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CXCR3 biased signaling, heteromerization and decoy properties

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Résumé

Le récepteur de chimiokine CXCR3 est un récepteur couplé à la protéine G (RCPG) exprimé, entre autre, sur les cellules T activées lors d'une réponse immune. CXCR3 est activé par trois ligands inductibles par l'interféron-γ (CXCL9, 10, 11) et, plus récemment, il a été découvert que CXCL4 liait CXCR3. Nous savons que CXCR3 joue un rôle dans la chimiotaxie des leucocytes, mais une attention limitée a été portée sur la signalisation biaisée induite par ces quatre ligands. Alors que l'homodimérisation entre récepteurs de chimiokine est un concept grandement observé, l'hétéromérisation entre deux récepteurs reste un domaine de recherche active. Enfin, certains récepteurs de chimiokine (*decoy*) jouent sur le gradient de chimiokines en les dégradant. À ce jour, aucune donnée n'a présenté CXCR3 en tant que récepteur *decoy*. La signalisation biaisée et l'hétéromérisation ont été testées grâce à la technique de bioluminescene resonance energy transfer (BRET) dans des cellules HEK293E. L'activité decoy de CXCR3 a été investiguée grâce à un essai de dégradation de chimiokines radio-marquées à l'iode 125. Nous présentons une caractérisation pharmacologique des quatre ligands de CXCR3 et démontrons l'hétéromérisation de CXCR3 avec CXCR4 et avec CXCR7. Nous démontrons que CXCR3 peut agir comme *decoy* en dégradant CXCL11 radio-marqué. Nos résultats suggèrent que les ligands de CXCR3 n'agissent pas de manière redondante. De plus, nos résultats de dégradation suggèrent l'absence de compétition entre les ligands de CXCR3. Enfin, nous montrons que CXCL12 n'affecte pas la dégradation de CXCL11 par CXCR3 ni par l'hétéromère CXCR3/CXCR4.

Mots clés

CXCR3, RCPG, Signalisation biaisée, Hétéromérisation, BRET, Récepteur *decoy*, CXCL11 radio-marquée.

Abstract

The chemokine receptor CXCR3 is a G-protein-coupled receptor (GPCR) rapidly induced on naïve T cells upon activation. CXCR3 is activated by three interferon-γ inducible ligands (CXCL9, 10, 11) and, more recently, CXCL4 has been discovered as a functional ligand for CXCR3. It is known that CXCR3 acts as a chemotactic receptor, but limited attention has been directed to the biased signaling induced by all four ligands. Chemokine receptor homodimerization is now a widely accepted concept, but the extent to which heterodimerization is prevalent remain matter of active research. Some chemokine (decoy) receptors have been reported to maintain/re-shape specific chemokine gradients by degrading chemokines. To date, CXCR3 has never been described as a decoy receptor.

In this work, biased signalling and heterodimerization were assessed with bioluminescence resonance energy transfer (BRET) in HEK293E cells. CXCR3 decoy properties were investigated by radiolabelled chemokine degradation assays. We present pharmacological characterization of all four ligands of CXCR3 and heterodimerization of CXCR3 with CXCR4 or CXCR7. Finally, we present CXCR3 decoy activities on radiolabelled CXCL11. Our results suggest that CXCR3 ligands are not redundant and that CXCR3 heterodimerizes with CXCR4 and with CXCR7. Our results also suggest that CXCR3 is able of CXCL11 scavenging. Our degradation assays demonstrated the absence of competition between ligands. Finally, CXCL12 did not affect CXCL11 scavenging neither by CXCR3 nor by CXCR3/4 heterodimer.

Key words

CXCR3, GPCR, Biased signalling, Heteromerization, BRET, Decoy receptor, Radiolabelled CXCL11.

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Abbreviations

AC	Adenylate cyclase
ACKR	Atypical chemokine receptor
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
ASM	Airway smooth muscle
BRET	Bioluminescence resonance energy transfer
BRET50	Half maximal bioluminescence resonance energy transfer value
BSA	Bovine serum albumin
C-terminal	Carboxy-terminal
Ca2+	Calcium ions
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHU	Centre hospitalier universitaire
COPSE	Comité d'organisation du programme des stages d'été
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle’s medium
EAE	Autoimmune encephalomyelitis

EC50	Half maximal effective concentration
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal regulated kinase
F	Forskolin
FRET	Fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
GFP10	Green fluorescent protein 10
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-O-[gamma-thio] triphosphate
HEK	Human embryonic kidney
HLMC	Human lung mast cells
HIV	Human immunodeficiency virus
HMVEC	Human microvascular endothelial cells
IFNγ	Interferon-gamma
IP-10	CXCL10, Interferon-gamma inducible protein 10
I-TAC	CXCL11, Interferon–inducible T cell alpha chemoattractant
L	Ligand
MAPK	Mitogen-activated protein kinase
MIG	CXCL9, Monokine induced by interferon-gamma
mRNA	Messenger ribonucleic acid
N-terminal	Amino-terminal
NK	Natural killer

MS	Multiple sclerosis
PBS	Phosphate buffered saline
PF-4	CXCL4, Platelet factor-4
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
RA	Rheumatoid arthritis
Rluc	Renilla luciferase
Rluc3	Renilla luciferase 3
RNA	Ribonucleic acid
SDF-1α	CXCL12, Stromal cell-derived factor 1 alpha
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TCA	Trichloroacetic acid
TNFα	Tumor necrosis factor α
T_{REG}	Regulatory T cell
wt	Wildtype
YFP	Yellow fluorescent protein
β-Arr	β-Arrestin

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1. Introduction

1.1 Chemokines and chemokine receptors

Chemokines form a large family of proteins that can be divided into sub-families based on their structures and on their functions.

1.1.1. Nomenclature of chemokines and chemokine receptors

Originally, chemokines have been named randomly without a clear system of nomenclature. Some chemokines were included in the large family of interleukins (e.g. IL-8), while others were given a name suggesting their function (e.g. I-TAC for Interferon-inducible T-cell Alpha Chemoattractant) [1].

More recently, members of the chemokine family were classified into four different groups according to their NH_2-terminal cysteine-motifs. There are now C chemokines, CC chemokines, CXC chemokines and finally, CX_3C chemokines [1]. For example, the first chemokine of a group having one residue between its two cysteines would be named CXCL1. Here, L stands for ligand.

Chemokine receptors are classified according to their ability to respond to a certain chemokine family. For instance, a chemokine receptor capable to interact with CXCL1 would be called a CXC Receptor, or CXCR.

The present master's thesis deals mainly with the chemokine receptor CXCR3 and with its interactions with the chemokine receptors CXCR4 and CXCR7.

1.1.2 Biological functions of chemokines

Chemokine are ***chemo***tactic cyto***kines*** involved in the directed migration of cells; a process called chemotaxis. Chemotaxis needs the formation of a concentration gradient of

chemokines. Cells that are attracted by chemokines move through the gradient from lower to higher concentration.

It has been established that some chemokines play essential roles in hematopoiesis and organ development [2]. For instance, CXCL12 is a constitutively expressed chemokine from bone marrow stromal cells. This homeostatic chemokine promotes proliferation of B cell progenitors [3]. CXCL12 also induces the migration of hematopoietic precursors to the bone marrow during embryogenesis [4, 5]. Of note, CXCL12 binds two different chemokine receptors: CXCR4 and CXCR7.

Chemokines are chemotactic, homeostatic, but also immunoregulatory proteins. In fact, chemokine were originally discovered as mediators of relocalization processes during inflammatory and immune responses. For example, some chemokines guide immune cells from tissues to the lymph nodes so they can act as antigen-presenting cells [2]. When microorganisms are phagocytosed by dendritic cells in peripheral tissues, these dendritic cells mature [2]. Then, the mature dendritic cells start to express the chemokine receptor CCR7. This chemokine receptor allows the dendritic cells to migrate in response to CCR7 ligands into the lymph nodes in order to present processed antigen to T cells [2].

During an immune response, different subsets of T cells are generated following the interaction of naïve T cells with antigen-presenting cells in the lymphoid compartment [6]. Once activated, these T cell subsets upregulate chemokine receptors, which guide them out of the lymphoid compartment towards sites of injury [6]. For instance, CXCR3 is upregulated on T cells following activation and is implicated in their migration.

1.2 Pathophysiological relevance of CXCR3 and its ligands

The expression of CXCR3 is associated with CD4+ type-1 helper and CD8+ cytotoxic lymphocytes [7-10] but is also expressed on various other cells types like B cells, NK cells, smooth muscle cells and endothelial cells [2].

CXCR3 is activated by three ligands: CXCL9 (**M**onokine induced by **I**FN-**γ** or MIG), CXCL10 (**I**FN-γ inducible **P**rotein **10** or IP-10) and CXCL11 (**I**nterferon-inducible **T**-cell **A**lpha **C**hemoattractant or I-TAC). In contrast to the constitutive chemokine CXCL12 secreted by the bone marrow stromal cells, the three ligands of CXCR3 are not constitutively expressed, but are up-regulated in an IFN-**γ** cytokine environment [11]. Neutrophils, monocytes/macrophages, dendritic cells, CD4+, CD8+, NK and NK-T cells, in response to IFN-γ, can secrete CXCL9, CXCL10 or CXCL11 [11-14].

Consequently, the role of CXCL9-10-11 is to recruit immune cells to inflammation sites [6, 15]. Therefore, a prevailing function in inflammatory and immune diseases has been suggested for CXCR3 and its ligands. For instance, it has been proposed that CXCR3 pathways are involved in local amplification loops of inflammation in targeted regions [6, 15].

We will briefly discuss the pathophysiological relevance of CXCR3 and its ligands in auto-immune/inflammatory diseases such as atherosclerosis, multiple sclerosis and asthma.

1.2.1 Role of CXCR3 and its ligands in atherosclerosis

The early phase of atherosclerosis, atherogenesis, is characterized by the adherence of blood circulating monocytes to the surface of arterial walls and by their migration to the sub-endothelial space where they undergo transformation into macrophages and causes fibrotic plaques [16, 17]. These plaques, called atheroma, are accumulated fatty substance and cells in

the inner layer of arterial walls. The worsening accumulation of cells and debris causes atherosclerosis.

These plaques contain the cytokine IFN-γ, considered as the master regulator of atherosclerosis [18]. The differential expression of the three IFN-γ–inducible chemokines CXCL9, CXCL10 and CXCL11 by atheroma-associated cells was reported [19]. These findings suggest that the expression of CXCR3 ligands by atheroma-associated cells act as recruiters and retainers of activated T lymphocytes within the vascular wall lesions in the course of atherogenesis [19]. Moreover, the expression CXCR3 by all T lymphocytes within human atherosclerotic lesions *in situ* was observed [19]. Since these cells persist at the site of the lesion, it has been suggested that they may play an important role in the development of atherosclerosis.

Combined treatment with two drugs, simvastatin with niacin, provided clinical benefits in patients suffering from atherosclerosis [20]. Simvastatin is an inhibitor of HMG-CoA reductase, it reduces low-density lipoproteins also called the "bad" cholesterol [21]. Niacin, also known as vitamin B3, appears to reduce the risk of cardiovascular disease [22]. However, since the complications of advanced atherosclerosis are chronic, there is an emerging need for alternative or complementary therapeutic interventions. For instance, these alternatives could target molecular mechanisms underlying the initiation of cell recruitment into the arterial wall's plaques [17]. It was reported that a CXCR3 antagonist attenuated atherosclerotic lesion formation by blocking direct migration of CXCR3+ effector cells from the circulation into the atherosclerotic plaque in mice [23]. Therefore, CXCR3 antagonists may be a possible therapy to inhibit inflammation-induced leukocyte migration and to subsequently reduce atherogenesis [23]. A better understanding of CXCR3 signalling is mandatory for better drug development in atherosclerosis.

1.2.2. Role of CXCR3 and its ligands in multiple sclerosis

Multiple sclerosis (MS) is an inflammatory disorder of the human central nervous system (CNS) [24]. This inflammatory disease largely involves mononuclear phagocytes and T cells [25]. These cells enter into the brain through a permissive brain-blood barrier [26] and attack myelin because they recognize it as an antigen [27]. Thus, it was argued that chemokine and chemokine receptors expressed on these cells may play a role in MS. However, other immunomodulatory cytokines, like tumor necrosis factor alpha (TNFα) or interferons-β (IFN-β), can also be implicated in MS [28].

It was reported that CXCL10 and CXCR3 co-localized in the inflamed CNS [29]. In addition, elevated levels of three chemokines, CXCL9, CXCL10 and CCL5 (the ligands of the chemokine receptors CCR1, CCR3 and CCR5) were observed in the cerebrospinal fluid of patients [25]. Compared with the circulation, the cerebrospinal fluid was significantly enriched in CXCR3+CCR5+ cells [25, 30]. These findings imply that specific chemokine–chemokine receptor interactions, and more precisely CXCR3 and CCR5 with their ligands, play important pathogenic roles in MS.

Current MS treatments include interferon immunosuppressive agents and antibodies [31]. However, these treatments often have unsatisfactory outcomes [31][32]. For example, the neutralizing antibodies persist in the body and reduce the biologic activity of IFN-β therapies. This persistence of neutralizing antibodies is also associated with a reduced treatment efficacy [32]. One promising avenue to overcome this issue is the CXCR3/CXCL10 axis. A better comprehension of CXCR3+ cells chemotaxis towards the CNS will surely gives us precious insight into how to block their migration. This could lead to an alternative treatment of MS patients that are now treated with antibodies and immunosuppressive agents.

1.2.3 Role of CXCR3 and its ligands in asthma

Asthma is an inflammatory lung disease characterized by airflow obstruction, airway contraction and hyper-responsiveness. The infiltration of airway smooth muscle (ASM) by mastocytes, also called mast cells, is a major determinant of the asthmatic phenotype [33].

The localization of mast cells within the airway structures is important in the pathophysiology of inflammatory lung disease [34]. Cytokine expression is also observed in airway pathology [35, 36]. Chemokines are likely candidates mediating mast cells migration into lung tissues.

Compared to mast cells localized elsewhere, human lung mast cells (HLMC) highly express CXCR3 [34]. In addition to CXCR3, it has been found that more than 10 % of *ex vivo* HLMC were expressing other chemokine receptors such as CCR3 and CXCR4 [34]. A fundamental question is how mast cells accumulate in the ASM in asthma. This is a key question because if this accumulation can be repressed, the symptoms can be attenuated [37]. It has been suggested that the interaction between CXCL10 derived from ASM and CXCR3+ mast cell may be the dominant pathway facilitating the migration of mast cells into the ASM bundles [37].

It has been demonstrated that the neutralization of CXCL10 strongly reduced allergic airway inflammation in a mouse model of asthma [38]. However, to date, no approved drug targeting CXCR3 is available. In fact, inhaled corticosteroids are the preferred treatment for long-term control of symptoms in asthmatic patients [39].

1.2.4 An example of a clinical trial

Even though CXCR3 is considered as a promising drug target [40], the outcome of clinical trials were disappointing. For instance, AMG487, a CXCR3 antagonist, has been tested for the treatment of psoriasis, a skin inflammatory disease in which CXCR3 plays an important role in the development of the pathology [41-43]. In preclinical studies, AMG-487 blocked immune-cell migration and showed excellent potency, selectivity and bioavailability [44]. In a phase 1 trial, the safety and tolerability of AMG487 was confirmed [45]. However, the phase 2 trial was discontinued due to a lack of clinical efficacy [45, 46]. This failure of using a small synthetic ligand of CXCR3 is one example among others that suggests that the CXCR3 mode of operation remains still insufficiently understood. Further investigations are needed to

develop new small molecule CXCR3 antagonists for the treatment of autoimmune diseases, including rheumatoid arthritis (RA) and MS.

1.3 Challenges and misconceptions in chemokine receptor drug design

Chemokine receptors are attractive therapeutic drug targets because they are central in many pathophysiological processes. To date, drugs inhibiting chemokine receptors (antagonists) are approved for the treatment of HIV infection and for stem cell mobilization [47]. However, no drugs have been approved yet for the treatment of inflammatory and autoimmune diseases [41, 47]. Why is that so? We will here briefly discuss the challenges of developing compounds antagonizing chemokine receptors.

The easiest way to explain our incapacity to successfully target these receptors in inflammatory diseases would be that we do not comprehend enough the chemokine receptor biology. Although this is true, this generality only superficially seizes the problem. It has been proposed that inappropriate target selection and animal models – and not the chemokine redundancy (see below) – are among the main hurdles to the use of chemokine receptor antagonists as anti-inflammatory treatments [41, 47].

1.3.1 Inappropriate target selection

Inappropriate target selection is a simple way to explain a complex reality: several chemokine receptor targets can exist for a given clinical condition but only some receptors can lead to clinical benefits. In addition, some animal models are irrelevant mirrors of human diseases in particular cases. We will use the case MS to explain these barriers to the design of drugs antagonizing chemokine receptors.

CCR1, CCR2, CCR3, CCR4, CXCR2 and CXCR3 have all been reported to be expressed in inflammatory diseases [47-51]. Although the presence of these receptors has been

demonstrated, the functions of cells expressing such receptors are not always crystal clear. Therefore, inhibiting one of these receptors might not necessarily lead to clinical benefits in a specific disease.

For instance, a CCR1 antagonist has been used in a phase II trial in patients suffering from MS without good clinical outcomes [41, 47]. The clinical study was stopped after the failure to show a reduction in the number of new inflammatory CNS lesions [52]. Was CCR1 a good target selection for MS? Some authors do believe it is [41, 52, 53]. However, even if evidence tends to demonstrate the implication of CCR1 in MS [41, 54, 55], there is no indication whether the expression of CCR1 is pathological, homeostatic or circumstantial [47]. In fact, the cells expressing CCR1 could either be aggressive, passive or regulatory cells. For instance, it has been shown that T_{REG} cells can express CCR1 [56]. Ultimately, the blockade of CCR1 expressing T_{REGS} by a CCR1 antagonist might worsen the condition instead of improving it.

1.3.2 Inappropriate animal models

In the case of animal disease models, some of them are not always predictive of human disease or even representative of human biology [57, 58]. There are several examples illustrating the unrecognized pitfalls of using an animal model to screen for potential treatments. This is especially true for the experimental autoimmune encephalomyelitis (EAE) model, an animal model of brain inflammation used to represent MS [41, 58].

First, tumour-necrosis factor inhibitors ameliorated the symptoms in animal models of MS but actually worsened the disease in patients [59, 60]. Second, CCR1 is constitutively expressed on neutrophils in rodents but is mainly expressed on monocytes and activated T cells in humans [41, 61]. According to these observations, it is possible that the inhibition of CCR1+ neutrophils could account for the beneficial effect of CCR1 antagonist in the EAE model [41]. However, CCR1-expressing neutrophils are not considered as a driving force of MS in humans [41]. This is in line with the inappropriate drug target choice mentioned earlier. In fact, both

arguments (inappropriate target and model) could account for the clinical failure of this chemokine receptor antagonist.

Finally, we see that the presence of multiple receptors can lead researchers to think they are all involved in a specific disease. This chemokine receptor overlap may reflect a certain redundancy of targets and surely causes problems in drug development. Therefore, the generation of drugs that can target and inhibit multiple receptors might represent a way to overcome this issue [41]. Also, animal models can mimic symptoms of a disease but only to a certain extent. In some cases, animal models can even be inappropriate.

We will now introduce another form of redundancy that misled researchers investigating the chemokine system.

1.3.3 The misconception of chemokine redundancy

The notion of redundancy, also referred to as ligand promiscuity, implies that a single receptor binds multiple ligands, and conversely, a single ligand can bind several receptors. The chemokine system displays considerable promiscuity with multiple ligands and chemokine receptors shared in common. This promiscuity led to the belief that several chemokines or chemokine receptors can carry out the same functions *in vivo* [47]. This perception of redundancy in the chemokine system may have developed for different reasons.

The redundancy model seems to be supported by the number of possible interactions between chemokines and their receptors. For instance, CXCR3 has three potential ligands (CXCL9, CXCL10, and CXCL11). However, CXCL11 is also a ligand for CXCR7. In addition, CXCR7 shares CXCL12 with CXCR4 (Figure 1).

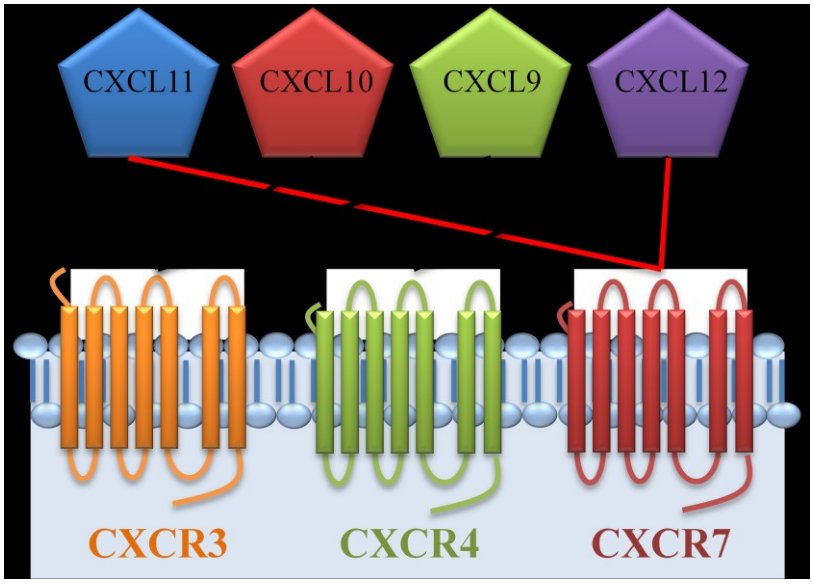

Figure 1. Schematic view of the possible interactions between different ligands and CXC-chemokine receptors.

The chemokine system is large and complex: about 50 chemokines for only 20 receptors [62]. The disparity between the number of chemokines and the limited number of receptors may have led researchers to oversimplify the biological functions of the chemokine system. This was especially true for early *in vitro* experiments describing chemokine activities [47, 63]. Indeed, in the early phase of chemokine discovery, many assessments of chemokines reported their ability to induce migration of given cell types *in vitro*. This led to the oversimplified conclusion that, because many chemokines could induce migration of the same cell type, they were redundant.

However, several reports indicate that different chemokines do not necessarily result in the same biological response on a given receptor or *vice versa* [47, 64, 65]. For instance, CCL5 is the ligand of three receptors: CCR1, CCR3 and CCR5. CCL5 induces very different patterns of receptor recycling to the cell surface on the different receptors. Specifically, CCR5 recycles to the cell surface [66]. CCR3 is partially restored to the cell surface and partially degraded in lysosomes [67]. Finally, CCR1 does not recycle at all [68].

In fact, several other examples demonstrated that redundancy does not exist at the molecular level of chemokine receptors (see also the section 1.4.3). Obviously, this includes CXCR3 [64, 69, 70]. Therefore, the failure of targeting chemokine receptors has nothing to do with the chemokine redundancy. The notion of redundancy rather suggests a fine-tuning of the chemokine system.

That being said, we will now elaborate on the molecular level of chemokine receptor functions. More precisely, we will focus on CXCR3.

1.4 Molecular basis of the chemokine system

1.4.1 Chemokine receptor signaling through G proteins and desensitization by β-arrestin

Chemokines are ligands that bind to members of the super-family of heptahelical G-protein-coupled receptors (GPCRs). As their name suggests, the signalization of GPCRs is through heterotrimeric G protein subunits (αβγ). Ligand binging on a GPCR causes subsequent conformational change in the receptor. This conformational change then activates the G protein by exchanging a GDP for a GTP. The Gα protein subunit, now bound to a GTP, dissociate from βγ subunits to further activate downstream signaling pathways (Figure 2, step 1). Most commonly, chemokine receptors are coupled to the Gαi subunit, which has as major function to modulate cAMP production by inhibiting adenylate cyclase (Figure 2, step 1). However, there is considerable evidence for alternative Gαq coupling that activates phospholipase C (Figure 2, step 1) [71, 72]. Finally, it has never been reported that a chemokine receptor stimulates adenylate cyclase through Gαs coupling (Figure 2, step 1).

One process that regulates GPCRs is desensitization when a receptor is exposed to its ligand for a prolonged time. Following receptor activation by a ligand, GPCR kinase (GRK) phosphorylates the cytoplasmic C-terminal of the agonist-bound receptor (Figure 2, step 2). This phosphorylation initiates impairment of the signaling and allows desensitization by the the subsequent recruitment of β-arrestin, which uncouples the receptor from further G protein

activation (Figure 2, step 3). In addition, β-arrestin recruitment may lead to internalization or other signalisation pathways (Figure 2, step 4).

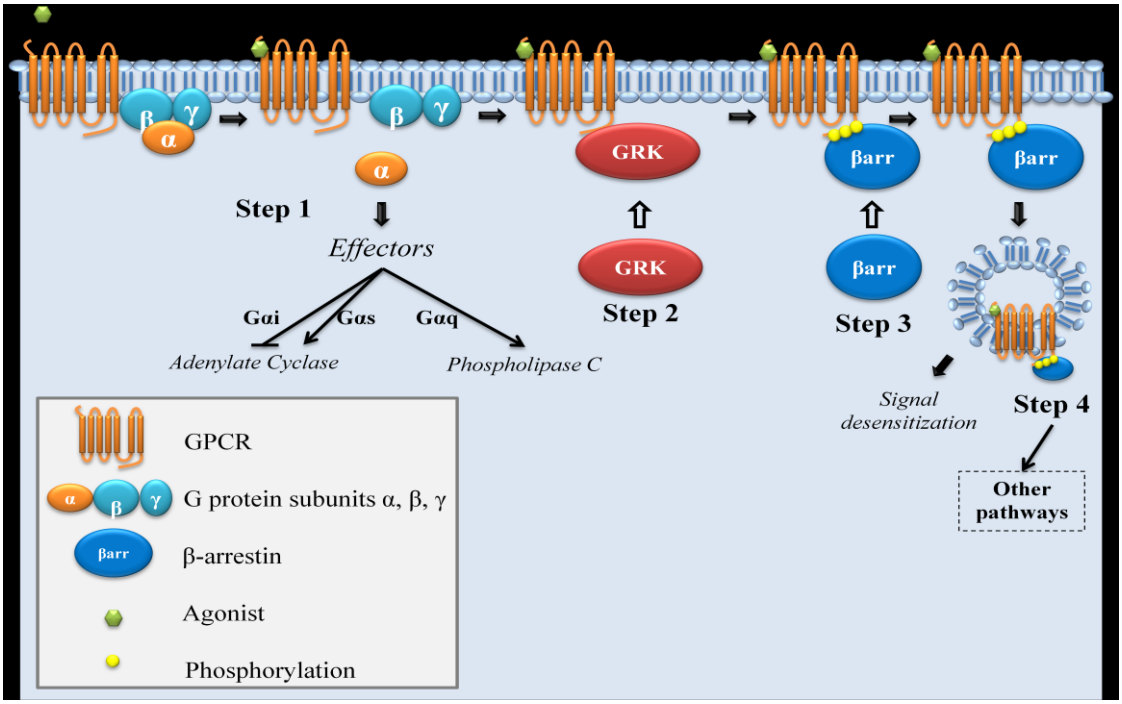

Figure 2. Schematic view of chemokine receptor signalisation, phosphorylation by GRK, desensitization and subsequent internalization by β-arrestin.
Inspired from Ma et al. Journal of Cell Science, 2007 [73].

1.4.2 Some principles of pharmacology

The present work mainly deals with CXCR3 at a pharmacogical level. Therefore, it is important to recall certain essential concepts in pharmacology such as affinity, efficacy, potency, agonist, partial agonist. These concepts will be especially useful in the section treating of functional selectivity, also called biased agonism (see section 1.4.3.).

Affinity, efficacy and potency are essential referential quantities used in drug discovery and in fundamental studies on receptors. Agonist affinity can be explained in terms of the

dissociation constant (Kd) for agonist binding to a receptor using *in vitro* techniques such as ligand binding assays [74]. The lower the dissociation constant is, the higher is the affinity of an agonist to its receptor.

Efficacy is associated to the maximal activity in a specific assay using a range of concentrations of the agonist (Figure 3). Therefore, the assay can also be referred to as a dose response experiment. In contrast to efficacy, potency is associated with the concentration of drug required to produce an effect of given intensity (Figure 3). The higher the potency is, the lower is the amount of drug required to produce a response. Figure 3 describes the difference between potency and efficacy.

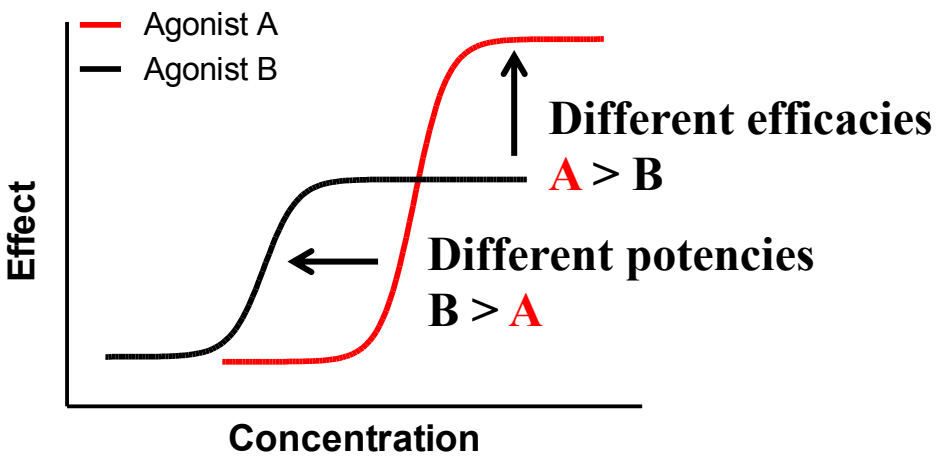

Figure 3. The principles of efficacy and potency in pharmacology.

Agonist A has a higher efficacy then agonist B, while agonist B has a higher potency then agonist A.

A full agonist is a ligand or a drug with high efficacy (defining maximal response). Generally, full agonists are defined as a reference ligand, most commonly associated to the endogenous ligand with the highest efficacy. For instance, CCR2 has been reported to bind seven natural ligands. However, CCL2 is the only ligand to elicit maximal response in the different

pathways [75]. Therefore, it is considered as the reference for the determination of relative agonist efficacy on CCR2. A partial agonist is a ligand or a drug that activates a given receptor but only has partial efficacy in comparison to a full agonist.

Noteworthy, the determination of a full agonist is pathway dependent. This implies that a full agonist in one assay can turn into a partial agonist in a different assay, assessing a different pathway. This is explained by the concept of functional selectivity.

1.4.3 The concept of functional selectivity (or biased agonism)

Over the past 20 years, several articles reported different agonists with different potencies and efficacies on their respective receptor [76]. In some cases, the different ligands of a same receptor can all be agonists for one pathway but with different potencies and efficacies. For example, CCL2 acts as a full agonist for the recruitment of β-arrestin on CCR2 [75]. Compared with CCL2, other CCR2 ligands were described as partial agonists with reduced efficacy and potency on the same pathway [75]. Moreover, the different ligands of a same receptor can be agonists for some pathways and antagonists for others. For instance, angiotensin II acts as an agonist of β-arrestin pathways but as an antagonist of G protein pathways on the angiotensin II type 1A receptor [77]. This phenomenon is named biased agonism and the ligands that display such behavior are called biased agonists[78]. These biased agonists have been classified as being functionally selective or biased toward certain response pathways compared with the other agonists [76].

This new concept provoked a paradigm shift in GPCR pharmacology, in that different ligands targeting the same receptor do not necessarily induce qualitatively similar intracellular signals. Different signalling axes, such as G-protein signalling versus β-arrestin signalling, are activated independently from one another. The promiscuous ligand-receptor relations in the chemokine system make chemokines one of the ideal systems to study “ligand-biased signalling”.

Biased agonism has been described for other chemokine receptors such as CCR2 [75]. Few articles reported the functional selectivity for CXCR3 and its ligands [64, 79]. For instance, it has been shown that CXCL11 was the most potent and efficacious inducer of CXCR3 internalization and chemotaxis in a leukemia cell line [79]. At the time the present work started, no data reported the biased agonism of CXCR3 and its ligands for Gαi and β-arrestin pathways. However, two groups now reported the biased agonism of CXCR3 [64, 80].

1.4.4 Chemokine receptor heteromerization

A factor further complicating the picture in the chemokine system is GPCR homo-and heteromerization. Before the introduction of the concept of receptor homo- or heteromerization, the results of GPCR signaling studies were attributed to the interaction of agonists with receptor monomers [81]. Now, GPCRs homo- and heteromerization have been assessed by a broad range of techniques: from co-immunoprecipitation, complementation, bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), to crystallography [81, 82].

While chemokine receptor homomerization is now widely accepted, the extent to which heteromerization is prevalent, and its functional consequences, remain matter of active research. One striking example of heteromerization effects is the negative modulation of CXCR4 signaling by heteromerization with CXCR7 [83]. Of note, these receptors share the common ligand CXCL12 (see Figure 1). On the functional side, CXCR7 attenuates CXCR4-mediated signals, and this may occur via CXCR4/7 heteromerization.

Potential heteromerization of CXCR7 with CXCR3 (with which it shares the CXCL11 ligand), and possible effects of CXCR7 on CXCR3 signalling have not been reported and remain to be tested. The same holds true for potential heteromerization of CXCR3 and CXCR4 (but see reference 56).

1.4.5 CXCR3 splicing

Alternative splicing is a crucial mechanism for gene regulation. It results from a single gene coding for multiple proteins. Chemokine receptor splicing affects the chemokine system by increasing its diversity.

For example, the *ccr2* gene encodes for two alternatively spliced transcript variants of the receptor: CCR2A and CCR2B. These two variants share common functions and differ only in their terminal carboxyl tails [84]. Both CCR2A and CCR2B mediate inhibition of adenylyl cyclase via coupling to Gαi [85]. Finally, both receptors are present on monocytes and macrophages but CCR2B is the predominant isoform [86].

Another example of splicing is CXCR3. Northern blot analysis revealed that CXCR3 mRNA was alternatively spliced to generate two variants: CXCR3A and CXCR3B [87]. The translation of CXCR3B mRNA generates a receptor containing a longer N-terminal extracellular domain, different from the CXCR3A sequence [87].

The two splice variants of CXCR3 are reported to have opposite functions. CXCR3A promotes cell growth and plays a major role in IFN-γ-inducible immune responses, whereas CXCR3B mediates apoptosis and inhibits cellular proliferation [87, 88]. In addition, the two variants are expressed on different cell types. CXCR3A is mainly expressed on T cells, B cells and NK cells, whereas CXCR3B is mainly expressed on endothelial cells [89]. The expression of variants is not exclusive to one cell type since both CXCR3 isoforms were detected in activated T lymphocytes [87]; CXCR3A being the predominant one [87, 90].

Another variant of CXCR3, named CXCR3-alt, results from alternative splicing via exon skipping [91]. CXCR3-alt has severe structural changes comparatively to CXCR3A but still localizes to the cell surface [91]. CXCR3-alt responds exclusively to CXCL11 [91].

The existence of CXCR3 splice variants is matter of debate. Campanella *et al.* suggested that CXCR3B does not even exist in mice [92]. They argued that an in-frame stop codon would terminate the CXCR3B splice variant in mice. This master's thesis focuses on CXCR3A and its ligands.

1.4.6 CXCL4 as a novel ligand for CXCR3

Platelets are cells involved in the process of blood clotting and act as reservoirs of some chemokines with inflammatory properties. For example, once activated, platelets release CXCL4 (or Platelet factor-4) in micromolar concentrations [93, 94]. For long, CXCL4 was considered as an orphan ligand since it acted as a chemoattractant for leukocytes via an unidentified receptor.

CXCR3 was described as a receptor for CXCL4 [87, 95]. For instance, it has been reported that CXCL4 acts as a chemoattractant on a murine cell line transiently expressing CXCR3A or CXCR3B. It also has been shown that CXCL4 can induce intracellular calcium release and the migration of activated human T lymphocytes [95].

The study of the CXCL4/CXCR3 axis is of a particular importance since it has been suggested that it was involved in T lymphocyte recruitment and the subsequent amplification of inflammation reported in diseases such as atherosclerosis [95]. In addition, studies suggested that platelets are involved in inflammatory phenomena, like bronchial asthma [96]. For instance, the plasma level of CXCL4 of patients suffering from asthma attacks is significantly higher than those of controls [97].

Although the discovery of CXCL4 as a ligand of CXCR3 was a step forward in elucidating the role of CXCL4, pharmacological characterizations of CXCL4 on CXCR3 are still missing. For

example, no publication assessed yet the recruitment of β-arrestin or the activation of Gαi on CXCR3 upon CXCL4 stimulation.

1.4.7 Chemokine gradient remodelling

During chemotaxis, cells that are attracted by chemokines move through the extracellular chemokine concentration gradients. During *in vitro* experiments, the gradient is diffused from a lower well, containing high concentrations of chemokines, to higher wells containing the cells. Therefore, cells are attracted by diffusion of chemokines. However, the chemokine gradient *in vivo* is much more complex.

In vivo maintenance and remodelling of these gradients require chemokine inactivation by extracellular proteases. However, cleavage of chemokine by proteases is another of layer of complexity since cleaved chemokine can bind to unexpected receptors. For instance, ligation of CXCR3 by proteolytically-processed CXCL12 has been implicated especially in brain diseases such as neuro-AIDS [98].

The maintenance of gradients *in vivo* can also happen through chemokine degrading receptors which internalize and, subsequently, degrade chemokines. These receptors, such as CXCR7, are commonly called **decoy** or atypical chemokine receptors (ACKRs) [99]. CXCR7 is atypical in that it does not mediate chemotaxis [100]. Also, unlike the well described CXCR3 and CXCR4 signalling (Gαi signalling followed by β-arrestin recruitment), CXCR7 does not mediate classical G-protein responses. No proximal intracellular CXCR7 signalling was known at all until our group discovered that CXCR7 does recruit β-arrestin in response to CXCL12 [101].

Atypical receptors like CXCR7 reshape gradients by constantly recycling to the cell surface. Until recently, this gradient remodelling was considered exclusive to ACKRs. To date, CCR2 is the only known typical receptor to have decoy properties [102]. However, it has been

reported that signalling chemokine receptors (typical), including CXCR3, could play a role in the clearance of chemokines from circulation and tissues [103].

1.5 Objectives

There are several lines of evidence suggesting a prevalent role of CXCR3 in different diseases. However, clinical trials investigating CXCR3 antagonists were often terminated due to a lack of efficacy [41, 45]. This suggests that the mode of operation of CXCR3 and its ligands is not sufficiently understood and warrants further exploration. There are thus unmet needs for better understanding in many aspects of CXCR3 biology. Theses aspects are illustrated in Figure 4.

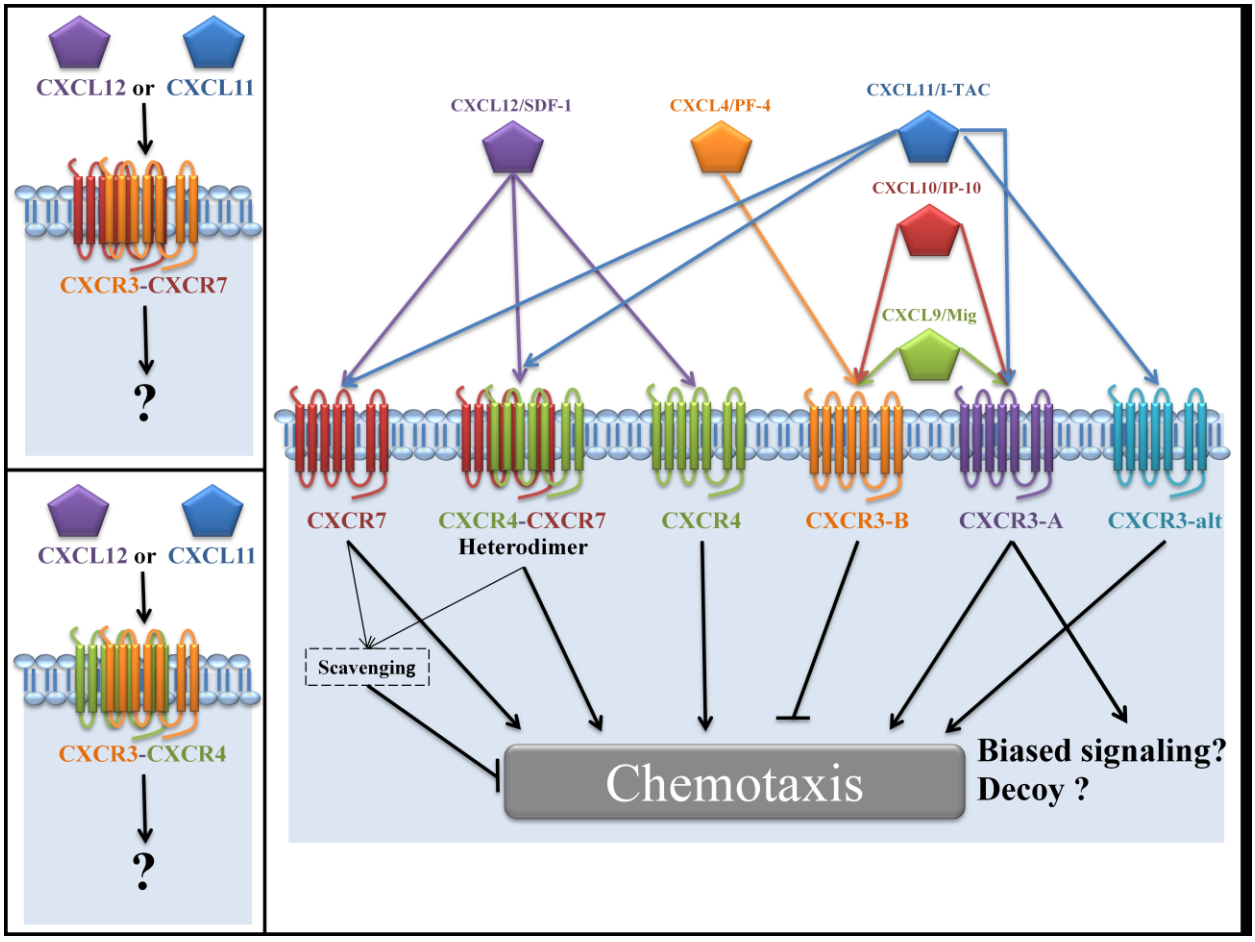

Figure 4. Overall overview of the chemokine system studied in this present work.
Biased agonist, decoy activity of CXCR3 and its heteromerization with CXCR4 or CXCR7 warrant further investigation. Figure inspired from Singh *et al*. [104].

In this present work, we propose to better delineate CXCR3A functions taking new developments such as new CXCR3 ligands, biased agonism, chemokine receptor heteromerization and decoy properties of classical (typical) chemokine receptor into account. We will achieve our goal by pursuing the following specific aims:

i) To test for agonist bias of CXCR3A ligands, including CXCL4 ;

ii) To demonstrate heteromerization of CXCR3A with CXCR4 and CXCR7;

iii) To address the effects of CXCR3A/CXCR4 or CXCR3A/CXCR7 heteromerization on CXCR3 biased signaling ;

iv) To demonstrate the decoy properties of the typical receptor CXCR3A ;

v) To address the effects of CXCR3A/CXCR4 or CXCR3A/CXCR7 heteromerization on CXCR3 decoy properties.

Taken together, this work adds new aspects to our knowledge on the molecular mechanism of CXCR3A function, a recognized potential drug target. Furthermore, this work provides fundamental insight into the organization of chemokine receptor heterodimers and potential mechanism underlying chemokine gradient shaping *in vivo*.

2. Materials and methods

2.1 Plasmids

The expression vectors containing CXCR3, CXCR4 and CXCR7 were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). CXCR3, CXCR4 and CXCR7 receptor sequences were subcloned to yield -YFP and -RLuc fusion proteins as described in *Berchiche et al* and in *Kalatskaya et al*.[101, 105] β-arrestin-RLuc was a generous gift from Dr. Michel Bouvier and the GFP10-EPAC-RLucIII was obtained as described in Leduc *et al*.[106]

2.2 Reagents

CXCR3 ligands (CXCL4, CXCL9, CXCL10 and CXCL11) were purchased from PeproTech (USA). They were dissolved as 100 μM stocks in phosphate buffered saline (PBS) 1 % bovine serum albumin (BSA) and used freshly diluted at the concentration indicated. Radiolabelled ligand (^{125}I-CXCL11) was purchased from Perkin Elmer (USA).

2.3 Cell culture and transfection

Human embryonic kidney (HEK) 293E (passage number 10 to 30) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (WisEnt), 100 units/mL penicillin/streptomycin (Invitrogen) and 200 μg/mL G418 and incubated at 37 ºC, 5% CO_2. Twenty-four hours before transfection, cells were plated at a density of 800,000 cells per well in 6-wells plates. Transient transfections were performed in 6-wells plates using the polyethylenimine method [107].

2.4 BRET measurements

Twenty-four hours post-transfection, transfected cells were plated in 96 wells plates pretreated with poly-D-lysine. Forty-eight hours post-transfection, DMEM media was changed for BRET buffer, containing PBS, 0.1% fetal bovine serum and 0.5 mM $MgCl_2$, at room temperature. Then, coelenterazine H ($BRET_1$) or coelenterazine 400a ($BRET_2$) were added to reach a final concentration of 10 uM. Total fluorescence and luminescence were measured with a Mithras LB940 luminometer (Berthold technologies) as described in Berchiche *et al* [105].

2.5 Gαi activity assays

Inhibition of forskolin-induced cAMP production was measured as previously in Leduc *et al*.[106]. Briefly, HEK293E cells were cotransfected with 1 μg of CXCR3 and 0.04 μg of an intramolecular GFP10-EPAC-RLucIII $BRET^2$ biosensor. Each condition was adjusted to 2 ug of DNA per well with the empty vector pcDNA3. Ten minutes after addition of the $BRET^2$ substrate coelenterazine 400a (Biotium, Hayward, CA), cells were stimulated with the indicated chemokine concentration in the presence or absence of 10 μM forskolin. Then, BRET measurements were performed. Experiments were carried out in triplicate and presented as mean ± SEM.

2.6 Arrestin recruitment assays

β2-Arrestin recruitment assays were conducted as $BRET_1$ experiments and protocols were previously described in Kalatskaya *et al*.[108] Briefly, HEK293E cells were cotransfected with 1 μg of CXCR3A-YFP and 0.05 μg of β2-Arrestin-RLuc. Each condition was adjusted to 2 μg of DNA per well with the empty vector pcDNA3. Cells were stimulated with the indicated chemokine concentration and then incubated for 5 min at 37ºC, 5% CO_2. Ten minutes after addition of the $BRET_1$ substrate coelenterazine H, BRET measurements were performed. BRET values were corrected to Net BRET by subtracting the background BRET signal detected when the β2-Arrestin-RLuc construct was expressed alone. Total fluorescence

and luminescence were used as a relative measure of total expression of the acceptor and donor proteins, respectively. Experiments were carried out in triplicate and presented as mean ± SEM.

2.7 BRET saturation assays

BRET titration experiments were done as described in Mercier et al.[109] Briefly, HEK293E cells were cotransfected with a constant amount of receptor-RLuc (0.03 ug) and increasing concentration of plasmids encoding YFP-tagged receptors, from 0 ug to 1.9 ug. BRET signal was determined by calculating the ratio of the receptor-YFP over the receptor-RLuc emission without ligand stimulation 10 minutes after coelenterazine H addition. Values were corrected to Net BRET by subtracting the background BRET signal detected when the receptor-RLuc construct was expressed alone. Saturation curves were obtained by plotting Net BRET values as a function of the [acceptor]/[donor] ratio. Total fluorescence and luminescence were used as a relative measure of total expression of the acceptor and donor proteins, respectively. Experiments were carried out in triplicate and presented as mean ± SEM. Each plotted point represents a different transfection.

2.8 Degradation assays

CXCR3 decoy properties have be assessed with radio-labelled ^{125}I-CXCL11 degradation assays. HEK293E cells were incubated with DMEM 0.1% BSA 50 pM ^{125}I-CXCL11 (Perkin Elmer) followed by 150 minutes incubation at 37 ºC, 5% CO_2. Then, supernatants were collected and cell surface–bound chemokines were removed by the addition of a 3M glycine solution (pH 2.7). Trichloroacetic acid (TCA) precipitation was used to distinguish between the radioactivity associated with intact chemokine (TCA-precipitable fraction) and degraded chemokine (TCA-non precipitable fraction). The TCA-precipitable fraction was dissolved in 250 µL PBS and collected. The cell layers (cell uptake) were harvested with PBS 1 % SDS. The radioactivity associated with all fractions was measured with a Cobra II gamma counter. Figures are represented either as in percent of the total input of radioactivity, or as the absolute

counts of degraded chemokines. Unlabelled ligand competitions (CXCL4-9-10-11) of ^{125}I-CXCL11 degradation have been tested by adding the indicating unlabelled chemokines. As controls of membrane expression of chemokine receptors, cell membrane staining assays were performed with antibodies coupled to fluorochromes. Data acquisition and analyses by flow cytometry were done on a BD FACSCalibur (BD biosciences).

2.9 Flow cytometry

HEK293E cells were washed once with PBS than stained (30 minutes, 4ºC) with the following antibodies: human (h) CXCR3-Phycoerythrin, hCXCR4-allophycocyanin, hCXCR7-allophycocyanin, murine (m) IgG2A-allophycocyanin, mIgG1-Phycoerythrin (R&D systems). Cells were washed twice and fixed with fixation buffer (PBS 2 % formaldehyde).

2.10 Data analysis

Data from BRET and degradation assays are the mean of independent experiments performed in triplicate and duplicate, respectively. Curve-fitting and statistical analyses were done with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). Statistical significance of the differences between more than two groups was calculated by one-way ANOVA, followed by Bonferroni's post-test.

3. Results

3.1 CXCR3A biased signaling

Although CXCR3A functions seem to be thoroughly described in the literature, few pharmacological characterizations have been done on its three endogenous ligands - CXCL9, CXCL10 and CXCL11. Studying the role of CXCR3A and its ligands on a pharmacological level will undoubtedly provide new insights on the regulation of T cell function [6], but also on every subsets of cells expressing CXCR3A [110-113]. Therefore, we investigated downstream CXCR3A signaling induced by its three ligands using bioluminescence resonance energy transfer (BRET). These assays investigate the activation of the Gαi subunit, and β-Arrestin recruitment to the receptor. Here, β-arrestin recruitment is assessed with $BRET_1$ and Gαi activation is assessed with $BRET_2$. The differences between both assays are explained in Figure 5 and Table 2.

To monitor the Gαi-dependent pathway in living cells, we used a $BRET_2$ version of the EPAC-based BRET sensor, for which the inactive cytosolic mutant form of human EPAC was inserted between GFP10 and RLucIII. This cAMP response assay has been previously described in Jiang *et al* and in Leduc *et al* [106, 114]. Briefly, cells were stimulated with a final concentration of 20 μM forskolin, which activates adenylate cyclase (AC) and leads to an increased concentration of cAMP. Bound to GFP10-EPAC-RLucIII, cAMP induces a conformational change that increases the distance between RLucIII and GFP, leading to a decrease in BRET signal. When stimulated with ligand, the Gα/i-CXCR3A complex inhibits AC. By inhibiting AC, Gα/i-CXCR3A signaling reduces cAMP production. This leads to a reduction of the forskolin-dependent decrease of the BRET signal (increase of BRET signal) (see Figure 6). Increase in BRET signal reflects inhibition of AC (and thus Gai activation), as represented in figure 2. Raw $BRET_2$ ratios have been normalized by using forskolin stimulated results as 0 % of inhibition of AC activity, and by using the non-stimulated (no ligand, no forskolin) results as 100 % of inhibition of AC.

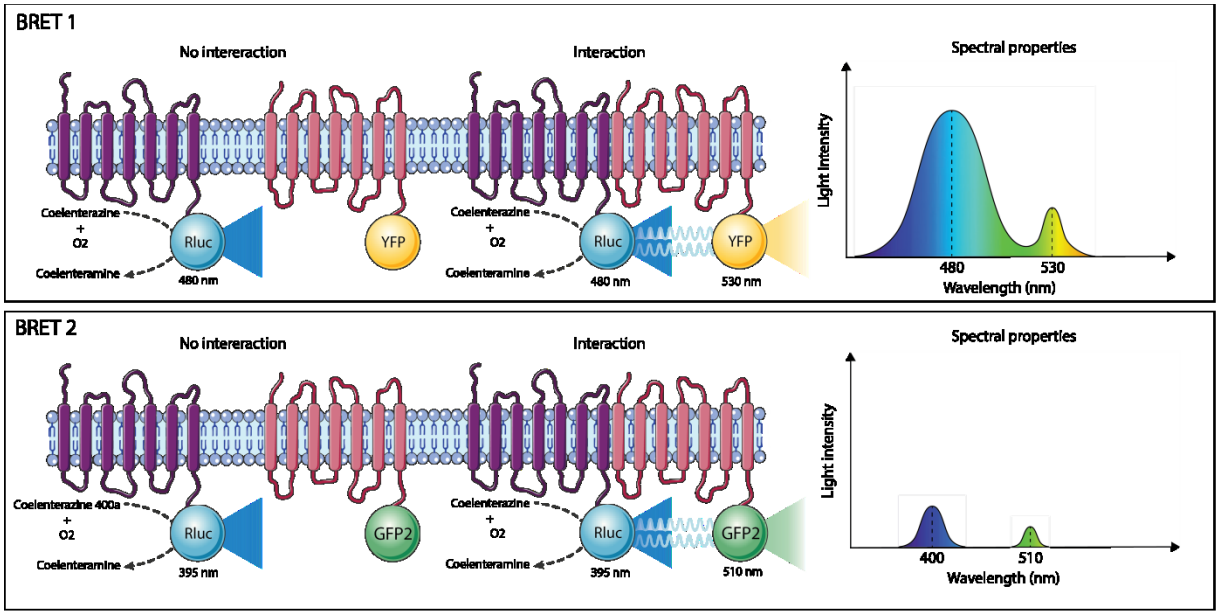

Figure 5. Schematic representation of $BRET_1$ and $BRET_2$ assays

Figure inspired from Institut Cochin website

Table 1. Distinction between $BRET_1$ and $BRET_2$ assays

	$BRET_1$	$BRET_2$
Energy donor	Rluc	Rluc
Energy acceptor	YFP	GFP2
Energy donor substrate	Coelenterazine	Coelenterazine 400a
Peak donor emission	480 nm	400 nm
Peak acceptor reemission	535 nm	510 nm
Advantage	Higher intensity of emitted light	Superior separation of donor and acceptor peaks

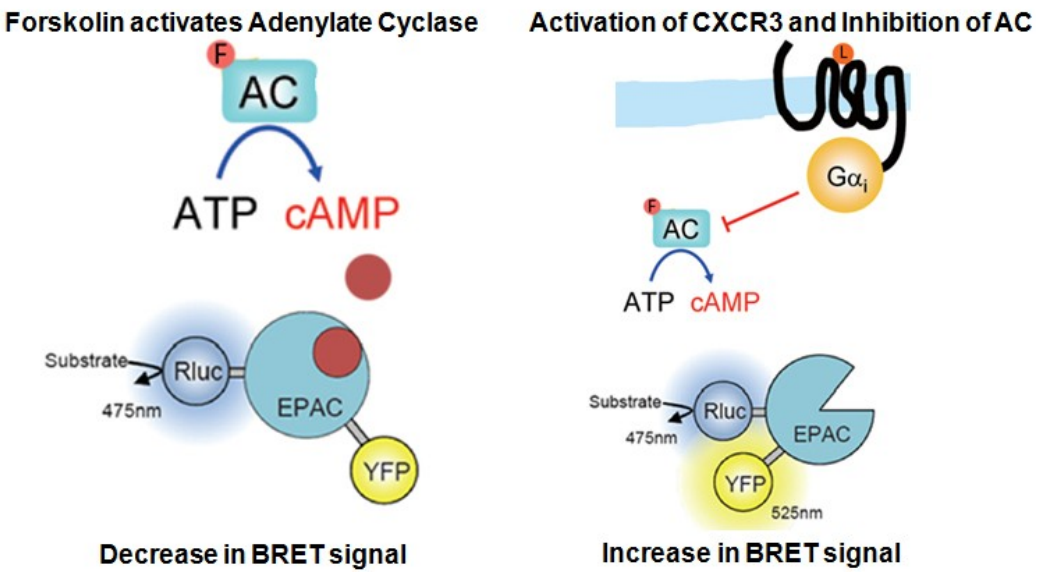

Figure 6. Schematic representation of EPAC BRET$_2$ assays.

After stimulation with forskolin, cAMP level increases in the cells. This leads to a conformational reorganisation of RLuc-EPAC-YFP biosensor. This reorganisation leads to a decrease of the BRET signal due to the increased distance and unfavorable orientation between the energy donor RLuc and the energy acceptor YFP. After ligand stimulation of Gα/i-CXCR3A, cAMP concentration decreases in the cells, leading to a conformational change in RLuc-EPAC-YFP biosensor. This reorganisation leads to an increase in BRET signal due to the reduced distance and favorable conformation between the energy donor RLuc and the energy acceptor YFP. Figures adapted from Salahpour *et al*., *Front Endocrinology*, 2012. [115] (F, Forskolin. AC, Adenylate cyclase. L, Ligand.)

Stimulation of HEK293E cells coexpressing this EPAC biosensor and CXCR3A receptor with its three ligands lead to different concentration-dependent cAMP responses (Figure 7 and Table 2). Out of the three ligands tested, CXCL11 was the most efficient and potent with an EC_{50} of 0.43 nM, leading to 53±2% inhibition of AC activity. Although CXCL10 shows

efficacy, with an EC_{50} of 5 nM, and it shows a smaller efficacy than CXCL11 with 22±1% inhibition of AC activity. As of CXCL9, it shows very little potency with an EC_{50} of 122 nM, but reaches an efficacy of 34±4 % inhibition of AC activity at a concentration of 1uM. Of note, CXCL9 $BRET_{max}$ and EC_{50} were derived from curved fitting. Therefore, the EC_{50} must be interpreted carefully.

To test β-arrestin recruitment to CXCR3A, we used a BRET-based proximity assay system in HEK293E cells transiently coexpressing the BRET donor β-arrestin-2-RLuc and CXCR3A fused to the BRET acceptor YFP. If both proteins, RLuc and YFP, are brought close enough for resonance energy transfer to occur, the bioluminescence energy generated by RLuc can be transferred to YFP, which then emits yellow light [116]. This light is detected as the BRET signal. Background BRET, which is the signal obtained from cells only expressing β-arrestin-2-RLuc, has been deduced from raw BRET. This leads to the plotted Net BRET. Dose-response experiments revealed the following potency rank order of the chemokines: CXCL11 > CXCL10 > CXCL9 (EC_{50} of 19 nM for CXCL11, 32 nM for CXCL10 and 207 nM for CXCL9) (Figure 8 and Table 2). As observed for AC inhibition assays, CXCL9 induced β-arrestin responses but did not reach saturation even at highest chemokine doses, leaving some uncertainty concerning the EC_{50} values, which had to be determined by curve fitting. CXCL11 showed the strongest efficacy and potency compared with CXCL10 and CXCL9. Here, we observed the same pattern of potency as in the cAMP assays; CXCL11 being the most potent and efficient of all three ligands of CXCR3A. Ligands with high affinities for a receptor can sometimes have low efficacies and/or potencies in different pathways. However, it is interesting to mention that our results are congruent with the different affinities of CXCR3 ligands described in Cox *et al.* [117]. The ligand with the highest affinity for CXCR3A (CXCL11) is also the ligand with the highest efficacy and potency [117].

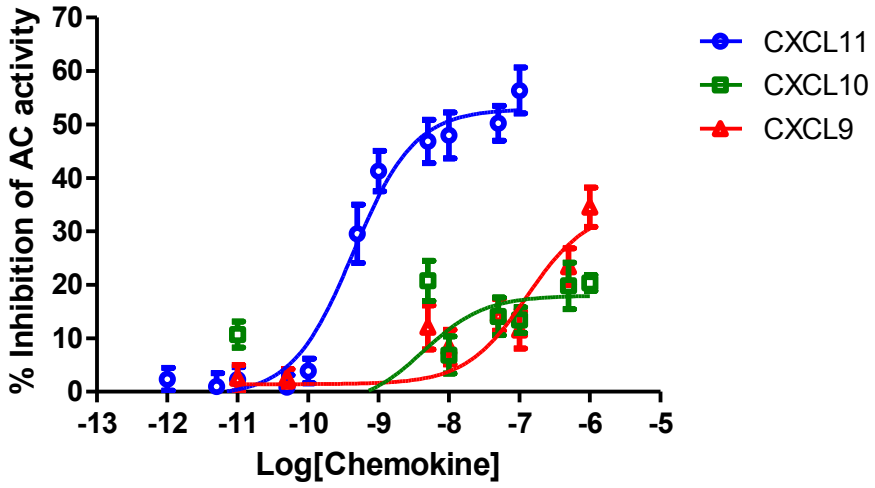

Figure 7. Inhibition of Adenylate cyclase activity followed by stimulation of CXCR3A by chemokine CXCL11, CXCL10 or CXCL9.

HEK293E cells coexpressing CXCR3A and EPAC reporter were incubated with indicated concentrations of ligand and resulting BRET was measured after 10 min at room temperature. **(Blue) ○ CXCL11**, **(Green) □ CXCL10, (Red) Δ CXCL9**. Data are means of three independent experiments performed in triplicate and presented as mean ± S.E.M.

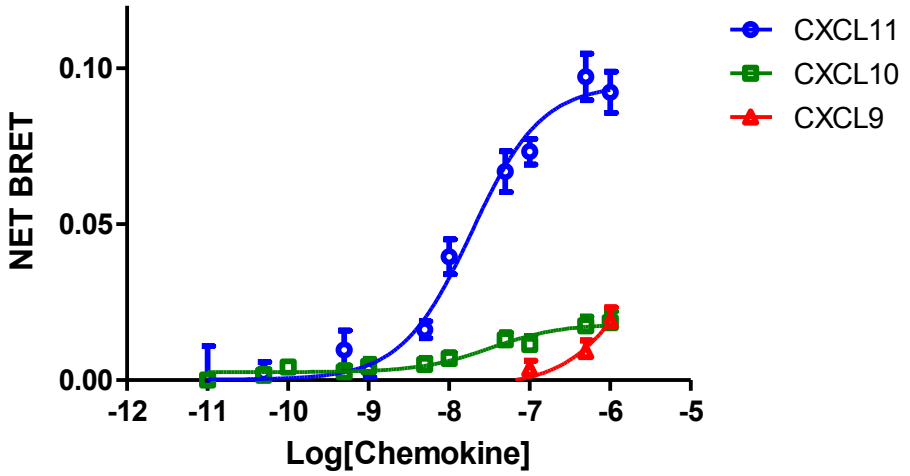

Figure 8. β-Arrestin-2 recruitment to CXCR3A followed by chemokine CXCL11 , CXCL10 or CXCL9 stimulation.

HEK293E cells transiently coexpressing CXCR3A-YFP and β-arrestin2-RLuc were incubated with indicated concentrations of ligand and resulting BRET was measured after 10 min at room temperature. **(Blue) ○ CXCL11**, **(Green) □ CXCL10, (Red) Δ CXCL9**. Data are means of three independent experiments performed in triplicate and presented as mean ± S.E.M.

Table 2. Pharmalogical parameters of CXCR3A biased signalling

	Ligand		
Adenylate cyclase Inhibition	**CXCL9***	**CXCL10**	**CXCL11**
n=3			
EC_{50} (nM)	$1.273e^{-7}$	$5.015e^{-9}$	$4.388e^{-10}$
$LogEC_{50}$	-6.90±0.18	-8.30±0.19	-9.358±0.103
$BRET_{max}$	34.22±3.79	21.69±1.45	52.90±1.897
B-Arrestin Recruitment			
n=3			
EC_{50} (nM)	$2.076e^{-6}$	$3.259e^{-8}$	$1.907e^{-8}$
$LogEC_{50}$	-5.68±0.69	-7.487±0.259	-7.720±0.105
$BRET_{max}$	0.061±0.001	0.018±0.002	0.094±0.004
Affinity (K_i)			
Cox et al. [117]	1.2±0.4 nM	0.033±0.006 nM	0.079±0.027 nM
Heise et al.[118]	45.2 nM	12.5 nM	0.069 nM

*$BRET_{max}$ and EC_{50} derived from curve fitting

3.2 CXCR3A Heterodimerization

CXCR3, CXCR4 and CXCR7 are implicated in many cancers and inflammatory and auto-immune diseases [119]. Previously, CXCR4 has been shown to form heterodimer complexes with CXCR7 [83]. Also, these receptors and their ligands are expressed in tumour microenvironment and on various immune and cancer cells [104]. Finally, because this receptor trio is an attractive target for therapeutic uses, we investigated whether CXCR3 could form heterodimer complexes with CXCR4 or with CXCR7.

We have characterized the relative propensities of CXCR3A to heterodimerize with CXCR4 and CXCR7 using a BRET-based saturation assay as described in Mercier *et al.* [109]. Constant (low) quantities of CXCR3A, CXCR4 or CXCR7 fused to RLuc were cotransfected with increasing quantities of CXCR3A or CXCR7 fused to YFP. The level of energy transfer detected for a given concentration of the RLuc (energy donor) rises with increasing concentration of the YFP (energy acceptor), until all RLuc fused molecules are engaged by a YFP fused molecule [109]. The level of energy transfer rises because more YFP acceptor molecules are expressed in the cells. Background BRET, which is the signal obtained from cells only expressing RLuc-fused receptor, has been deduced from raw BRET. This leads to the plotted Net BRET. The concentration of acceptor yielding 50% of the maximal energy transfer ($BRET_{50}$) can be interpreted as a measure of the relative propensity of two proteins to interact (Figure 9) [109].

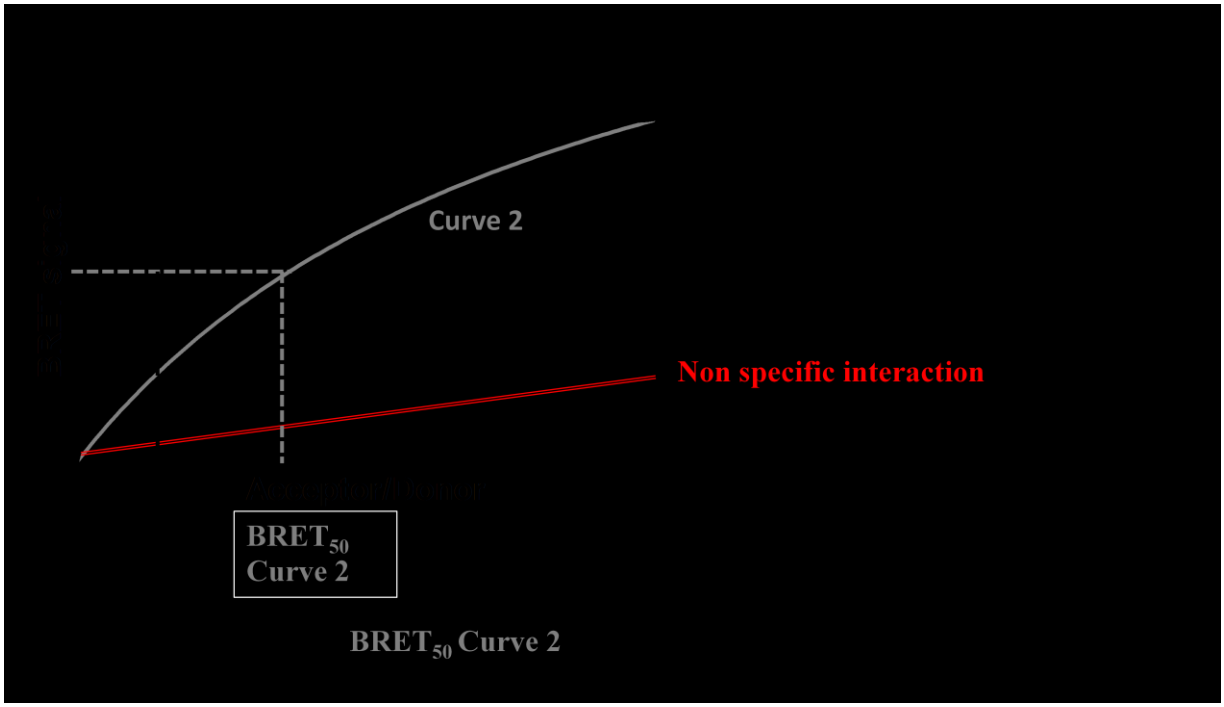

Figure 9. Interpretation of BRET saturation curves

The two proteins interacting in curve 1 **(black)** have a higher affinity towards each other comparatively to the two proteins interacting in curve 2 **(grey)**. The straight line **(red)** represents a non specific interaction between two proteins.

In cells expressing RLuc-fused CXCR4 (Figure 10), CXCR3A (Figure 12) or CXCR7 (Figure 10 and 12), increasing the concentration of CXCR3A receptor tagged with YFP resulted in BRET signals that increased hyperbolically, reaching an asymptote when all RLuc-tagged receptors are associated with those fused to YFP (BRET max). In contrast, we observed non-specific interactions between CXCR3A-RLuc and CXCR7-YFP that led to a linear BRET signal increasing with YFP/RLuc ratios in cells coexpressing the YFP and RLuc-tagged receptors (Figure 9-13). Interestingly, an exchange between the two fusion proteins in the two receptors (CXCR3A and CXCR7) led to an unfavorable proximity or orientation for energy

transfer to occur. These arrangements (CXCR3A-RLuc/CXCR7-YFP) led to a straight linear BRET signal increasing with YFP/RLuc ratios. Constructions known to lead to straight linear BRET signal are often used as negative controls for BRET saturation curves since they represent non specific interaction (Figure 9). That being said, the two first saturation curves presented here lack negative controls. For example, experiments could be reproduced with RLuc-tagged receptor with increasing concentration of YFP alone or any unrelated receptor (for instance, GABA receptor) tagged with YFP.

The $BRET_{50}$, which is an instrument-dependent relative value of the YFP/RLuc ratio when half of the BRET max is reached, represents the propensity of two proteins to interact. A smaller $BRET_{50}$ reflects a greater propensity of two proteins to interact (Figure 9). $BRET_{50}$ values were not significantly different for both heterodimers CXCR3A-CXCR4 and CXCR3A-CXCR7 (Figure 10). However, $BRET_{50}$ values for the CXCR7 homodimer were lower than for the CXCR3A-CXCR7 heterodimer, even though this difference did not reach statistical significance (Figure 14). It is tempting to suggest that this difference reflects the preference of CXCR7 to form homodimers, instead of heteromers with CXCR3A. As for the CXCR3A homodimer, the $BRET_{50}$ values cannot be compared to those of CXCR3A-CXCR7 heterodimer since saturation curves values did not produce a hyperbole (Figure 13). By comparing the calculated $BRET_{50}$, we cannot speculate that CXCR3A also has a propensity for homomerization over heteromerization with CXCR7 (Figure 14). Intriguingly, Watts *et al*. found comparable $BRET_{50}$ values for all CXCR3 and CXCR4 homo- and heterodimers, suggesting that CXCR3 and CXCR4 have comparable propensities to form homo- and heterodimer complexes [80]. In the same order of idea, Levoye *et al.* found comparable $BRET_{50}$ for CXCR4 and CXCR7 homodimers and CXCR4/7 heterodimer [83]. This suggests that heterodimerization of CXCR4 and CXCR7 occurs with the same efficiency as receptor homodimerization. Of note, Levoye *et al.* performed the saturation curves for CXCR4/7 heterodimers with two distinct constructions (CXCR4-RLuc-CXCR7-YFP and CXCR7-RLuc-CXCR4-YFP) [83]. This might explain the difference observed for the published $BRET_{50}$ of CXCR4/7 heterodimers[83]. This will be further discussed in the discussion.

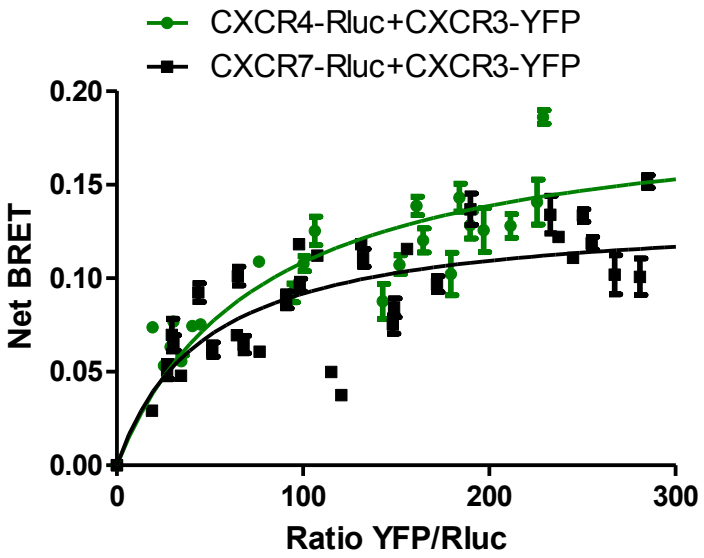

Figure 10. CXCR3A forms heterodimer with CXCR7 and with CXCR4 in HEK293E cells.

Saturation curves were generated in cells expressing a constant amount of CXCR7/CXCR4-RLuc and an increasing amount of CXCR3A-YFP without stimulation. $BRET_{max}$ and $BRET_{50}$ were calculated by nonlinear regression curve fit base on the model of one site total. Saturation curves represent pool data of 3 experiments performed in triplicate and presented as mean ± SEM. Each point represents a different transfection. $BRET_{50}$ of CXCR3/4 is 77±9. B $BRET_{50}$ of CXCR3/7 is 47±9. $BRET_{50}$ of the two heterodimers are not significatively different. **(Black)** ■ CXCR3A-YFP + CXCR7-RLuc, **(Green)** ● CXCR3A-YFP + CXCR4-RLuc.

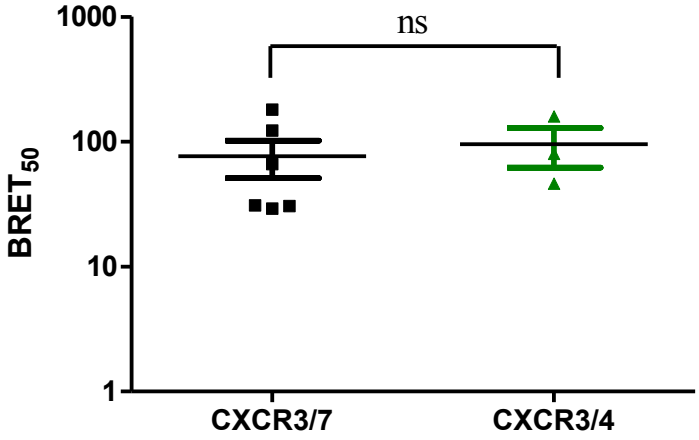

Figure 11. CXCR3 has no preference for heterodimerization with CXCR4 or CXCR7.
$BRET_{50}$ values of chemokine receptor dimerization were extracted from three curves. Saturation curves were generated in cells expressing a constant amount of CXCR7-RLuc or CXCR4-RLuc and an increasing amount of CXCR3A-YFP without ligand stimulation. $BRET_{50}$s were calculated by nonlinear regression curve fit (based on the model of one site total binding). Data are from three to six experiments, each performed in triplicate and presented as mean ±SEM. Unpaired T test was performed and the difference between the $BRET_{50}$ values did not reach significance (p value of 0.6772).

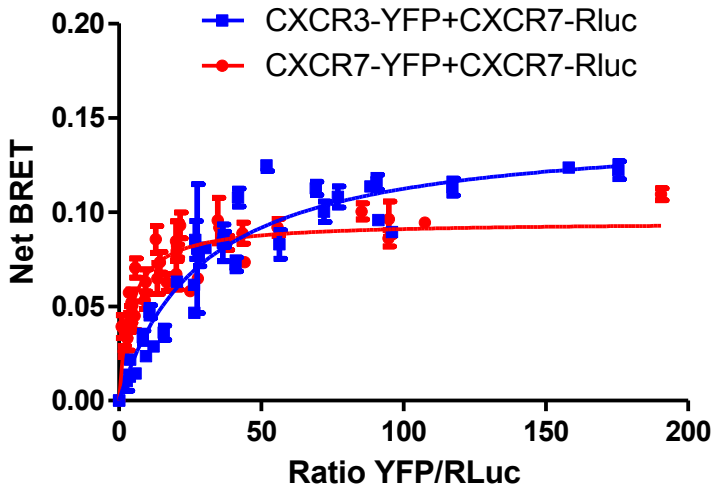

Figure 12. CXCR7 forms homodimers and heterodimers with CXCR3A in HEK293E cells. Saturation curves were generated in cells expressing a constant amount of CXCR7-RLuc and an increasing amount of Receptor-YFP (CXCR3A and CXCR7) without stimulation. $BRET_{max}$ and $BRET_{50}$ were calculated by nonlinear regression curve fit base on the model of one site total. Saturation curves represent pool data of 3 experiments performed in triplicate and presented as mean ± SEM. Each point represents a different transfection. $BRET_{50}$ of CXCR3/7 is 29±4. $BRET_{50}$ of CXCR7/7 is 4.0±0.5. $BRET_{50}$ of homodimer is not significatively lower than the $BRET_{50}$ of the heterodimer. **(Blue)** ■ CXCR3A-YFP + CXCR7-RLuc, **(Red)** ● CXCR7-YFP + CXCR7-RLuc.

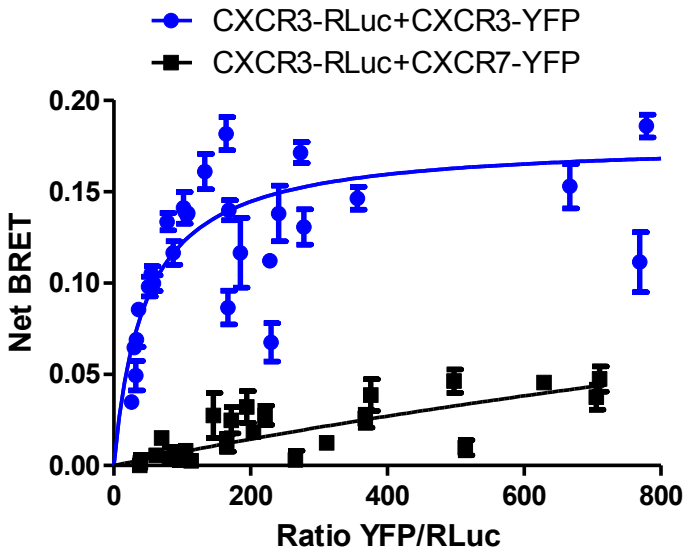

Figure 13. CXCR3 forms homodimers in HEK293E cells.

Saturation curves were generated in cells expressing a constant amount of CXCR3A-RLuc and an increasing amount of Receptor-YFP (CXCR3A and CXCR7) without stimulation. $BRET_{50}$ were calculated by nonlinear regression curve fit base on the model of one site total. Saturation curves represent pool data of 3 experiments performed in triplicate and presented as mean ± SEM. Each point represents a different transfection. $BRET_{50}$ of CXCR3/7 is 29±4. $BRET_{50}$ of CXCR7/7 is 4.0±0.5. **(Blue)** ■ CXCR3A-RLuc + CXCR3-YFP, **(Black)** ● CXCR3-RLuc + CXCR7-YFP

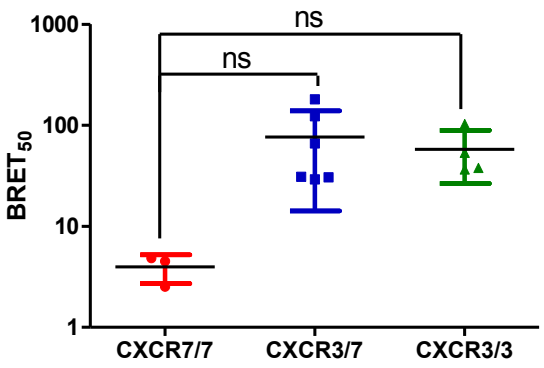

Figure 14. CXCR7 tendency for homodimerization.

$BRET_{50}$ values of chemokine receptor dimerization were extracted from three to six curves. Saturation curves were generated in cells expressing a constant amount of CXCR3A-RLuc or CXCR7-RLuc and an increasing amount of Receptor-YFP (CXCR3A and CXCR7) without stimulation. $BRET_{50}$ were calculated by nonlinear regression curve fit base on the model of one site total. Data are from three to six experiments performed in triplicate and presented as mean ±SEM. One way ANOVA with Bonferroni's multiple comparison tests were performed and $BRET_{50}$ values did not reach significance.

3.3 CXCR3A Decoy properties

Some chemokine receptors, commonly called decoy receptor, act as modulators of specific chemokine gradients by degrading or remodeling gradients involved in the biological process of chemotaxis. It is known that CXCR3A acts as a conventional chemotaxis receptor (typical receptor) via their interaction with CXCL9, CXCL10 and CXCL11. Considering that the typical chemokine receptor CCR2 also acts as a decoy receptor [102], we wondered whether CXCR3A had similar decoy proprieties as CCR2. This is of particular relevance given that chemokine degradation plays an important role in regulation of chemotaxis and termination of inflammatory states.

CXCR3 decoy properties have been assessed using radiolabelled ^{125}I-CXCL11 in chemokine degradation assays. HEK293E cells were incubated with 50 pM radiolabelled chemokines. After two hours of incubation, supernatants were precipitated with TCA. Proteins degraded or cleaved do not undergo TCA precipitation, while undegraded chemokines precipitate. Cell-associated radioactivity, TCA precipitated (pellet) and unprecipitated chemokine degradation products (supernatant) was measured with a Cobra II gamma counter. Results from degradation assays are presented as the percentage of total input of ^{125}I-CXCL11 (Figures 15, 16, 18, 19, 21, 26). For ligand competition with radio-labelled chemokines, 100 nM of the indicated unlabelled chemokines were added. As controls for chemokine receptor expression at the plasma membrane, cell surface staining assays were performed with antibodies coupled to fluorochromes and analyzed by flow cytometry (Figures 17, 20, 23).

HEK293E cells transiently overexpressing CXCR3A are capable of higher CXCL11 scavenging compared to HEK293E cells transfected with an empty vector, plotted as control (Figure 15). Of note, HEK293E cells constitutively express a high level of CXCR3. This endogenous expression could account for the basal degradation of CXCL11 we observed. It has been reported that CXCR7 is capable of CXCL11 and CXCL12 scavenging [120], therefore we used CXCR7 as a positive control for ^{125}I-CXCL11 scavenging. We observed between 35 to 40 % of ^{125}I-CXCL11 total input associated with the CXCR3A expressing cells;

such uptake was between 25 to 30% for CXCR7 expressing cells. In addition, we observed that 35% of ^{125}I-CXCL11 was degraded when cells expressed CXCR7, and between 20 and 25% for CXCR3A expressing cells (Figures 13 and 14). As expected, addition of unlabelled CXCL12 interferes with CXCL11 degradation by CXCR7, but not by CXCR3A (Figures 15 and 16).

We also investigated whether unlabelled ligand could impede radiolabelled ligand degradation (Figures 18 and 19). After adding 100 nM of the different ligand of CXCR3A, we observed that only unlabelled CXCL11, but not unlabelled CXCL9 or CXCL10, lowered ^{125}I-CXCL11 degradation via CXCR3A. As proposed in Cox et al. [117], these results suggest differential binding properties of CXCR3 ligands. It was also suggested that CXCR3 ligands are have allotopic properties. This implies that these three ligands bind to distinct, non-overlapping, recognition sites on CXCR3.

We then investigated the effect of coexpression of CXCR3A and CXCR4 on radiolabelled CXCL11 degradation. As expected, cells expressing CXCR4 do not show decoy properties (Figures 21 and 22). Furthermore, we did see a significant effect on CXCL11 scavenging when both receptors are co-expressed and incubated with CXCL12. This could suggest an effect on CXCL11 scavenging by CXCR3A via the CXCR4/CXCL12 axis. However, the difference in degraded ^{125}I-CXCL11 between CXCR3A and CXCR3A/CXCR4 transfected cells (Figure 22) may alternatively simply result from different CXCR3A expression levels. Indeed, in flow cytometry expression experiments, a reduction of CXCR3A in co-transfected cells is seen, compared to single receptor-transfected cells (Figure 23). The difference in receptor surface expression may be explained by the saturation of the transcription and translation machineries of the cells, which were transfected with double the quantity of DNA coding for receptors (2 μg of receptor instead of 1μg of receptor complemented with 1 μg of empty vector). Finally, as expected, CXCR4 has no CXCL11 scavenging properties. We conclude that decoy CXCR3A is not affected by the presence of the CXCR4/CXCL12 axis.

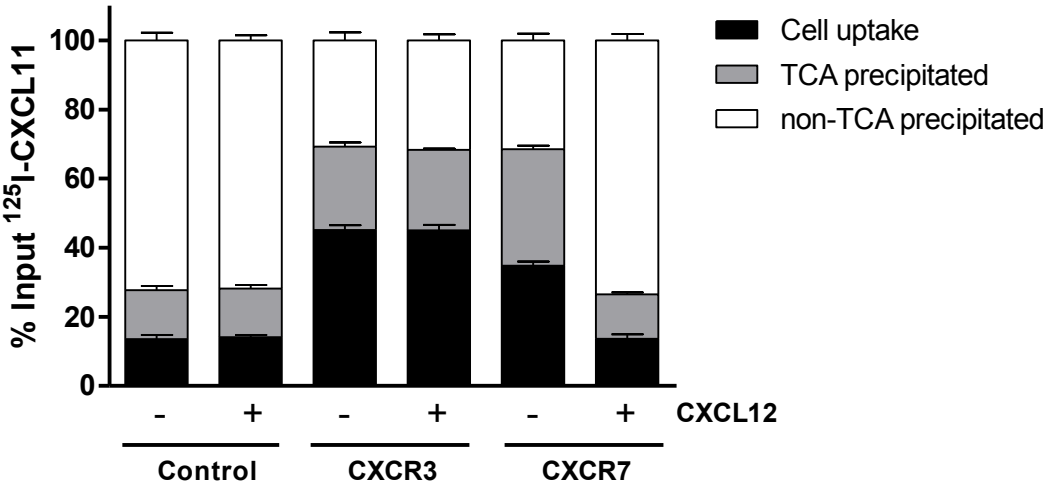

Figure 15. The typical chemokine receptor CXCR3 acts as scavenger for CXCL11.

HEK293E cells transiently expressing pcDNA3 (control), CXCR3A or CXCR7 were incubated 2h30 with 50 pM ^{125}I-CXCL11with or without 100 nM CXCL12. After incubation, cells were spun out, and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as the percentage of the total input of radioactivity. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM. (Black) cell uptake, (Grey) TCA precipitated, (White) non-TCA precipitated.

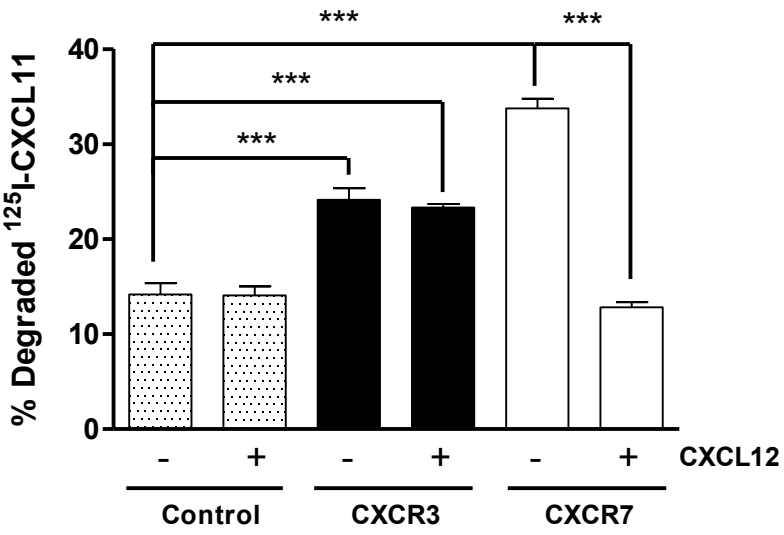

Figure 16. CXCL11 is degraded by CXCR3 and CXCR7.

^{125}I-CXCL11 scavenging via CXCR3 is significantly higher than control condition (empty vector without 100 μM CXCL12) (p value < 0.001). ^{125}I-CXCL11 scavenging via CXCR7 without CXCL12 reached significance compared to the condition with 100 μM CXCL12 (p value < 0.001). One way ANOVA tests with Bonferroni's multiple comparison tests were performed. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

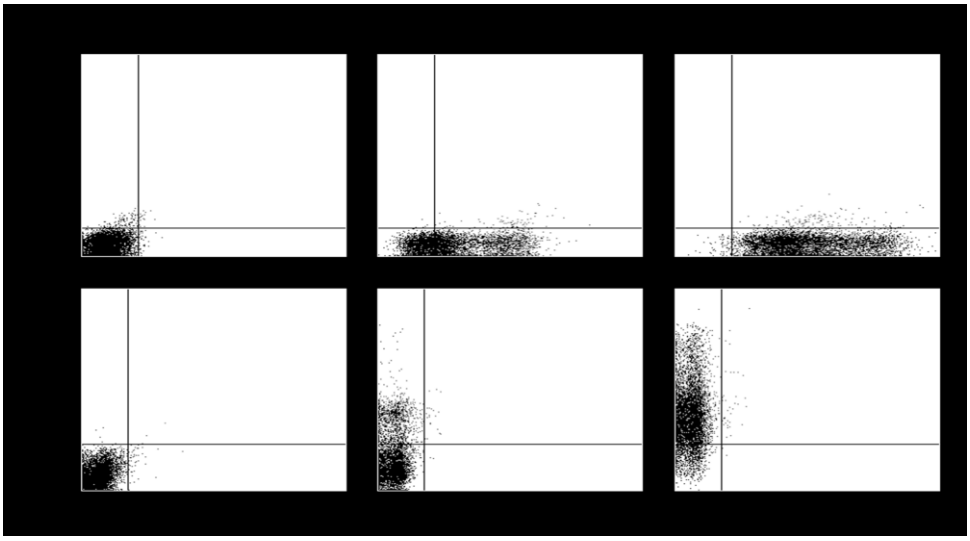

Figure 17. Membrane expression of CXCR3A and CXCR7.

Flow cytometry analysis of membrane expression of CXCR3A and CXCR7 in HEK293 cells. Upper left, non specific staining with IgG1- Phycoerythrin (isotype control); Upper Middle, endogenous expression stained with CXCR3A-Phycoerythrin. Upper right, transfected expression stained with CXCR3A-Phycoerythrin. Lower left nonspecific stain IgG2a-Allophycocyanin, Lower Middle endogenous expression stained with CXCR7-Allophycocyanin. Lower right, transfected expression stained with CXCR7-Allophycocyanin. Data show one representative out of three experiments.

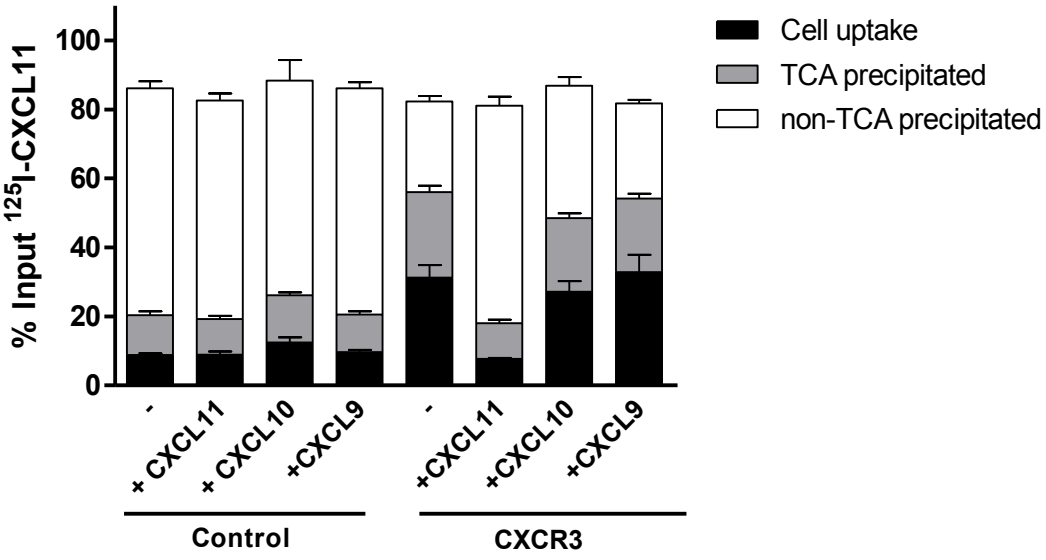

Figure 18. Decoy properties of chemokine receptors CXCR3A in the presence or absence of other ligands.

HEK293E cells transiently transfected with pcDNA3 (control) or CXCR3A were incubated 2h30 with 50 pM ^{125}I-CXCL11 in the presence or absence of 100 nM CXCL11, CXCL10, or CXCL9. After incubation, cells were spun out, and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as a percentage of the total input of radioactivity. Samples of each condition were done in duplicate. (Black) cell uptake, (Grey) TCA precipitated, (White) non-TCA precipitated. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

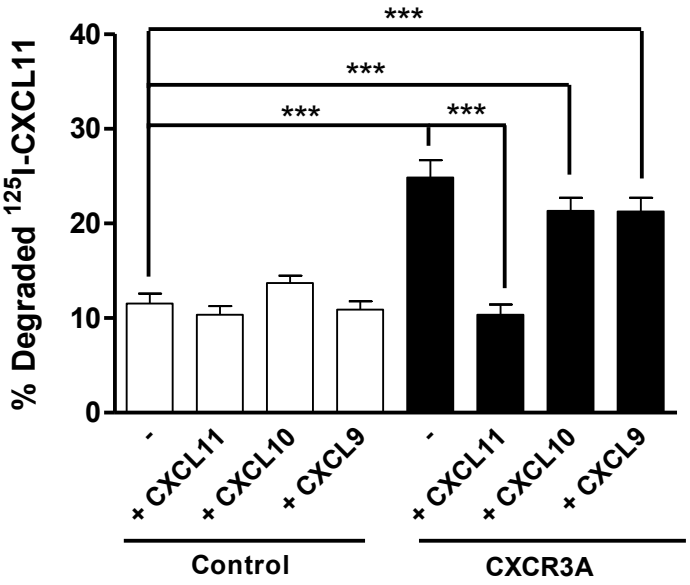

Figure 19. CXCR3A ligands have allotopic properties.

^{125}I-CXCL11 scavenging via CXCR3 is significantly higher than control condition (empty vector without chemokine) (p value < 0.001). ^{125}I-CXCL11 scavenging via CXCR3 with 100 nM CXCL10 or CXCL9 is significantly higher than control condition (p value < 0.001).). ^{125}I-CXCL11 scavenging via CXCR3 is significantly reduced in presence of 100 µM CXCL11. One way ANOVA tests with Bonferroni's multiple comparison tests were performed. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

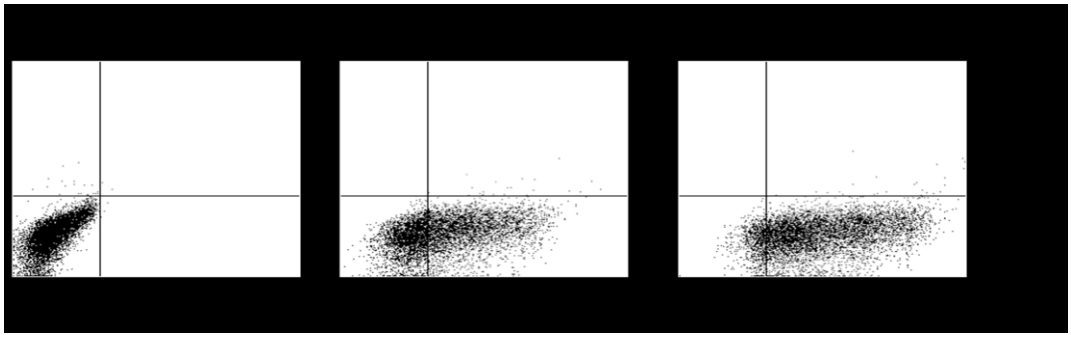

Figure 20. Membrane expression of CXCR3A.

Flow cytometry analysis of membrane expression of CXCR3A in HEK293 cells Left, IgG1-Phycoerythrin. Middle, non-transfected labelled with CXCR3A-Phycoerythrin. Right, transfected and labelled with CXCR3A-Phycoerythrin.

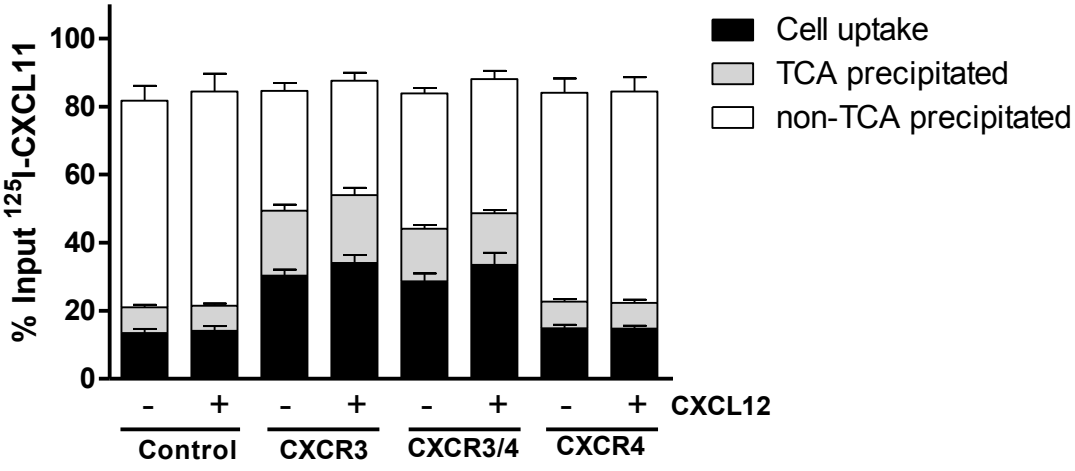

Figure 21. Decoy properties of chemokine receptors CXCR3A, CXCR4 and the CXCR3A/CXCR4 heterodimer in the presence or absence of CXCL12.

HEK293E cells transiently expressing pcDNA3, CXCR3A, CXCR4 or CXCR3A+CXCR4 were incubated 2h30 with 50 pM ^{125}I-CXCL11 in the presence or absence of 100 nM CXCL12. After incubation, cells were spun out, and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as the percentage of the total input of radioactivity. Samples of each condition were done in duplicate. (Black) cell uptake, (Grey) TCA precipitated, (White) non-TCA precipitated. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

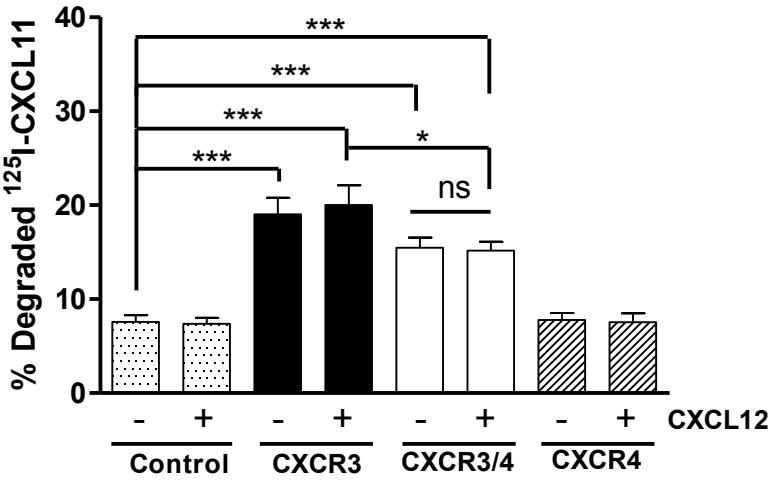

Figure 22. CXCL12 does not affect CXCL11 scavenging neither by CXCR3A nor CXCR3A/4.

^{125}I-CXCL11 scavenging via CXCR3 with or without 100 μM CXCL12 is significantly higher than control condition (empty vector without 100 μM CXCL12) (P value < 0.001). ^{I125}-CXCL11 scavenging via CXCR3/4 heterodimer in presence or absence of 100 uM CXCL12 is significantly higher than control condition (P value < 0.001). ^{125}I-CXCL11 scavenging via CXCR3 is significantly higher than via the CXCR3/4 heterodimer in presence of 100 μM CXCL12 (P value < 0.001). One way ANOVA tests with Bonferroni's multiple comparison tests were performed. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

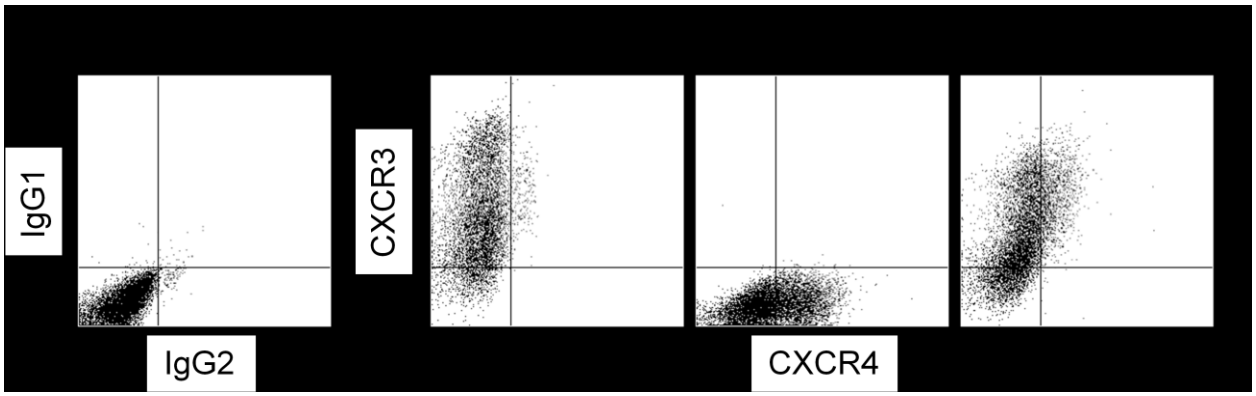

Figure 23. Membrane expression of CXCR3A, CXCR4 and CXCR3A/4.

Flow cytometry analysis of membrane expression of CXCR3A and CXCR4 in HEK293 cells Left, IgG1-Phycoerythrin and IgG2-Allophycocyanin. Right, transfected cells labelled with CXCR3A-Phycoerythrin, CXCR4-Allophycocyanin and CXCR3A-Phycoerythrin+CXCR4-Allophycocyanin.

3.4 Effects of CXCL4 on CXCR3A

Platelets play a role in hemostasis, but also participate in the inductive phase of inflammatory responses [121]. CXCL4 was long time considered as an orphan ligand. Only recently, it has been found to bind CXCR3A with a low affinity (order of μM) and its role is still not well understood [87]. CXCL4 is a platelet-associated chemokine that modulates tumor angiogenesis, inflammation within the tumor microenvironment, and turn tumor growth [122]. Released at micromolar concentrations upon platelet activation, CXCL4 represent the most abundant protein contained within platelet α-granules [123, 124]. Considering that CXCL4 is released in high concentration, we hypothesized that CXCL4 might block chemotaxis via CXCR3A. The effect of synthetic ligand on CXCL4 binding to CXCR3A remains unexplored, as do the functional consequences of CXCL4 binding to CXCR3A. This is potentially of high importance given that plasma CXCL4 levels are increased in acute asthma attacks [97].

Therefore, to establish a better understanding of CXCL4-CXCR3A signalling, we investigated downstream CXCR3A signaling induced by CXCL4 using BRET assays. These assays investigate AMPc concentration and β-Arrestin recruitment. We also investigated whether CXCL4 could block degradation of radiolabelled CXCL11 by CXCR3A.

Out of our BRET assays, we cannot conclude that CXCL4 activates the Gαi subunit (inhibiting adenylate cyclase) (Figure 24), nor recruit β-arrestin (Figure 25). The EC_{50} value for CXCL11 was approximately 1.8 nM, which is a little bit smaller than the value obtained previously (Table 1), but still is in the single digit nanomolar. In addition, we stimulated cells transfected with an empty vector with 100 nM of chemokine and did not observe an significant increase in inhibition of adenylate cyclase, showing that what we observed due to overexpression of CXCR3A alone (Figure 24). Concerning the chemokine degradation assay, a massive concentration of CXCL4 (1 μM) was still not able to compete for radiolabelled CXCL11 (Figures 26 and 27).

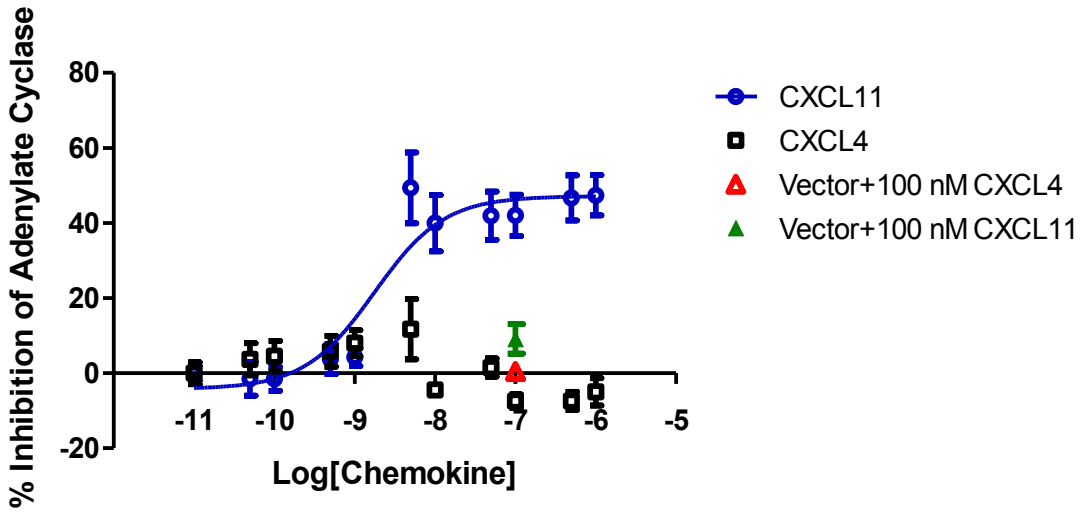

Figure 24. Inhibition of adenylate cyclase following stimulation of CXCR3A by chemokine CXCL11 or CXCL4.

HEK293E cells coexpressing CXCR3A and the EPAC reporter were incubated with indicated concentrations of ligand and resulting BRET measured after 10 min at room temperature. **(Blue) ○ CXCL11**, **(Black) □ CXCL4**, **(Red) Δ vector + 100 nM CXCL4**, **(Green) Δ vector + 100 nM CXCL11**. Data are means of three independent experiments performed in triplicate and presented as mean ± S.E.M.

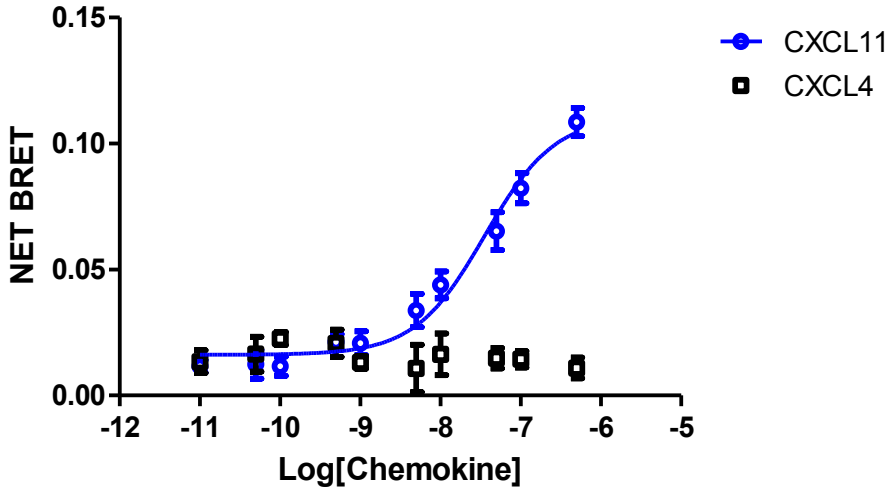

Figure 25. β-Arrestin-2 recruitment to CXCR3A followed by chemokine CXCL11 or CXCL4 stimulation.

HEK293E cells transiently coexpressing CXCR3A-YFP and β-arrestin2-RLuc were incubated with indicated concentrations of ligand and resulting BRET measured after 10 min at room temperature. **(Blue) ○ CXCL11**, **(Black) □ CXCL4**. Data are means of two independent experiments performed in triplicate and presented as mean ± S.E.M

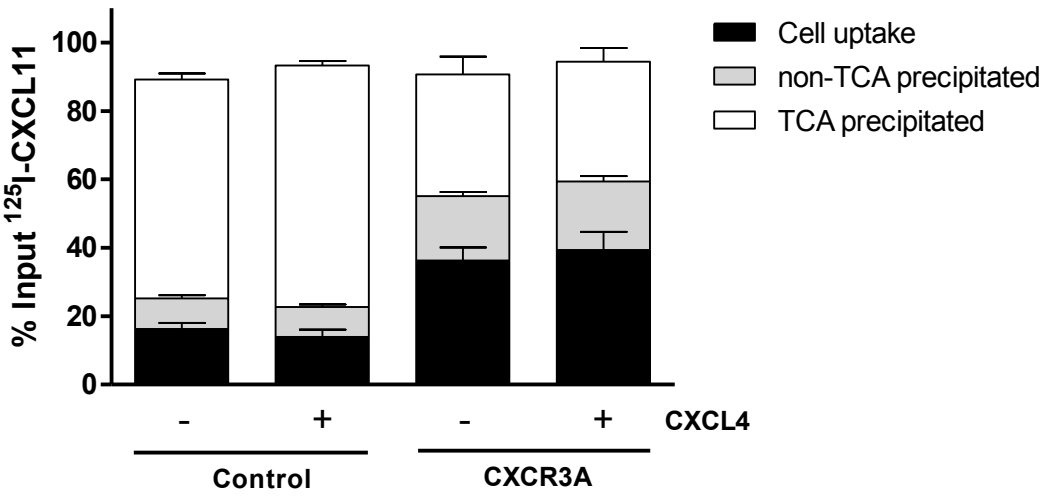

Figure 26. Decoy properties of chemokine receptor CXCR3A in the presence or absence CXCL4.

HEK293E cells transiently expressing pcDNA3 or CXCR3A were incubated 2 h 30 with 50pM ^{125}I-CXCL11 in the presence or absence of 1 μM CXCL4. After incubation, cells were spun out, and the supernatant was subjected to TCA precipitation. Radioactivity associated with the TCA pellet, the non-TCA precipitable fraction, and the cells themselves was counted and is presented as a percentage of the total input of radioactivity. Samples of each condition were done in duplicate. (Black) cell uptake, (Grey) TCA precipitated, (White) non-TCA precipitated. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

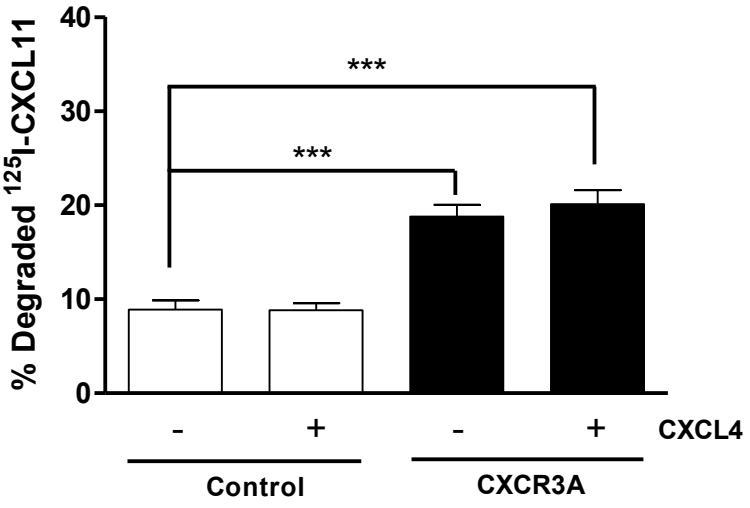

Figure 27. High concentration of CXCL4 does not block CXCL11 scavenging by CXCR3A.

^{125}I-CXCL11 scavenging via CXCR3 with or without 1 μM CXCL4 is significantly higher than control condition (empty vector without chemokine) (p value < 0.001). One way ANOVA tests with Bonferroni's multiple comparison tests were performed. Pooled data from 3 experiments performed in duplicate and presented as mean ± SEM.

4. Discussion

Since our work investigated different aspects of chemokine receptor biology, we decided to discuss these different aspects separately. We are first going to discuss CXCR3A biased signaling, followed by the concept of heteromerization and its importance in chemokine receptor biology. The potential physiological role and relevance of CXCR3A decoy properties will then be covered and finally, we will close the discussion with CXCL4 as a novel ligand for CXCR3A.

4.1 CXCR3A biased signaling

Over the past few years, it became clear that GPCRs are able to activate many different signaling pathways. It has also been demonstrated that different ligands of a same receptor may have different potencies and efficacies for different pathways. This paradigm, called biased agonism, has first been described as a consequence of GPCR complexity and was considered as a property of synthetic drugs. This phenomenon has been studied in various systems such as the serotonin, opioid, dopamine, vasopressin, and adrenergic receptor systems [125-128]. However, whether biased signalling was a property inherent to endogenous systems was still unknown. For long, the different endogenous ligands of a same receptor, like CXCL9, CXCL10 and CXCL11 for CXCR3, were seen as redundant. Biased signaling has extensively been addressed with synthetic drugs, but cannot be investigated in many endogenous systems. Because of the promiscuity between ligands and their receptors, the chemokine and chemokine receptors represent a unique endogenous system to study biased signaling. Now, an increasing number of studies report biased signaling as a mechanism used by chemokine receptors for the fine-tuning of different response associated with different ligands [64, 75, 129, 130].

In the present study, we have addressed whether the different ligands of CXCR3A were biased towards different signalisation pathways (Gαi vs β-arrestin). Compared to the full agonist CXCL11, we found that CXCL10 and CXCL9 were partial agonists with reduced efficacy

and/or potency. We showed that despite the fact that CXCL10 is more potent than CXCL9, CXCL9 is more efficacious than CXCL10. However, we did not observe ligand rank order shifts in between our BRET assays, contrary to what was previously described for other chemokine receptors such as CCR10 [64]. Consistent with others studies investigating calcium mobilization, GTPγ^{35}S binding assays and migration assays [79, 95, 117, 118], we report the following potency rank order: CXCL11 > CXCL10 > CXCL9. We also report the following efficacy rank order CXCL11 > CXCL9 > CXCL10. Hence, we observe a difference between potency and efficacy rank orders in both assays (Gαi signaling and β-arrestin recruitment).

While the present work was under way, another group also investigated the different responses of CXCR3A to its ligands [64]. Using enzyme fragment complementation assays, they showed CXCL11 was the most potent and efficacious ligand in Gαi signalling and β-arrestin recruitment [64]. Consistent with their results, we did observe CXCL11 as the most potent and efficient ligand. Moreover, our results concur with their potency rank order for Gαi signalling and β-arrestin recruitment (CXCL11 > CXCL10 > CXCL9) (Figures 5 and 6, Table 1) [64]. Interestingly, we also observed a different efficacy rank order in both readouts (CXCL11 > CXCL9 > CXCL10) (Figures 7 and 8, Table 2) [64]. Rajagopal *et al.* stated that a subtle form of biased agonism was present because their relative ligand efficacies (compared to the maximal response) changed for different signalling pathways [64]. We also observed such a difference in efficacies in between ligands. However, the classical view of biased signalling involves a rearrangement in rank order of ligand efficacy for different assays. Therefore, we cannot conclude we observed biased agonism since we do not observe any efficacy rank order shift in between our BRET assays (see EC_{50} in Table 2).

Compared to CXCL11, we concluded that CXCL9 and CXCL10 were partial agonists on both BRET readouts. Partial agonists are ligands that activate a given receptor but have only partial efficacy relative to a full agonist. Comparatively to the full agonist CXCL11, we claim that CXCL9 and CXCL10 are partial agonists on CXCR3A. It has been reported that CXCL11 has the highest affinity, followed by CXCL10 and CXCL9 [117]. This may in part explain our

potency rank order. The difference in signalling efficacy may be explained by the receptor-ligand interactions in which different ligands stabilize different receptor conformations. It has been reported that CXCR3A ligands bind differentially to their receptor [117]. Therefore, it is plausible to suggest that CXCR3A adopts different conformations depending on the bound ligand. The different conformations adopted by the receptor could imply different potencies and efficacies to transduce signal to downstream effectors, like Gαi subunit or β-arrestin.

Even if we did not observe an efficacy rank order shift, our results are compatible with the concept of functional selectivity: different ligands can stabilize different receptor conformations leading to different efficacies on various downstream signalling pathways [131]. In the case of our β-arrestin recruitment assays, BRET signals depend on orientation and distance between the two proteins of interest. Therefore, we could speculate that the orientation and the distance between the BRET donor and acceptor were not favorable upon CXCL9 and CXCL10 binding to CXCR3A in this specific assay. In other words, CXCR3A-CXCL10 or CXCR3A-CXCL9 complexes might not be stabilized in a favorable way for energy transfer to occur between β-arrestin and the receptor. This may in part explain the observed difference in efficacy in between ligands. To support this explanation, it has been shown that CXCL10 and CXCL11 have differential binding to CXCR3A receptor states, depending if it is coupled or uncoupled to G protein [117]. CXCL10 and CXCL11 also have disparate binding sites [70], which strongly suggest they are allotopic ligands of CXCR3A.

In light of our results, further investigation the CXCR3 biased signaling is required. Chemokine receptors are best described as coupled to Gαi subunit. However, chemokine receptors are also able of Gαq coupling, leading to PLC activation and an increase of intracellular Ca^{2+} upon receptor activation [132]. In fact, it has been demonstrated that CXCR3 was able to activate PLC once stimulated with CXCL10 or CXCL11 [133]. Considering this, it would be interesting to investigate CXCR3A biased signaling with the intracellular calcium biosensor obeline in order to validate previously published data about CXCR3A calcium mobilization and PLC activation [79, 133, 134]. We could expect the following potency rank order for PLC activation: CXCL11 > CXCL10 > CXCL9. Furthermore, no studies have yet

investigated biased signalling of CXCR3B. The difference between splice variants could be considered as a logical continuation of our investigation of CXCR3 biased signalling. Nevertheless, our results suggest that CXCR3A ligands should not be considered redundant *in vivo* since CXCL9 and CXCL10 are partial agonists: they do not quantitatively induce the same signalling as CXCL11.

4.2 CXCR3 homo- and heteromerization

Chemokine receptors are potential drug targets considering their role in autoimmune and inflammatory diseases [15, 135]. Unfortunately for drug development, chemokine receptors can form heterodimers or higher order oligomer complexes. In addition, few approaches can study GPCR high order oligomers. For instance, a sequential three-color FRET was used to study oligomeric complexes of the α_{1b}-adrenoceptor [136]. Lopez-Gimenez *et al.* showed that the α_{1b}-adrenoceptor was able to form high order oligomers and that disruption of effective oligomerization had profound consequences on cell surface expression and function [136].

Homo/hetero/oligomerization adds another level of complexity, as these structures may be harder to target individually and, thus, specifically. Initially, the concept of receptor heterodimerization was not as consensual as it is today. In fact, the first widely accepted evidence that GPCR heterodimerization could play an important role in GPCR signaling came from the $GABA_B$ receptors in the 1990's [137]. The relevance of receptor dimerization for their signaling is now a widely accepted. Also, this concept is studied in multiple models such as $GABA_B$, taste, adrenergic, opiod, somatostatin, purinergic, and chemokine receptors [137, 138].

CXCR3 and CXCR7 share a common ligand, CXCL11. CXCR3 and CXCR4 are co-expressed on activated T-cells, natural killer cells, dendritic cells and cancer cells [139, 140]. Considering this, we hypothesized that CXCR3 might form heterodimers with CXCR4 or with CXCR7. We also hypothesized that CXCR3 heterodimerization with CXCR4 or CXCR7 would modulate its signaling. A good example of GPCR heteromerization affecting chemokine receptor signalisation was described by Levoye *et al*. [83]. They reported that CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling [83].

Here, using BRET titration saturation assays, we demonstrated that CXCR3 could form homo- and heterodimer complexes with CXCR4 and CXCR7. Although this did not reach statistical

significance, the $BRET_{50}$ values of CXCR7/7 homodimer was lower than CXCR3/7 heterodimer and CXCR3/3 homodimer. If $BRET_{50}$ values were different, we could conclude that CXCR7 prefers to form homodimer complexes. The population of heterodimers would then be marginal. In some cases, GPCR heteromerization has been shown to be essential for the formation of a functional receptor [141]. For instance, the heterodimerization of γ-aminobutyric acid receptors ($GABA_{B1}$ and $GABA_{B2}$) is essential in the formation of a functional $GABA_B$ receptor [142]. Other receptors, like dopamine receptors, have different relative affinities to form homo- and heterodimers. Indeed, it has been showed that dopamine receptors D4R form oligomers with different affinities with other dopamine receptors [143]. However, this preference for homodimerization over heterodimerization has never been described for chemokine receptors. The similarity in our $BRET_{50}$ values between homo- and heterodimers is in accordance with *Levoye et al.* [83]. They showed similar $BRET_{50}$ values for CXCR7 and CXCR4 homodimers versus CXCR4/CXCR7 heterodimer using BRET titration saturation assays [83]. While the present work was under way, Watts *et al.* published similar results assessing heterodimerization between CXCR3/CXCR4 using co-immunoprecipitation, time-resolved fluorescence resonance energy transfer, saturation BRET and GPCR-heteromer identification technology approaches [80]. In that study, they found comparable $BRET_{50}$ values for all CXCR3 and CXCR4 homo- and heterodimer combinations [80]. They also found evidence for specific β-arrestin2 recruitment to CXCR3-CXCR4 heteromers in response to agonist stimulation [80]. The impact of heteromerization on β-arrestin recruitment or G protein activation has not been studied in the present work.

We are now going to discuss the BRET saturation assay specificity. In the case of CXCR3A/7 (Figure 11) heterodimer, we observed a straight line for CXCR3A-RLuc/CXCR7-YFP, which is interpreted as the absence of heteromers. Once the energy donor and acceptor were inverted, we observed a hyperbolic curve for CXCR3A-YFP/CXCR7-RLuc, suggesting heteromerization, and thus seemingly at odds with the first result. Even if the CXCR4 crystal structure has been reported, it is unknown whether all chemokine receptor C-terminals have a determined structure [144]. It is possible that RLuc or YFP fused to the C-terminal of a receptor can affect its interaction with other proteins, especially with other receptors nearby.

Therefore, we cannot exclude the possibility that fusion proteins impair the formation of heterodimers, as their sizes are relatively big and restricting. Still, this observation adds specificity and conclusiveness to the BRET saturation assays because it shows that not all configurations yield hyperbolic curves (which represent specific interaction between two proteins). Thus, the CXCR3A-Rluc/CXCR7-YFP configuration can be seen like a negative control. Therefore, we can conclude that not all configurations between energy donor and acceptor are permissive and that BRET saturation assays are useful and specific tools to assess receptor dimerization.

What are the biological implications of chemokine receptor heterodimerization? It has been shown that CCR2/CCR5/CXCR4 could form heterodimer complexes and that negative binding cooperativity occurred between the binding pockets of these chemokines receptors highly expressed on leukocytes [145]. Sohy *et al.* also showed that CCR2 and CCR5 antagonists inhibit cell migration via the CXCR4/CXCL12 axis, reinforcing the concept of cross-inhibition between receptors in the context of heterodimers [145]. Taken together, these results illustrate how antagonists targeting one receptor can regulate functional responses of another receptor to which they do not bind directly [146]. It has been demonstrated that AMD3100, a highly selective CXCR4 antagonist [147, 148], has clinical benefit in the treatment of asthma [149]. Interestingly, CXCR4 is not an inflammatory but a homeostatic receptor; its anti-inflammatory effects are not quite understood [150]. Of note, CXCR4 is widely expressed on T-lymphocytes, B-lymphocytes, monocytes and macrophages, neutrophils [151]. For instance, AMD3100 had significant effect in down-regulating the inflammation and pathophysiology of the allergen-induced response in a mouse model of asthma [149]. It is tempting to suggest that AMD3100 benefits are mediated through CXCR3/CXCR4 heterodimers. Considering this hypothesis, questions arise. For example, could inhibitors of CXCR4 reduce asthma symptoms strongly related to CXCR3+ mast cells [152, 153]?

What role can CXCR3/CXCR4 heterodimers play in others auto-immune diseases? For instance, what role does CCR2/CCR5/CXCR4 plays in RA, an auto-immune disease in which

CXCR3 is also strongly involved [154]? It has been reported that most T lymphocytes in RA synovial fluids were CXCR3 and CXCR4 positive [155, 156]. Furthermore, several papers support the involvement of CXCL12 and its receptor CXCR4 in memory T cell migration in the inflamed RA synovium [155]. Interestingly, T140, a CXCR4 antagonist, significantly ameliorated clinical severity in collagen-induced arthritis in mice [157]. As perspectives to investigate the possible role of chemokine receptor heterodimers in diseases, it could be interesting to assess negative binding cooperativity or trans-inhibition between CXCR3/CXCR4 complexes. For instance, we could investigate whether AMD3100 or T140 could interfere with CXCR3 signalling. In the same order of idea, we could investigate whether CXCR4/CXCL12 axis can influence CXCR3 signalling through its endogenous ligands. The same holds true for CXCR3/CXCR7 heterodimers. The influence of CXCR3/CXCR4 heteromerization will further be discussed in the *CXCR3 decoy activities* section.

4.3 CXCR3 decoy activities

Chemotaxis is defined as the directed locomotion of a cell from a lower to a higher concentration of chemoattractant. Cells reshape this gradient to maintain their locomotion, but the mechanisms underlying gradient remodelling are not well understood and are still a matter of active research. In fact, this process is studied in various fields from immunology to embryogenesis [158-160].

Here, we asked whether the typical chemokine receptor CXCR3A was capable of CXCL11 scavenging. We found that CXCR3A is able to degrade CXCL11 to a lower extent than the atypical receptor CXCR7. However, in contrast with CXCR3A, CXCR7 is constantly recycled to the cell membrane [120]. We will discuss this difference in trafficking below. We were also wondered whether CXCR3A ligands could inhibit CXCL11 scavenging. We only found that CXCL11 was able to displace ^{125}I-CXCL11. Since CXCR3A and CXCR4 are co-expressed on activated T-cells, natural killer cells, dendritic cells and cancer cells [139, 140, 161], we asked whether the CXCR4/CXCL12 axis could interfere with CXCL11 scavenging via CXCR3A. Finally, we did not observe such reduction of CXCL11 degradation in CXCR3A/CXCR4 co-expressing cells. Previously, only one report indicated CCR2 as a typical receptor capable of chemokine scavenging [102], which was recently confirmed by another study [162]. Therefore, the discovery of CXCL11 scavenging by CXCR3A is very promising since it is the second typical receptor to possess such decoy properties. The biological relevance of chemokine scavenging by CXCR3A will be discussed below.

Since CXCL11 scavenging is preeminent in CXCR7 transfected cells, it is important to think about the underlying mechanism of chemokine degradation by a typical chemokine receptor. The scavenging of CXCL11 via CXCR3A may be the consequence of CXCR3A degradation, followed by chemokine stimulation. Previous findings reported CXCL11 as the most potent and efficacious ligand to down-regulate CXCR3A surface expression [163]. On one hand, chemokine-induced CXCR3A downregulation occurs rapidly with CXCL11 [163]. On the other hand, chemokine receptors are usually recycled to the cell surface within an hour [163,

164]. However, in the case of CXCR3A, it has been shown that cell surface replenishment of CXCR3A occurred only after several hours in T lymphocytes and CXCR3 transfectants [163]. This replenishment was dependent on mRNA transcription, *de novo* protein synthesis and transport through the ER and Golgi [163]. It is tempting to suggest that CXCL11 scavenging is the indirect product of CXCR3A degradation. To validate this hypothesis, future experiments should use cycloheximide, a protein synthesis inhibitor, in our degradation assays. Contrary to the constant recycling of CXCR7 [120], we can suppose that the fate of CXCR3A is to be directed towards degradation. Therefore, in the presence of cycloheximide, we would expect to observe a diminution over time of CXCL11 scavenging via CXCR3A but not via CXCR7.

We wondered whether CXCR3A ligands could inhibit CXCL11 scavenging. We observed homologous but not heterologous competition (Figures 16, 17). It may not be possible to observe heterologous ligand competition because CXCR3A ligands are allotopic (different binding sites on CXCR3A) [117]. This implies that, unable to displace CXCL11 from its binding pocket, neither CXCL10 nor CXCL9 can inhibit CXCL11 scavenging. It will be interesting to use radiolabelled CXCL9 and CXCL10 to further assess the decoy activity of CXCR3A. Of note, these radiolabelled ligands are not commercially available. Since CXCR3A ligands are allotopic, we could expect the same pattern of homologous competition in CXCL9 and CXCL10 degradation assays. However, the inability of CXCR3A to scavenge two of its three classical ligands would have tremendous biological implication. It has been reported that distinct domains of CXCR3 mediate its functions [95]. For instance, the C-terminal domain was predominantly required by CXCL9 and CXCL10 for β-arrestin recruitment, and the third intracellular loop was predominantly required by CXCL11 for internalization [95]. This CXCR3 biased trafficking could explain the hypothetical case in which CXCR3A only scavenges CXCL11. If this happens to be true, it could in part explain the pathological abundance of CXCL9 and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma [165] or in systemic sclerosis [166].

We are now going to discuss the CXCR3/CXCR4 heterodimer in the context of chemokine degradation. Cross-inhibition between chemokine receptors has been observed [145], and we

demonstrated CXCR3 heterodimerization with CXCR4. Hence, we asked whether the CXCR4/CXCL12 axis could interfere with CXCL11 scavenging via CXCR3A. Considering the similar $BRET_{50}$ values observed for CXCR3A and CXCR4 homo and heterodimer [80], we would expect a population composed of half heterodimer (CXCR3A/4, CXCR4/3A), one quarter CXCR4 homodimers and one quarter CXCR3A/3A homodimers in cells transfected with equal amounts of each plasmid (Figure 28). Consequently, we expected CXCL12 to block or, at least, reduce scavenging by CXCR3A/4 heterodimers. We did not observe such reduction (Figures 19 and 20). This suggests the incapacity of CXCR4 to impede CXCR3A of CXCL11 scavenging. In perspective, it would be insightful to confirm binding of CXCL11 to CXCR3A/4 heterodimers. Since the CXCR4/CXCL12 axis does not interfere with CXCR3A, we do not expect to find any difference of CXCL11-binding between CXCR3A or CXCR3/4 cells. We could also look whether CXCR3A/4 heterodimers are capable of CXCL12 scavenging. As for the CXCR3/7 heterodimer, it is less tempting to investigate since they both have decoy activities.

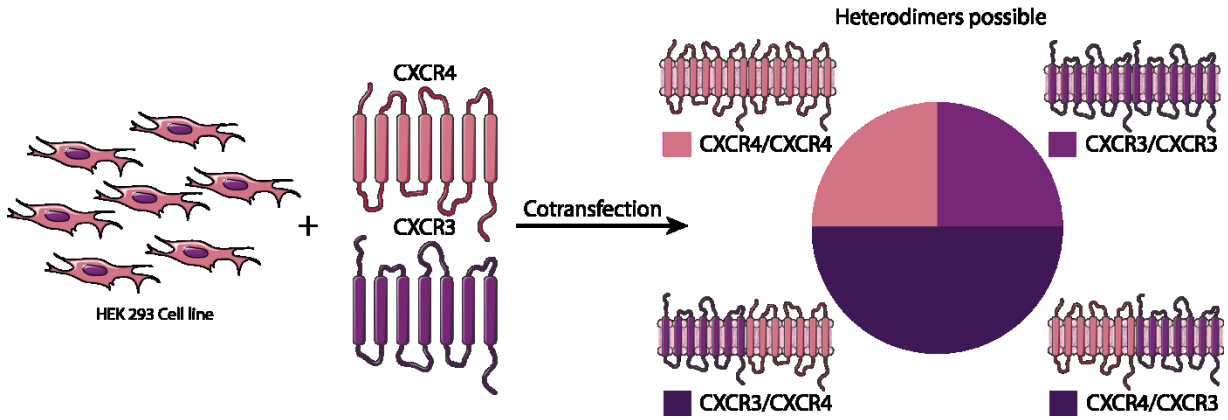

Figure 28. Probabilities of homo- and heterodimers formation in co-transfected cells.

What is the relevance of gradient remodelling by CXCR3A expressing cells? For instance, CXCR7 self-generates a gradient of CXCL12 during embryogenesis. This gradient acts as the predominant mechanism by which cells generate robust collective migration [160]. Another publication suggested that CXCR7 continuously cycles between the plasma membrane and intracellular compartments, while the chemokine cargo is targeted for degradation [120]. In

light of these results, CXCR3A decoy properties seem to be different than the ones of CXCR7. It is tempting to suggest that CXCR3A expressing cells reshape their own chemokine gradient to migrate towards injured sites during the inflammatory/immune response. Moreover, does this unusual chemokine degradation occur only in migrating cells, or does it also occur in endothelial cells? What is the role played by CXCR3+ endothelial cells highly expressing the CXCR3B variant [87]? We could imagine that endothelial cells are involved in the termination of the chemoattractant signal. To support this statement, it has been shown that CXCR3B is able to inhibit cell migration [87]. We suggest that this inhibition is most probably due to chemokine clearance, which affects the concentration gradient necessary for the chemotaxis process. In summary, we suggest that endothelial cells are a source of chemokines until enough inflammatory cells reached the site of injury. Then, endothelial cells terminate the chemoattractant signal in order to limit the inflammatory response and further cell migration that could be potentially harmful.

The decoy properties of cells endogenously expressing CXCR3, most likely activated T cells isolated from human peripheral blood, should be further investigated. Finally, it would also be important to investigate chemokine degradation in cells expressing endogenously the CXCR3B splice variant.

4.4 Characterization of CXCL4, a novel ligand of CXCR3

Following platelet activation, CXCL4 is released at high (micromolar) concentrations from α-granules and promotes leukocytes recruitment [93, 95]. The receptor by which CXCL4 signals was for long unknown and its underlying signaling pathways remain to be elucidated.

Lasagni *et al*. were first to demonstrate CXCR3B as a functional receptor for CXCL4 [87]. They showed CXCL4 able to transduce, at high concentration, apoptotic, but not chemotactic signals in human microvascular endothelial cells (HMVEC) [167]. Another group showed CXCR3-mediated migration of activated T lymphocytes toward CXCL4 [95]. This CXCL4-induced chemotaxis was inhibited by CXCR3 antagonist and pretreatment of cells with pertussis toxin, a Gαi inhibitor, suggesting the role of the CXCR3 GPCR [95]. Other studies showed activation of p38 MAPK and Src kinase in CXCR3A transfected cells followed by CXCL4 stimulation [168]. Furthermore, CXCL4 slightly enhanced forskolin-stimulated cAMP production in HMVEC [168]. These studies confirmed that CXCL4 activates both CXCR3A and CXCR3B. In the light of these results, we hypothesized that CXCL4 could induce a cAMP response and β-arrestin recruitment in CXCR3A transfected cells. In addition, we investigated whether CXCL4 could inhibit CXCR3A mediated CXCL11 degradation.

We did not observe any Gαi activation or β-arrestin recruitment to CXCR3 followed by CXCL4 stimulation in our BRET assays. Since CXCL4 enhanced forskolin-stimulated cAMP production, it would be interesting to investigate whether the CXCR3A/CXCL4 axis signals through the Gαs subunit. For instance, the cAMP BRET assay in the absence of forskolin might be a good way to assess Gαs signaling. CXCR3/CXCL4 signalling has been observed in HMVEC and may not be observable in HEK293E cells. As an alternative, it could be useful to consider primary cell lines in order to validate our results. Also, we cannot exclude the possibility that conformational changes in the receptor upon CXCL4 binding do not favor energy transfer between the donor and acceptor in our BRET assays. As an alternative, we could invert YFP and RLuc from both proteins, in the case of β-arrestin2 recruitment assays, to see if we can detect such recruitment.

Once released from platelets, CXCL4 is present in micromolar concentrations [93]. CXCL4 avidly binds to glycosaminoglycans but only binds to CXCR3 with a low affinity [87]. CXCL4 is ten times less potent than CXCL11: 1 μM CXCL4 is necessary to induce migration when it only needs 100 nM for CXCL11 [95]. However, the biological relevance of CXCR3/CXCL4 interaction is still important considering the high concentration of *in situ* CXCL4 following platelet activation. Considering this, the competition of CXCL11 with 1μM CXCL4 was far from excessive. Previously, it has been described that CXCL10 could not compete with CXCL4 in binding assays, suggesting both ligands are allotopic [95]. This might explain why we did not see a blockade by CXCL4 on CXCL11 scavenging (Figures 12A and 12B).

The CXCR3B variant is specifically expressed on endothelial cells, and most probably involved in gradient termination and angiostatic effects during inflammatory states [87]. It was suggested that the CXCR3/CXCL4 axis may play a role in T lymphocyte recruitment and the subsequent amplification of inflammation [95]. Moreover, it is known that CXCR3B overexpression in prostatic cancer cells blocked chemokine-induced cell motility and invasion [113]. Therefore, it is tempting to suggest that CXCR3B is capable of CXCL4 scavenging. The investigation of CXCL4 scavenging and, most probably, CXCL4 scavenging via CXCR3B is surely a promising avenue for further investigations.

5. Conclusion

So far, no drugs efficiently target chemokine receptors for the treatment of inflammatory and/or auto-immunes diseases [47]. However, this is not a sufficient reason to claim that chemokine and chemokine receptors are inappropriate targets [47]. With different examples and at different levels, we showed how much more complex the chemokine biology is comparatively to what we previously though. Obviously, in order to overcome the challenges we face in drug discovery, a better understanding of chemokine and chemokine receptor is mandatory.

In this present work, we illustrated the complexity of this particular system. For instance, we showed that chemokines can act differentially on the same chemokine receptor. We demonstrated that different ligands of CXCR3 act differentially by being full agonists (CXL11) or partial agonists (CXCL9, CXCL10) in different pathways. In contrast to what was previously reported [64], the chemokine receptor CXCR3A does not seem to elicit biased agonism in our readouts.

We also illustrated and discussed about chemokine heteromerization and its tremendous potential implications in chemokine biology. For instance, we discussed that different receptors can be expressed on a same cell type and that, instead of the classical monomers, these receptors could form higher-order complexes. For instance, CXCR3 can form homodimers, but also heterodimers with CXCR4 or with CXCR7.

We also demonstrated that chemokine receptors can endorse diverse biological functions such as chemokine scavenging. According to the literature, the chemokine gradient remodelling is almost exclusive to atypical chemokine receptors (ACKRs). Since ACKRs do not mediate chemotaxis or classical signaling, their assigned role was to clear or reshape the gradient. However, we showed here that the classical chemokine receptor CXCR3A can share this function with the ACKR CXCR7 by degrading CXCL11.

6. References

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