, A. M., Tus, K., Subramanian, S., Liu, Y., Lin, F., Igarashi, P., Zhou, X. J., Batteux, F., Wong, D., Wakeland, E. K., & Mohan, C. (2009). CXCR4/CXCL12 hyperexpression plays a pivotal role in the pathogenesis of lupus. *Journal of immunology (Baltimore, Md. : 1950)*, *182*(7), 4448–4458. https://doi.org/10.4049/jimmunol.0801920

Wang, C., Chen, W., & Shen, J. (2018). CXCR7 Targeting and Its Major Disease Relevance. Frontiers in pharmacology, 9, 641. https://doi.org/10.3389/fphar.2018.00641

Wang, J. and Knaut, H. (2014), 'Chemokine signaling in development and disease', *Development* **141**, 4199–4205.

Wang, L., Chen, W., Gao, L., Yang, Q., Liu, B., Wu, Z., Wang, Y., & Sun, Y. (2012). High expression of CXCR4, CXCR7 and SDF-1 predicts poor survival in renal cell carcinoma. World journal of surgical oncology, 10, 212. https://doi.org/10.1186/1477-7819-10-212

Ward, R. J., Pediani, J. D. and Milligan, G. (2011), 'Ligand-induced internalization of the orexin OX1and cannabinoid CB1receptors assessed via N-terminal SNAP and CLIP-tagging', *British Journal of Pharmacology* **162**(6), 1439–1452.

Ward, R. J., Pediani, J. D., Marsango, S., Jolly, R., Stoneman, M. R., Biener, G., Handel, T. M., Raicu, V., & Milligan, G. (2021). Chemokine receptor CXCR4 oligomerization is disrupted selectively by the antagonist ligand IT1t. *The Journal of biological chemistry*, *296*, 100139. https://doi.org/10.1074/jbc.RA120.016612

Watts AO, van Lipzig MM, Jaeger WC, Seeber RM, van Zwam M, Vinet J, van der Lee MM, Siderius M, Zaman GJ, Boddeke HW, Smit MJ, Pfleger KD, Leurs R, Vischer HF. Identification and profiling of CXCR3-CXCR4 chemokine receptor heteromer complexes. Br J Pharmacol. 2013 Apr;168(7):1662-74. doi: 10.1111/bph.12064. PMID: 23170857; PMCID: PMC3605874.

Wescott, M. P., Kufareva, I., Paes, C., Goodman, J. R., Thaker, Y., Puffer, B. A., Berdougo, E., Rucker, J. B., Handel, T. M., & Doranz, B. J. (2016). Signal transmission through the CXC chemokine receptor 4 (CXCR4) transmembrane helices. Proceedings of the National Academy of Sciences of the United States of America, 113(35), 9928–9933. https://doi.org/10.1073/pnas.1601278113

Wess, J. (1997), 'G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition.', *FASEB journal* **11**(5), 346–54. **URL:** http://www.fasebj.org/content/11/5/346.abstract%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/914150

Weston, C., Winfield, I., Harris, M., Hodgson, R., Shah, A., Dowell, S. J., Mobarec, J. C., Woodlock, D. A., Reynolds, C. A., Poyner, D. R., Watkins, H. A., & Ladds, G. (2016). Receptor Activity-modifying Protein-directed G Protein Signaling Specificity for the Calcitonin Gene-related Peptide Family of Receptors. The Journal of biological chemistry, 291(42), 21925–21944. https://doi.org/10.1074/jbc.M116.751362

Wettschureck, N. and Offermanns, S. (2005), 'Mammalian G Proteins and Their Cell Type Specific Functions', *Physilological Review* **85**, 1159–1204.

Wetzel-Strong SE, Li M, Klein KR, Nishikimi T, Caron KM. Epicardial-derived adrenomedullin drives cardiac hyperplasia during embryogenesis. Dev Dyn. 2013;243:243–256.

Whalen, E. J., Rajagopal, S., & Lefkowitz, R. J. (2011). Therapeutic potential of β-arrestin- and G protein-biased agonists. *Trends in molecular medicine*, *17*(3), 126–139. https://doi.org/10.1016/j.molmed.2010.11.004

Wilden, U., Hall, S. W. and Kuhn, 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. **URL:** *http://www.pnas.org/cgi/doi/10.1073/pnas.83.5.1174*

Wijtmans, M., Maussang, D., Sirci, F., Scholten, D. J., Canals, M., Mujić-Delić, A., Chong, M., Chatalic, K. L., Custers, H., Janssen, E., de Graaf, C., Smit, M. J., de Esch, I. J., & Leurs, R. (2012). Synthesis, modeling and functional activity of substituted styrene-amides as small-molecule CXCR7 agonists. European journal of medicinal chemistry, 51, 184–192. https://doi.org/10.1016/j.ejmech.2012.02.041

Wojdasiewicz, P., Poniatowski, _L. A., Kotela, A., Deszczy´nski, J., Kotela, I. and Szukiewicz, D. (2014), 'The Chemokine CX3CL1 (Fractalkine) and its Receptor CX3CR1: Occurrence and Potential Role in Osteoarthritis', Archivum Immunologiae et Therapiae Experimentalis**62**(5), 395–403.

Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F. C., Hamel, D. J., Kuhn, P., Handel, T. M., Cherezov, V., & Stevens, R. C. (2010). Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science (New York, N.Y.)*, *330*(6007), 1066–1071. https://doi.org/10.1126/science.1194396

Xing, J.Z.; Zhu, L.; Jackson, J.A.; Gabos, S.; Sun, X.J.; Wang, X.B.; Xu, X. Dynamic monitoring of cytotoxicity on microelectronic sensors. *Chem. Res. Toxicol.* **2005**, *18*, 154–161.

Xu, C., Zhao, H., Chen, H., & Yao, Q. (2015). CXCR4 in breast cancer: oncogenic role and therapeutic targeting. Drug design, development and therapy, 9, 4953–4964. https://doi.org/10.2147/DDDT.S84932

Xu, L., Li, Y., Sun, H., Li, D., & Hou, T. (2013). Structural basis of the interactions between CXCR4 and CXCL12/SDF-1 revealed by theoretical approaches. *Molecular bioSystems*, *9*(8), 2107–2117. https://doi.org/10.1039/c3mb70120d

Xu, Z. Q., Zhang, X., & Scott, L. (2007). Regulation of G protein-coupled receptor trafficking. *Acta physiologica (Oxford, England)*, *190*(1), 39–45. https://doi.org/10.1111/j.1365-201X.2007.01695.x

Yagi, H., Tan, W., Dillenburg-Pilla, P., Armando, S., Amornphimoltham, P., Simaan, M., Weigert, R., Molinolo, A. A., Bouvier, M. and Gutkind, J. S. (2011), 'Cancer biology: A synthetic biology approach reveals a CXCR4-G13-rho signaling axis driving transendothelial migration of metastatic breast cancer cells', Science Signaling **4**(191).

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

Yen YC, Schafer CT, Gustavsson M, Eberle SA, Dominik PK, Deneka D, Zhang P, Schall TJ, Kossiakoff AA, Tesmer JJG, Handel TM. Structures of atypical chemokine receptor 3 reveal the basis for its promiscuity and signaling bias. Sci Adv. 2022 Jul 15;8(28):eabn8063. doi: 10.1126/sciadv.abn8063. Epub 2022 Jul 13. PMID: 35857509; PMCID: PMC9278869.

Yu, L., Cecil, J., Peng, S. B., Schrementi, J., Kovacevic, S., Paul, D., Su, E. W., & Wang, J. (2006). Identification and expression of novel isoforms of human stromal cell-derived factor 1. *Gene*, *374*, 174–179. https://doi.org/10.1016/j.gene.2006.02.001

Yu S, Crawford D, Tsuchihashi T, Behrens TW, Srivastava D. The chemokine receptor CXCR7 functions to regulate cardiac valve remodeling. Dev Dyn. 2011;240(2):384–93.

Zagzag D, Lukyanov Y, Lan L, Ali MA, Esencay M, Mendez O, Yee H, Voura EB, Newcomb EW. Hypoxia-inducible factor 1 and VEGF upregulate CXCR4 in glioblastoma: implications for angiogenesis and glioma cell invasion. Lab Invest. 2006 Dec;86(12):1221-32. doi: 10.1038/labinvest.3700482. Epub 2006 Oct 30. PMID: 17075581.

Zaitseva, M., Blauvelt, A., Lee, S., Lapham, C. K., Klays-Kovtun, V., Mostowski, H., Manischewitz, J. and Golding, H. (1997), 'Expression and Function of CCR5 and CXCR4 on human Langerhans cells and Macrophages: implications for HIV primary infection', Nature Medicine 3(12), 1369–1375. URL: http://www.ncbi.nlm.nih.gov/pubmed/9585240

Zarca, A., Perez, C., van den Bor, J., Bebelman, J. P., Heuninck, J., de Jonker, R. J. F., Durroux, T., Vischer, H. F., Siderius, M., & Smit, M. J. (2021). Differential Involvement of ACKR3 C-Tail in β-Arrestin Recruitment, Trafficking and Internalization. *Cells*, *10*(3), 618. https://doi.org/10.3390/cells10030618

Zhang, D., Zhao, Q. and Wu, B. (2015), 'Structural studies of G protein-coupled receptors', Molecules and Cells 38(10), 836–842.

Zhang, X., Dong, S. and Xu, F. (2018), 'Structural and Druggability Landscape of Frizzled G Protein-Coupled Receptors', Trends in Biochemical Sciences pp. 1–14. URL: https://doi.org/10.1016/j.tibs.2018.09.002

Zheng H, Worrall C, Shen H, Issad T, Seregard S, Girnita A, Girnita L. Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor. Proc Natl Acad Sci U S A. 2012 May 1;109(18):7055-60. doi: 10.1073/pnas.1118359109. Epub 2012 Apr 16. PMID: 22509025; PMCID: PMC3345003.

Zhu P, Stanisheuski S, Franklin R, Vogel A, Vesely CH, Reardon P, Sluchanko NN, Beckman JS, Karplus PA, Mehl RA, Cooley RB. Autonomous Synthesis of Functional, Permanently Phosphorylated Proteins for Defining the Interactome of Monomeric 14-3-3ζ. ACS Cent Sci. 2023 Apr 10;9(4):816-835. doi: 10.1021/acscentsci.3c00191. PMID: 37122473; PMCID: PMC10141581.

Zhuo, Y., Crecelius, J. M., & Marchese, A. (2022). G protein-coupled receptor kinase phosphorylation of distal C-tail sites specifies βarrestin1-mediated signaling by chemokine receptor CXCR4. *The Journal of biological chemistry*, *298*(9), 102351. https://doi.org/10.1016/j.jbc.2022.102351

Zhuo, W., Jia, L., Song, N., Lu, X. A., Ding, Y., Wang, X., Song, X., Fu, Y. and Luo, Y. (2012), 'The CXCL12 -CXCR4 chemokine pathway: A novel axis regulates lymphangiogenesis', *Clinical Cancer Research* **18**(19), 5387–5398.

Zlotnik, A., Burkhardt, A. M., & Homey, B. (2011). Homeostatic chemokine receptors and organ-specific metastasis. *Nature reviews. Immunology*, *11*(9), 597–606. https://doi.org/10.1038/nri3049

Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012 May 25;36(5):705-16. doi: 10.1016/j.immuni.2012.05.008. PMID: 22633458; PMCID: PMC3396424.